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Ghrelin's second life: From appetite stimulator to glucose regulator

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Abstract

Ghrelin, a 28 amino acid peptide hormone produced by the stomach, was the first orexigenic hormone to be discovered from the periphery. The octanoyl modification at Ser³, mediated by ghrelin O-acyltransferase (GOAT), is essential for ghrelin's biological activity. Ghrelin stimulates food intake through binding to its receptor (GRLN-R) on neurons in the arcuate nucleus of the hypothalamus. Ghrelin is widely expressed throughout the body; accordingly, it is implicated in several other physiological functions, which include growth hormone release, gastric emptying, and body weight regulation. Ghrelin and GRLN-R expression are also found in the pancreas, suggesting a local physiological role. Accordingly, several recent studies now point towards an important role for ghrelin and its receptor in the regulation of blood glucose homeostasis, which is the main focus of this review. Several mechanisms of this regulation by ghrelin have been proposed, and one possibility is through the regulation of insulin secretion. Despite some controversy, most studies suggest that ghrelin exerts an inhibitory effect on insulin secretion, resulting in increased circulating glucose levels. Ghrelin may thus be a diabetogenic factor. Obesity-related type

2 diabetes has become an increasingly important health problem, almost reaching epidemic proportions in the world; therefore, antagonists of the ghrelin-GOAT signaling pathway, which will tackle both energy- and glucose homeostasis, may be considered as promising new therapies for this disease.

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Key words: Ghrelin; Blood glucose; Pancreas; Diabetes; Insulin

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INTRODUCTION

Ghrelin, a 28 amino acid peptide hormone predominantly produced by the stomach^[1], is the endogenous ligand of the growth hormone secretagogue receptor 1a isoform (GHS-R1a), presently renamed ghrelin receptor (GRLN-R)^[2]. Soon after the identification of ghrelin^[3], it became clear that ghrelin's effect extends beyond the stimulation of growth hormone (GH) secretion. Ghrelin's involvement in appetite stimulation was discovered as a side-effect in a study investigating the effect of ghrelin injection on GH-release in healthy humans^[4]. Ghrelin was soon identified to be the only known orexigenic hormone from the periphery, which stimulates food intake in a dose-dependent manner in rodents^[5-8] and humans^[9,10]. Many papers have been dedicated to ghrelin's orexigenic

effect, which has long been considered its main physiological function. More recent research now supports the idea that ghrelin may play an equally or even more important role in the regulation of blood glucose homeostasis. This review will give a summary of research data pointing out the importance of ghrelin in the regulation of blood glucose homeostasis. We will start with a general introduction about ghrelin and its expression throughout the body, including the pancreas. The relation with insulin will be discussed, followed by an overview of possible therapeutic implications resulting from these findings. The main goal of this review is not to provide an integral overview of previous research, but to indicate the importance of ghrelin in the regulation of blood glucose and implications for the treatment of disorders like diabetes.

GHRELIN AND THE GHRELIN RECEPTOR

Ghrelin is mainly produced by the X/A like cells, a distinct endocrine cell type found in the mucosal layer of the stomach and, to a lesser extent, in the small and large intestines^[1]. Smaller amounts are also detected in the pancreas, hypothalamus, heart, kidneys, lungs, testes, liver, and thyroid^[11,12].

Active ghrelin contains a unique posttranslational modification, an n-octanoyl group on the third serine residue, performed by ghrelin O-acyltransferase (GOAT), the 4th and highly conserved member of the membrane-bound O-acyltransferases superfamily (MBOAT4)^[13,14]. GOAT is localized to the gastric ghrelin cells, suggesting that ghrelin becomes octanoylated at its production site^[15]. GOAT transcripts predominantly occur in the stomach and pancreas in humans, while in mice they are found in the stomach, small intestine, colon, and, to a lesser extent, in the testis^[13,14]. According to the latest reports, acyl ghrelin makes up only 25% of the total amount of circulating ghrelin^[16]. The amount of circulating acylated ghrelin does not always parallel total ghrelin concentrations, suggesting that ghrelin acylation and secretion are regulated separately^[17,18]. In addition to n-octanoyl ghrelin, other acyl groups can be transferred to ghrelin, giving rise to other forms, such as decanoylated ghrelin, the second most abundant form of acylated ghrelin in circulation. The acyl groups added to ghrelin reflect the fatty acid content of the diet^[18], as enrichment of the diet with heptanoic acid causes ghrelin to be preferentially acylated with heptanoate, rather than with octanoic acid^[19]. The non-modified, unacylated form of ghrelin, which is the most abundant form of circulating ghrelin, does not bind to the ghrelin receptor and was first believed to be biologically inactive. Several studies have since established a physiological role for unacylated ghrelin^[20-22], probably through binding to a distinct, but unidentified, receptor^[22,23].

Alternative splicing of the *GRLN-R* gene results in the formation of two receptor forms, the full length *GRLN-R1a*, which is believed to be the biologically active form, and the truncated *GRLN-R1b*^[24,25]. *GRLN-R1a* is activated by octanoyl ghrelin and synthetic growth

hormone secretagogues while the *GRLN-R1b* is not activated by ghrelin and its physiological function remains unknown, despite its wide expression. *GRLN-R1a* is also widely expressed throughout the body. The highest expression levels of *GRLN-R1a* have been found in the hypothalamus^[26], where *GRLN-R1a* is expressed on growth hormone releasing hormone-expressing neurons^[27,28] and NPY/AgRP neurons in the arcuate nucleus^[29], consistent with its role in growth hormone release and appetite stimulation. Other expression sites of *GRLN-R1a* mRNA include the pituitary, thyroid, pancreas, spleen, myocardium, the adrenal gland, and the intestinal myenteric plexus^[11,30]. *GRLN-R* immunoreactivity was also observed within neuronal cell bodies and fibers in the human stomach and colon^[31].

The variety of ghrelin and *GRLN-R* expression sites already suggests a broad range of different physiological functions. In addition to its stimulatory effects on food intake in humans^[9,10] and rodents^[6,32,33], ghrelin also stimulates body weight gain, not only by promoting food intake, but also through an increased adipogenesis^[8,34]. Ghrelin also has prokinetic properties in the gastrointestinal tract. Peripheral ghrelin administration accelerates gastric emptying in conscious rats^[35,36] and mice^[37,38]. Ghrelin infusion stimulates gastric emptying in healthy volunteers^[39] and in patients with idiopathic^[40], diabetic^[41] and neurogenic^[42] gastroparesis, and induces hunger contractions in the fasting state^[43]. In addition, ghrelin has many other physiological functions, including cardiovascular effects^[44] and stimulatory effects on learning and memory^[45,46]. Recently, more and more attention has been given to the regulatory effects of ghrelin on glucose homeostasis^[47,48].

To unravel the physiological functions of ghrelin signaling, different transgenic and knockout mice models have been developed. Mice with a genetic deletion of ghrelin^[49,51], the ghrelin receptor^[52,53], or GOAT^[19] display only subtle phenotypic changes on a standard laboratory diet and a lower body weight compared to wild-type mice on a high fat diet, probably due to less accumulation of body fat^[19,53,54]. Ablation of ghrelin in *ob/ob* mice markedly improved glucose tolerance by increasing serum insulin levels, but did not result in a reduced body weight^[55]. Another study reported that ghrelin or ghrelin receptor ablation did not prevent diet-induced obesity in adult mice, but increased insulin sensitivity^[56]. When these mice were subjected to 50% caloric restriction, ghrelin and *GRLN-R* knockout mice had lower blood glucose levels than their wild-type littermates, as also observed in a recent study using GOAT knockout mice^[57], suggesting that ghrelin is involved in the counterregulatory glucose response during negative energy balance. While several compensatory mechanisms on food intake and body weight may have evolved in these knockout mice, these studies support the hypothesis that ghrelin and the *GRLN-R* may be non-essential regulators of appetite. It seems that ghrelin and its receptor have an important role in the regulation of blood glucose ho-

meostasis, which may represent a more important physiological function than the earlier reported effects on food intake regulation.

EXPRESSION OF GHRELIN AND THE GHRELIN RECEPTOR IN THE PANCREAS

The endocrine cells of the pancreas are assembled in dispersed islets containing traditionally four different types of cells, the insulin releasing β -cells, which make up the major cell population within an islet, the glucagon producing α -cells, the somatostatin producing δ -cells, and the pancreatic polypeptide containing PP cells. Moreover, the pancreas is an important source of ghrelin. Release of ghrelin by the pancreas was assessed by comparing the ghrelin concentration in the pancreatic vein with that in the pancreatic artery in rats. Acylated and unacylated ghrelin levels were found to be significantly higher in the pancreatic vein, indicating that the pancreas not only produces ghrelin^[58], but also expresses GOAT, the enzyme responsible for its acylation^[14].

The exact location and cellular origin of pancreatic ghrelin has been a matter of debate, and conflicting results have appeared. The first study to report that the pancreas is an important production site for ghrelin^[59] also stated that ghrelin production is restricted to the insulin producing β -cells of the human pancreas. In contrast, Date *et al.*^[47] reported that ghrelin is exclusively expressed in the glucagon producing α -cells in rats and humans, supported by the overlapping immunohistological stainings for ghrelin and glucagon observed in another study^[58]. The existence of a fifth separate islet cell population in the human pancreas, producing ghrelin and devoid of any other islet hormones, was postulated for the first time by Wierup *et al.*^[60] and is no longer debated. These pancreatic cells were found to be quite numerous (up to 10% of the endocrine cells) from midgestation to early postnatally, sometimes forming an almost continuous layer at the islet periphery^[60]. Only a few ghrelin cells remain visible on the mantle of the islets in adult pancreata of humans^[60,61] and rats^[62]. Therefore, the major source of ghrelin in fetal circulation is probably the pancreas, not the stomach^[63]. Normal mouse pancreas also contains a small population of ghrelin-producing cells, which were named “epsilon” cells. In the neonatal mouse pancreas, about 30% of the ghrelin cells were found to coexpress glucagon, whereas two-thirds (67%) of the ghrelin cells represented a unique islet cell population^[64].

Deletion of Nkx2.2 or Pax4 in mice, two transcription factors involved in the differentiation of insulin-producing cells, leads to an enormous increase in ghrelin-producing ϵ cells^[64]. Based on these results, it has been postulated that insulin-producing β -cells and ghrelin-producing ϵ cells share a common precursor^[64].

Guan *et al.*^[26] first demonstrated a weak GRLN-R mRNA signal in the rat pancreas during *in situ* hybridization. Indeed, not only transcripts for ghrelin, but also for its receptor are expressed in pancreatic tissue of both

humans^[11,59,65] and rats^[3,47]. Immuno-histochemical studies in rat pancreatic tissue revealed that the GRLN-R is localized to most of the α -cells and to some, but not all, β -cells^[66]. The latter was confirmed in human pancreatic islets^[67], supporting the idea of an autocrine/paracrine response of both β - and α -cells to ghrelin.

EFFECTS OF GHRELIN ON INSULIN SECRETION AND VICE VERSA

Glucose homeostasis is controlled by two key processes: insulin secretion by the pancreatic β -cells and insulin sensitivity of the peripheral tissues. The presence of the GRLN-R on pancreatic β -cells already suggested a role for ghrelin in the function of the β -cell, leading to the hypothesis that ghrelin also has a regulatory role in insulin secretion.

The observed inverse relationship between the circulating levels of ghrelin and insulin in healthy humans^[68] suggested inhibitory feedback between ghrelin and insulin. Indeed, insulin is able to suppress circulating ghrelin concentrations, independent from changes in glucose concentrations^[69]. The decrease of plasma ghrelin is induced by hyperinsulinemia and not by the resulting plasma glucose decrease, because plasma ghrelin was similarly suppressed when glucose was kept constant in a euglycemic study^[70]. A direct effect of physiological insulin concentrations on ghrelin secretion was also shown in the isolated perfused rat stomach^[71]. The inhibitory effect of insulin on ghrelin secretion was confirmed in several other studies^[72,73], while some reports did not confirm this observation, probably because of the different experimental conditions^[74,75].

Ghrelin, in turn, has been proven to affect insulin secretion, which was first demonstrated by Broglio *et al.*^[76], who showed that acute ghrelin administration in healthy volunteers resulted in prompt increases in blood glucose levels, followed by a decrease in insulin levels, independent from GH. Numerous other studies have investigated ghrelin's (acylated, unless otherwise indicated) effects on glucose and insulin metabolism, sometimes showing ambiguous results. An overview is given in the following paragraphs.

In vitro studies

Exogenous ghrelin: Studies on the effect of exogenous ghrelin on insulin release *in vitro* are summarized in Table 1.

Studies with β -cell lines, isolated β -cells, and pancreatic perfusion experiments considerably contributed to the understanding of the basic mechanisms governing the role of ghrelin in glucose and insulin metabolism. Date *et al.*^[47] reported an increased cytosolic Ca^{2+} concentration and stimulated insulin release in isolated rat islets treated with ghrelin (1 pmoL/L) in the presence of a stimulatory (8.3 mmol/L), but not basal (2.8 mmol/L) glucose concentration^[47]. Ghrelin at 1 pmoL/L modestly potentiated glucose-induced Ca^{2+} responses in a small portion of β -cells, but ghrelin had a clear inhibitory ef-

Table 1 Summary of *in vitro* and *in vivo* studies investigating the effect of exogenous ghrelin on insulin release

Study	Species	Dose	Effect on insulin
<i>In vitro</i> studies			
Isolated rat islets			
Date <i>et al</i> ^[47] , 2002	Rat	1 pmol/L ghrelin +2.8 mmol/L glucose	No effect
		1 pmol/L ghrelin +8.3 mmol/L glucose	Increase
Dezaki <i>et al</i> ^[58] , 2004	Rat	1 pmol/L-0.1 nmol/L ghrelin +8.3 mmol/L glucose	No effect
		10 nmol/L ghrelin +8.3 mmol/L glucose	Decrease
		10 nmol/L ghrelin +2.8 mmol/L glucose	No effect
Colombo <i>et al</i> ^[79] , 2003	Rat	1 pmol/L-1 μmol/L ghrelin +16.7 mmol/L glucose	Dose-dependent decrease
Qader <i>et al</i> ^[80] , 2008	Rat	10 nmol/L-1 μmol/L ghrelin +8.3 mmol/L glucose	Decrease
Reimer <i>et al</i> ^[81] , 2003	Mouse	10 nmol/L ghrelin +3.5-5.5 mmol/L glucose	No effect
		0.01-1 nmol/L ghrelin +8.3-22.2 mmol/L glucose	Decrease
Qader <i>et al</i> ^[80] , 2008	Mouse	1 pmol/L ghrelin +12 mmol/L glucose	Decrease
		10 nmol/L-1 μmol/L ghrelin +12 mmol/L glucose	Increase
Pancreas perfusion			
Egido <i>et al</i> ^[85] , 2002	Rat	10 nmol/L ghrelin +5.5 mmol/L glucose	No effect
		10 nmol/L ghrelin +5.5-9 mmol/L glucose	Decrease
Dezaki <i>et al</i> ^[77] , 2006	Rat	10 nmol/L ghrelin +8.3 mmol/L glucose	Decrease
Desacyl ghrelin			
Dezaki <i>et al</i> ^[77] , 2006	Rat	10 nmol/L desacyl ghrelin +8.3 mmol/L glucose	No effect
Pancreatic tissue fragments			
Adeghate <i>et al</i> ^[86] , 2002	Rat	1 nmol/L ghrelin	Increase
β cell lines			
Wierup <i>et al</i> ^[62] , 2004	INS-1	0.1-100 nmol/L ghrelin +3 mmol/L glucose	No effect
		0.1-100 nmol/L ghrelin +15 mmol/L glucose	Decrease
Gauna <i>et al</i> ^[87] , 2006	INS-1	10 nmol/L ghrelin +20 mmol/L glucose	Increase
Doi <i>et al</i> ^[83] , 2006	MIN 6	1-10 nmol/L ghrelin +3.3 mmol/L glucose	No effect
Wang <i>et al</i> ^[84] , 2010		1-10 nmol/L ghrelin +22.2 mmol/L glucose	Decrease
Granata <i>et al</i> ^[67] , 2007	HIT-T15	100 nmol/L ghrelin +1.25 mmol/L glucose	No effect
		100 nmol/L ghrelin 7.5-15 mmol/L glucose	Increase
Desacyl ghrelin			
Gauna <i>et al</i> ^[87] , 2006	INS-1E	10 nmol/L desacyl ghrelin +20 mmol/L glucose	Increase
Granata <i>et al</i> ^[67] , 2007	HIT-T15	100 nmol/L desacyl ghrelin +1.25-15 mmol/L glucose	Increase
<i>In vivo</i> studies			
Dezaki <i>et al</i> ^[58] , 2004	Mouse overnight fasted	1-10 nmol/kg (<i>ip</i>) ghrelin +1 g/kg (<i>ip</i>) glucose	Decrease
Reimer <i>et al</i> ^[81] , 2003	Mouse 3 h fasting	5 nmol/kg (<i>iv</i>) ghrelin	No effect

		+1 g/kg (<i>iv</i>) glucose	
		50-150 nmol/kg (<i>iv</i>) ghrelin	Decrease
		+1 g/kg (<i>iv</i>) glucose	
Cui <i>et al</i> ^[89] , 2008	Rat overnight fasted	0.3 pmol/kg/mL (40 min, 1 mL/h, <i>iv</i> portal vein or <i>ip</i> femoral vein) ghrelin +13.3 mg/kg/min (10-40 min, <i>iv</i> portal vein or <i>ip</i> femoral vein)	Decrease (portal vein) No effect (femoral vein)
Broglio <i>et al</i> ^[76] , 2001	Healthy humans overnight fasted	0.3 nmol/kg (<i>iv</i>) ghrelin	Decrease
Akamizu <i>et al</i> ^[91] , 2004	Healthy humans overnight fasted	0.3-1.5 nmol/kg (<i>iv</i>) ghrelin	Decrease after 90 min only with 1.5 nmol/kg
Lucidi <i>et al</i> ^[92] , 2005	Healthy humans overnight fasted	7.5-15 pmol/kg/min (<i>iv</i> , 2 h infusion)	No effect
Gauna <i>et al</i> ^[93] , 2004	Adult-onset GH-deficient patients overnight fasted	0.3 nmol/kg ghrelin, <i>iv</i>	No effect
Tong <i>et al</i> ^[48] , 2010	Healthy humans, 10-12 h fast	0.3-1.5 nmol/kg/h (<i>iv</i> , 65 min) 0.3-1.5 nmol/kg/h (<i>iv</i> , 65 min) +11.4 g/m ² body surface area glucose (<i>iv</i> , after 55 min)	No effect Dose-dependent decrease
Desacyl ghrelin			
Gauna <i>et al</i> ^[90] , 2007	Rat overnight fasted	3-30 nmol/kg desacyl ghrelin (<i>iv</i>) +1 g/kg (<i>iv</i>) glucose	Increase
Broglio <i>et al</i> ^[94] , 2004	Healthy humans overnight fasted	0.3 nmol/kg desacyl ghrelin (<i>iv</i>)	No effect
Gauna <i>et al</i> ^[93] , 2004	Adult-onset GH-deficient patients overnight fasted	0.3 nmol/kg desacyl ghrelin (<i>iv</i>)	No effect

iv: Intravenous; *ip*: Intraperitoneal; GH: Growth hormone.

fect at relatively high concentrations (10 nmol/L)^[58,77], which is consistent with the majority of literature reports. Although this concentration is higher than that of circulating ghrelin, ranging from 100 pmol/L to 3 nmol/L^[78], it is generally conceived that the level of hormone working in a paracrine or autocrine manner is higher than that working in an endocrine manner. Acylated ghrelin could also dose-dependently suppress glucose-induced insulin secretion in isolated adult rat and mouse islets^[79-81], in isolated islets from rat neonates^[82], and in β -cell lines^[62,83,84]. In a pancreas perfusion study, an *in vitro* system that retains the intact circulation in pancreatic islets excluding the influence of other organs, the infusion of ghrelin into the isolated pancreas also inhibited the insulin response to increasing glucose concentrations, arginine, and carbachol^[77,85].

Only a few studies reported a stimulatory effect of ghrelin on insulin secretion. Ghrelin could stimulate insulin release in pancreatic tissue fragments from normal and diabetic rats^[86]. Accordingly, both acylated (AG) and unacylated ghrelin (UAG) could exert an insulinotropic effect in the INS-1E rat^[67,87] and HIT-T15 hamster^[67] insulinoma derived β -cell lines in the presence of a static glucose concentration.

In addition its effect on insulin secretion, both acylated and unacylated ghrelin promote cell proliferation and counteract apoptosis of pancreatic β -cells in INS-1E β cell lines and in human islets independent from the GRLN-R^[67].

In vitro studies

Endogenous ghrelin: To examine the effects of endogenous ghrelin produced in the islets, isolated rat islets and pancreata were treated or perfused, respectively, with GRLN-R antagonists or an antiserum against active ghrelin, both resulting in increased glucose-induced insulin re-

lease in the absence of exogenous ghrelin^[77]. In the same study, glucose-induced insulin release from isolated islets of ghrelin knockout mice was significantly greater than that of wild-type mice, despite the similar density and size of the islets. These results indicate an insulinostatic effect of endogenous ghrelin in the islets. In contrast, the GRLN-R antagonist YIL-781 only blocked the inhibitory effect of ghrelin on glucose-induced insulin secretion in dispersed pancreatic islets, but had no effect in the absence of ghrelin^[88].

In vivo studies

Exogenous ghrelin: Studies on the effect of exogenous ghrelin on insulin release *in vivo* are summarized in Table 1.

Systemic administration of exogenous ghrelin decreases glucose-induced insulin secretion in mice^[58,81] and rats^[89]. In mice fasted for 3 h, intraperitoneal (*ip*) injection of ghrelin diminished the 1-min insulin response after administration of an intravenous glucose load (1g/kg)^[81], which was confirmed in overnight fasted mice^[58]. When ghrelin was simultaneously injected with glucose, the glucose levels at 30 min and 60 min were higher and the insulin levels at 5 min and 10 min were markedly attenuated in comparison to control values. Overnight fasted mice also displayed significantly elevated glucose levels at 30 min after *ip* administration of ghrelin, an effect that could be blocked completely by simultaneous administration of the GRLN-R antagonist, [D-Lys³]GHRP-6, indicating that ghrelin increases blood glucose *via* specific interaction with the GRLN-R^[58]. Accordingly, ghrelin infusion into the hepatic portal vein inhibited glucose-induced insulin secretion in rats^[89]. In contrast, UAG infusion at pharmacological doses enhanced the insulin response to an intravenous glucose load potently and dose-dependently, an effect that was abolished by co-administration of AG^[90].

There is little and inconsistent information concerning the effect of ghrelin on insulin release in humans. The first study with exogenous ghrelin administration in healthy humans confirmed that acute administration of ghrelin (1 µg/kg) after an overnight fast resulted in prompt increases in blood glucose concentrations, followed by a slight, but significant, decrease in insulin levels, which may further increase blood glucose^[76]. A decrease in insulin levels was also observed in a study from Akamizu *et al.*^[91]. However, Lucidi *et al.*^[92] could not confirm these changes in insulin and glucose levels when physiological increases (two to three-fold increments) in plasma ghrelin levels were reached, indicating that ghrelin only affects glucose metabolism at pharmacological doses.

Until now, only a few studies investigated the effect of exogenous ghrelin administration on glucose-stimulated insulin secretion. A recent study reported a reduction in glucose-induced insulin in an intravenous glucose tolerance test after exogenous ghrelin administration^[48] in healthy humans. However, because ghrelin infusion raised ghrelin levels by about 4.5, 15.4, and 22.6 fold, the clinical relevance of the latter study can be questioned.

Administration of ghrelin not only affects insulin release, but also insulin sensitivity. In adult-onset GH-deficient patients, ghrelin reduced insulin sensitivity up to 6 h after administration, whereas co-administration of AG ghrelin and UAG ghrelin neutralized the insulin desensitizing effects of AG administration and even improved insulin sensitivity^[93]. Broglio *et al.*^[94] also showed that administration of UAG alone did not affect insulin and glucose levels, but antagonized the effects of AG on insulin secretion and glucose levels in humans. That the relationship between AG and UAG may have an impact on metabolism has been suggested from clinical studies, which show an indirect relationship between circulating AG/UAG ratio and insulin resistance^[95], and a decreased AG/UAG ratio in fasting, relatively insulin sensitive, subjects^[17].

In vivo studies

Endogenous ghrelin: Intraperitoneal injection of the GRLN-R antagonist, [D-Lys³]GHRP-6, in mice markedly decreased fasting glucose concentrations, and resulted in an attenuated glucose elevation and enhanced insulin response after an *ip* glucose challenge^[58]. Similarly, injection of [D-Lys³]GHRP-6 in overnight fasted rats resulted in an enhanced insulin response during an intravenous glucose tolerance test (GTT)^[90]. In gastrectomized rats, which have dramatically lower acylated ghrelin levels (around 16% of control animals), [D-Lys³]GHRP-6 increased plasma insulin concentrations to a similar extent as in normal rats, indicating that locally produced ghrelin in the pancreas is the major regulator of insulin release^[77].

Numerous studies were performed in ghrelin, ghrelin receptor and recently also in GOAT knockout mice, to investigate whether endogenous ghrelin can influence blood glucose homeostasis. Ghrelin knockout mice presented normal blood glucose and plasma insulin levels in

the fed and fasted state on a standard laboratory diet^[49,50]. However, during a GTT, ghrelin knockouts had reduced blood glucose and increased insulin levels compared to the wild-types. In addition, an insulin tolerance test showed a more pronounced decrease in glucose levels occurring 30 min after insulin injection in the ghrelin knockout animals on a standard diet, pointing towards increased insulin sensitivity compared to the wild-types. The latter was confirmed by an increased performance in euglycemic hyperinsulinemic clamp studies^[55]. When placed on a high fat diet immediately after weaning, wild-type mice became glucose intolerant and insulin resistant, while ghrelin knockout animals were able to maintain normal glucose levels because of a markedly enhanced insulin secretion^[54,77]. Their body weight and body fat percentage were lower.

Similar results were obtained in GRLN-R knockout mice, which showed resistance to diet-induced obesity and enhanced insulin sensitivity when exposed to a high fat diet immediately after weaning^[53,96]. In addition, RQ values were found to be higher in GRLN-R knockout mice indicating a preference for carbohydrates as fuel, regardless of the diet^[96].

Whereas one study found no difference in glucose tolerance during an *ip* GTT in GOAT knockout mice^[19], Zhao *et al.*^[57] showed that GOAT knockout mice, during caloric restriction (fasted for 16 h), experience an improved glucose tolerance and an increased insulin secretion during an oral GTT. Accordingly, mice that were pretreated during an *ip* glucose challenge with the bisubstrate analog, GO-CoA-Tat, which antagonizes GOAT, showed a significant increase in insulin response that was accompanied by a reduction in blood glucose^[97]. This effect was not observed in ghrelin knockout mice, indicating that the effect on glucose regulation is mediated by acyl ghrelin.

MECHANISMS INVOLVED IN THE INHIBITORY EFFECT OF GHRELIN ON INSULIN SECRETION

Insulinoma-associated protein 2β (IA-2β), a membrane glycoprotein localized to secretory granules, is a β cell autoantigen for type 1 diabetes, and about 50% of newly diagnosed patients have autoantibodies against IA-2β^[98,99]. Administration of ghrelin increased IA-2β mRNA expression in mouse pancreas, brain, and insulinoma (MIN6 and βTC3) cell lines. Moreover, both ghrelin administration and stable overexpression of IA-2β could attenuate glucose-induced insulin secretion. These findings strongly suggest that ghrelin exerts its inhibitory effects on insulin secretion at least partly through enhancement of IA-2β expression^[83]. Another recently identified pathway implicated in the effect of ghrelin on glucose-stimulated insulin secretion (GSIS) and independent from IA-2β, is the AMPK-uncoupling protein 2 (UCP2) pathway^[84], which is also involved in the control of food intake in

the hypothalamus^[100]. UCP2, which uncouples oxidative phosphorylation from ATP synthesis, diminishes the glucose-stimulated insulin secretion in β cells^[101]. The link between ghrelin and UCP2 was hypothesized based on the observation that ghrelin knockout mice, which have an enhanced glucose-induced insulin secretion, also displayed reduced expression of UCP2 mRNA in the pancreas^[55]. Conclusive evidence was provided by down-regulating UCP2 with the siRNA technique in MIN6 cells, which enhanced GSIS and blocked ghrelin's inhibitory effect on GSIS^[84].

GHRELIN RECEPTOR AS A THERAPEUTIC TARGET FOR THE TREATMENT OF DIABETES

Diabetes mellitus (DM) is a chronic disease with increasing worldwide prevalence. In 2000, 171 million individuals were estimated to have diabetes and this is expected to increase to 366 million by 2030^[102]. Currently, more than 220 million people worldwide have diabetes. There are two main types of DM: type 2 diabetes or non insulin-dependent diabetes mellitus (NIDDM) accounts for 90% of patients, whereas type 1 or insulin-dependent diabetes mellitus (IDDM) accounts for most other diabetic patients and generally appears before the age of 40 years. Both types of diabetes are characterized by fasting hyperglycemia and abnormal glucose excursion after administration of a glucose load. In the following paragraphs, the role of GRLN-R as a therapeutic target for type 1 and 2 diabetes will be discussed. For both types of diabetes, it is important to preserve and protect viable β -cells. Type 2 diabetes is also characterized by insulin resistance, which means that improving insulin sensitivity is important for the outcome of this disease.

DM type 1

Type 1 diabetes results from autoimmune destruction of the β -cells, leading to a dependency of exogenous insulin administration for maintaining blood glucose homeostasis in these patients. Inverse patterns of plasma ghrelin and insulin concentrations have been described in a 24 h observation period in normal subjects^[68]. Likewise, fasting insulin and ghrelin concentrations were found to be negatively correlated in both lean and obese subjects^[103]. However, plasma ghrelin data obtained from type 1 diabetic patients are often conflicting, probably dependent on whether these patients are treated or not with insulin in addition to the timing of sampling. In newly diagnosed children with type 1 diabetes, ghrelin levels were low prior to insulin treatment and did not respond to meal tests^[104]. This was confirmed in another study, which reported that total and acylated ghrelin concentrations were decreased, compared to healthy children^[105]. Another study observed higher plasma total ghrelin concentrations in type 1 diabetic patients and these levels declined by 29% after insulin treatment^[106]. Murdolo *et al.*^[107] showed

that the lack of insulin in untreated type 1 diabetic patients prevented post-prandial ghrelin suppression, which may contribute to the hyperphagia that is often observed in these patients. Accordingly, gastric and plasma ghrelin concentrations were reported to be increased in untreated streptozotocin (STZ)-induced diabetic rats, an animal model of type 1 diabetes^[108-110]. Conclusive evidence for the contribution of ghrelin signaling pathway to the development of STZ-induced diabetic hyperphagia has been provided in two studies with ghrelin^[111] and ghrelin-receptor knockout mice^[112].

Therapeutic applications of ghrelin receptor antagonists in type 1 diabetes

Treatment of mice, five days after the induction of STZ-induced diabetes, with a GRLN-R antagonist reduced blood glucose levels and normalized plasma glucagon levels^[111]. In addition, daily food intake was reduced. However, in STZ-induced diabetic GHS-R or ghrelin knockout mice hyperphagia was reduced, but no differences in blood glucose levels were observed compared with wild-type mice^[111,112]. These data suggest that ghrelin receptor antagonists may only be efficient in the treatment of type 1 diabetes when a small percentage of insulin-producing β -cells remain intact, particularly at early onset.

Therapeutic applications of ghrelin receptor agonists in type 1 diabetes

Growing evidence exists for a potential therapeutic role for ghrelin receptor agonists during type 1 diabetes, at least at an early stage. Ghrelin administration for 7 d was able to promote β -cell regeneration in newborn rats treated with STZ, thereby preventing the development of hyperglycemia at an adult age^[113]. Surprisingly, similar protective effects were observed after treatment with unacylated ghrelin, which resulted in an increased area and number of pancreatic islets in STZ-treated rats^[114]. Likewise, daily exogenous ghrelin injections in 90% pancreatectomized rats enhanced endocrine and exocrine pancreatic regeneration. Acylated ghrelin treatment increased the number of β -cells, resulting in increased insulin production and an improved glucose tolerance^[115]. However, twice daily ghrelin administration for 4 wk, in adult mice 8 wk after STZ-diabetes induction, did not change body weight, food intake, blood glucose levels, or plasma insulin levels, indicating that ghrelin did not improve or worsen diabetic conditions^[116]. This strongly suggests that ghrelin may only affect diabetes type 1 in its earliest stage, when some viable β -cells remain. Ghrelin administration may then be able to prevent further progression of the disease, while later administration may have the opposite effect and may further inhibit insulin secretion. This indicates that the timing of administration is very important. Long-term treatment may further impair the function of the remaining β -cells; therefore, it is also important to take the duration of treatment into account.

In *in vitro* studies, both acylated and unacylated ghrelin have been demonstrated to promote proliferation and to

inhibit apoptosis (induced by serum-starvation or cytokines) of HIT-T15 and INS-1E β cell lines and human islets of Langerhans^[67,117]. Acylated ghrelin prevents lipotoxicity-induced apoptosis in MIN6 β -cells^[118]. The comparable effects of both acylated and unacylated ghrelin on β -cell survival may be mediated *via* an as yet unidentified receptor, because high-affinity binding sites for both peptides were identified on the cell membrane of HIT-T15 cells, which do not express the ghrelin receptor^[67].

Ghrelin also stimulates the expression of islet associated protein 2 β (IA-2 β) mRNA, a major auto-antigen for type 1 diabetes, in mouse pancreas, brain and insulinoma (MIN6 and β TC3) cell lines^[83].

DM type 2

The key pathogenic feature of type 2 DM is insulin resistance leading to a compensatory hypersecretion of insulin, ultimately leading to β -cell dysfunction. Type 2 diabetes, which is far more common than type 1 diabetes, is tightly associated with obesity, making it difficult to distinguish between the effects of diabetes alone, or in conjunction with obesity. Obese subjects have decreased circulating ghrelin levels^[103] and several studies have found that low ghrelin levels are associated with elevated fasting insulin concentrations and the prevalence of type 2 diabetes and insulin resistance^[119]. Indeed, total plasma ghrelin, as well as unacylated ghrelin, concentrations were found to be lower in insulin-resistant obese adults relative to equally obese insulin-sensitive controls^[120], indicating a link between ghrelin and insulin sensitivity.

Therapeutic applications of ghrelin receptor antagonists in type 2 diabetes

Although inconclusive data about the effects of ghrelin on insulin secretion were reported, most studies observed an inhibitory effect of ghrelin on glucose-induced insulin secretion *in vitro* using isolated pancreatic islets, β -cell lines, or perfused pancreata, which was confirmed *in vivo* in rodents and humans (Table 1). Transgenic mice over-expressing ghrelin showed glucose intolerance caused by decreased insulin secretion^[121]. Another study revealed that MK-677, a ghrelin mimetic, decreased insulin sensitivity and increased fasting blood glucose in aged healthy volunteers in a 1 year study^[122].

Accordingly, improved insulin sensitivity was observed in ghrelin-receptor knockout mice^[53,96,123] and the high fat diet-induced glucose intolerance is largely prevented by an enhanced insulin release in ghrelin knockout mice^[77]. Ghrelin deletion in ob/ob mice also promoted insulin sensitivity and insulin release in response to a glucose challenge^[55], by decreasing pancreatic UCP2 expression. By deleting the insulinostatic effect of ghrelin, the maximum capacity of glucose-induced insulin release may be increased enabling the β -cells to secrete more insulin to meet the increased insulin demand during obesity. Oral glucose tolerance testing in GOAT knockout mice, which lack the active form of ghrelin, showed a markedly better glucose tolerance and enhanced insulin secretion

on a regular and a high fat diet, compared to their wild-type littermates^[57].

Immunoneutralization of endogenous ghrelin with anti-ghrelin antiserum, or by GRLN-R antagonism, resulted in an enhanced glucose-induced insulin release in perfused pancreas^[77] and in isolated islets^[58]. *In vivo*, the ghrelin receptor antagonist, YIL-781 improved body weight and glucose tolerance in rats with insulin resistant diet-induced obesity^[88]. The GOAT-inhibitor, GO-CoA-Tat improved glucose tolerance and reduced weight gain in wild-type mice, but it remains to be investigated whether this compound is also effective in animal models of type 2 diabetes^[97]. The first non-peptidic, small molecule antagonists of GOAT, have recently been discovered^[124].

Unacylated ghrelin prevented the ghrelin-induced decrease in insulin secretion and insulin sensitivity, but did not induce any changes in these parameters when it was given alone^[94]. It is therefore unlikely that this compound will find any application in patients with type 2 diabetes, who have low plasma ghrelin levels.

All these results indicate that ghrelin is a diabetogenic factor and that counteraction of ghrelin augments glucose-dependent insulin secretion in pancreatic β -cells and improves insulin sensitivity in peripheral tissues, with beneficial effects on body weight. This reinforces the concept that ghrelin antagonists and/or GOAT inhibitors may provide a good therapeutic option for the treatment of type 2 diabetes and obesity.

Table 2 gives an overview of the ghrelin receptor antagonists that are currently under development. It is intriguing to observe that, despite 10 years of ghrelin research, the development of ghrelin antagonists is still in its infancy. One of the problems may be the discrepancy obtained with ghrelin antagonists *in vivo* and *in vitro*^[125] or the restraint of pharmaceutical companies to develop drugs which target a multifactorial disease such as obesity related type 2 diabetes, where the redundancy in orexigenic signals may lead to unpredictable results.

CONCLUSION

Besides ghrelin's well-described effects on food intake and growth hormone secretion, more attention has been given recently to its contribution to the regulation of blood glucose levels in the body. Although this effect may be mediated by direct mechanisms, most evidence suggests indirect regulation through insulin. Despite controversial results, many studies point out that ghrelin is able to inhibit insulin secretion *in vitro* and *in vivo*, which provides excellent therapeutic perspectives for type 2 diabetes. Ghrelin receptor antagonists for example, could be used to improve insulin release and insulin sensitivity. However, only a few antagonists are currently under investigation in clinical studies and the development of new potent and selective antagonists of the ghrelin receptor are warranted. Moreover, antagonists of ghrelin-O-acyltransferase have recently been discovered and their development may provide new perspectives.

Table 2 Ghrelin antagonists under development for the treatment of obesity and type 2 diabetes

Ghrelin antagonists Drug	Company	Target	Status
TZP-301	Tranzyme Pharma	Obesity, metabolic syndrome	Lead optimization
Two families of ghrelin antagonists	Helsinn Pharmaceuticals	Obesity, metabolic syndrome	Lead optimization
AEZS-123	Aeterna Zenartis	Obesity, alcohol abuse	Preclinical
Ghrelin antagonist	Novartis (Elixir)	Type 2 diabetes, obesity	Preclinical

Ghrelin agonists may be a promising therapeutic avenue to preserve and improve the function of the remaining β -cells in both type 1 and type 2 diabetes, by promoting β cell regeneration at an early stage of the disease. In addition, ghrelin agonists are useful to accelerate diabetic gastroparesis, which often impairs quality of life in diabetic patients. Long-term studies will be needed to investigate whether the developed ghrelin agonists do not induce desensitization of the ghrelin receptor. To prevent this issue, the half-life of these agonists should not be too long. Moreover, ghrelin has a wide range of other physiological functions, such as cardiovascular effects, anxiety, sleep, memory, and mood, which may lead to unwanted side effects. Additional research is needed to further address these issues.

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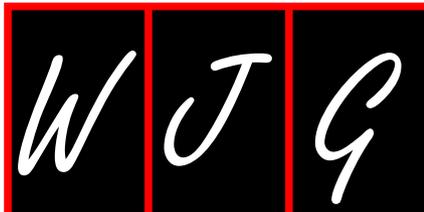
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Can zinc enhance response interferon therapy for patients with HCV-related liver disease?

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Abstract

Patients with liver disease may be at risk of zinc depletion. Zinc supplementation has been shown to contribute to inhibition of liver fibrosis and improvement in hepatic encephalopathy. However, little is known about the anti-inflammatory effect of zinc on hepatitis C virus (HCV)-related chronic liver disease. The standard of care for chronic HCV has improved markedly since the approval of interferon (IFN) therapy more than a decade ago. Over the past 20 years, IFN therapy has improved to more effectively eliminate the virus, progressing from single IFN therapy to combination therapy with ribavirin (RBV) and finally to pegylated IFN (PEG-IFN) therapy. However, even combined therapy with PEG-IFN and RBV for 48 wk is unable to eliminate the virus in some 40% of hepatitis C cases, particularly those with genotype 1b and high viral load. Treatment options for patients who have relapsed or are refractory to treatment with PEG-IFN and RBV therefore need to be critically assessed. This paper overviews the relationship between chronic liver disease and zinc metabolism.

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Key words: Chronic hepatitis C; Zinc; Interferon therapy

LIVER DISEASE AND ABNORMAL METAL METABOLISM

Abnormal metal metabolism, which is related to liver disease, has long been a subject of pathological study, particularly storage diseases such as iron metabolism in hemochromatosis^[1] and copper metabolism in Wilson's disease^[2].

As nutrition science has advanced, it has recently been pointed out that trace metallic elements in blood play essential roles, and abnormal metal metabolism involving metal deficiency in various diseases has been studied.

Zinc is an essential trace element in the human body, with approximately 2 g distributed throughout the body of a healthy adult, including many organs^[3-5]. *In vivo*, this element stimulates the activity of as many as 300 metal enzymes and metal-activated enzymes, and is crucial for nucleic acids and protein metabolism. Zinc plays an important role in the activity of many enzyme proteins, and a deficiency of zinc causes various pathologic disorders in the human body.

Regarding zinc metabolism abnormality in liver disease, in 1951 Vikbladh pointed out that the zinc content in serum was low in the case of various liver diseases^[6], and conjectured that serum contains albumin loosely

bound to zinc and globulin firmly bound to zinc, and that the albumin is associated with a lower zinc content. In 1957, Bartholomay *et al*^[7] claimed that hypozincemia in liver disease might be associated with increased urinary zinc excretion. In 1979, Sullivan *et al*^[8] pointed out that decreased zinc absorption was observed in cases of alcoholic cirrhosis, and in 1988, Grüngreiff *et al*^[9,10] reported that poor zinc absorption was observed in cases of non-alcoholic cirrhosis.

It is known that among liver disease cases, those with chronic hepatitis C show lower zinc concentrations in blood as their pathologic condition worsens from chronic hepatitis, to compensated cirrhosis, to decompensated cirrhosis, to hepatocellular carcinoma (HCC). Serum and hepatic zinc concentrations are decreased in chronic liver diseases, and zinc depletion has been suggested to be a cause of liver fibrosis^[11,12]. Particularly, cases with liver failure^[13] or cases of HCC^[14,15] are known to show conspicuous hypozincemia, and zinc supplementation therapy improves liver disease.

CORRELATION OF HEPATITIS C VIRUS WITH ZINC

A study of the relationship of the existence of hepatitis C virus (HCV) with zinc concentration in serum suggested that zinc concentration was not related to HCV genotypes or HCV-RNA values, and thus the existence of HCV did not affect the serum zinc concentration. However, it was reported that the serum zinc concentrations of untreated asymptomatic carrier (ASC) cases were significantly lower than those of healthy people^[16]. From this report, it can be inferred that the existence of HCV may be related to the serum zinc concentration even if the former does not directly affect the latter.

It has recently been reported that nonstructural 3 proteinase^[17-20], which is involved in the replication of HCV, is a zinc-containing enzyme, and that the nonstructural 5A protein is a zinc metalloprotein^[21]. Accordingly, it is necessary to examine and control the serum zinc concentration when treating cases of refractory chronic hepatitis C. In addition, in ASC cases, their progress may need to be observed by monitoring the serum zinc concentration and other means.

It has been reported that zinc in blood is closely related to hepatic fibrosis in cases of liver disease. It has also been reported that drug therapy or gene therapy that increases the serum zinc concentration can inhibit the progress of hepatic fibrosis^[22]. Many studies, including those noted above, suggest that pathologic conditions of the liver are closely related to zinc in blood. However, although there have been basic researches, its relationship with hepatic fibrosis has not been clinically studied.

CHRONIC HEPATITIS C AND ZINC

Some 60% to 70% of cases of hepatitis C progress to

chronic hepatitis, and then to cirrhosis and hepatocellular carcinoma over 20 years to 30 years. Following the 1986 report of Hoofnagle *et al*^[23] on the effects of interferon (IFN) on HCV infection in patients with chronic hepatitis, IFN therapy has been employed throughout the world to treat chronic hepatitis C. The treatment of choice for chronic hepatitis C is antiviral therapy using IFN^[24,25].

In Japan, many cases of hepatitis C have been treated with IFN since 1992, when treatment using IFN drugs became covered by the public health insurance system. IFN monotherapy, which was used initially, successfully resulted in low sustained HCV-negative condition of all cases treated^[26]. Sustained virological response (SVR) was gained in merely 6% of patients with HCV-1b in high viral loads when the practice of administering a 6-mo regimen of natural IFN was started in 1992; it increased to 20% with the 6-mo regimen of standard IFN combined with ribavirin (RBV) implemented in 2001^[27,28]. Finally, the introduction of the 12-mo regimen of pegylated IFN (PEG-IFN) and RBV approved in 2004 achieved SVR in 50%^[29,30].

The viral factors that affect the effectiveness of IFN therapy include HCV genotype and HCV-RNA quantity, and the host factors that do so include the histological condition of the liver, age and degree of fatness.

Generally, genotype I b virus is more resistant to IFN than genotype II a or II b viruses. In Japan, genotype I b in high viral loads accounts for more than 70% of HCV infections, making it difficult to treat patients with chronic hepatitis C^[27,28].

With regard to the effect of the combination therapy of oral administration of zinc with IFN for chronic hepatitis C, Nagamine *et al*^[31] reported on the roles of zinc and metallothionein in IFN- α therapy for hepatitis C in 1997. Takagi *et al*^[32] treated 68 cases of chronic hepatitis C with genotype I b virus of an HCV-RNA quantity of at least 100 Kcopy/mL with a dose of 10 million units of IFN- α once daily for four weeks, and then the same dose three times weekly for the subsequent 20 wk, by intramuscular injection. They combined the IFN administration with an oral dose of 150 mg of polaprezinc (Promac; Zeria Pharmaceutical, Company Ltd., Tokyo, Japan) daily (equivalent to 34 mg of zinc) in 32 of the 68 cases. The effects of the treatment were evaluated six months after completion, with the criteria of complete response (CR) for disappearance of HCV-RNA and normal alanine aminotransferase (ALT) levels; incomplete response (IR) for HCV-RNA that did not disappear, but normal ALT levels; and non-responder for other cases. The IFN monotherapy group (IFN) was compared with the IFN and zinc combination group (IFN + Zn) in terms of those criteria, with the following results. The IFN group showed CR: 11.1% (4/36) and IR: 11.1% (4/36), totaling 22.2% (8/36), while the IFN + Zn group showed CR: 37.5% (12/32) and IR: 18.8% (6/32), totaling 56.3% (18/32). Thus, they concluded that the zinc combination group had a significantly higher effective rate^[32].

Murakami *et al*^[33] treated cases of chronic hepatitis C with a high virus quantity of genotype I b with the PEG-IFN/RBV therapy, and analyzed the proportion of cases that became HCV-RNA negative after eight weeks of treatment by using groups with and without combination with zinc administration with an oral dose of 150 mg of polaprezinc (containing 34 mg of zinc) daily. They reported that the proportion of cases in whom ALT lowered to 35 U/L or less by the eighth week was 91% (10/11) in the zinc combination group and 58% (7/12) in the non-zinc administration group; however, the proportions of cases that became HCV-RNA negative were 18% (2/11) and 25% (3/12) respectively, showing no significant difference. Furthermore, they continued to observe the cases, and reported that all the cases (9/9) in the group with combination with zinc administration and 67% (8/12) of the cases in the group without combination with zinc administration showed normal ALT levels after 24 wk, and that all the cases (7/7) in the group with zinc and 60% (6/10) of the cases in the group without zinc did so after 48 wk. Interferon therapy for chronic hepatitis C has improved in many ways such as combination with ribavirin, and the rate of virus disappearance resulting from such therapy has increased conspicuously in comparison with the initial results. This therapy now achieves an SVR of approximately 50% even for cases with a high virus quantity of genotype I b, and a viral clearance rate of nearly 90% for other cases. However, this therapy has shown poor results for cases with severe hepatic histological fibrosis or thrombocytopenia, or for elderly patients, so its combination with zinc administration should be attempted in refractory cases.

HEPATIC FIBROSIS AND ZINC

In cirrhosis, the quantity of hydroxyproline in liver tissues increases while that of zinc decreases. Boyett *et al*^[34] reported that the concentration of zinc per unit nitrogen not derived from collagen was correlated with the concentration of zinc in liver tissues. Himoto *et al*^[35] examined the effects of zinc administration on inflammatory activity and fibrosis of the liver in patients with HCV-related chronic liver disease (CLD). Treatment with polaprezinc significantly decreased serum aminotransferase levels (aspartate aminotransferase: 92 ± 33 IU/L *vs* 63 ± 23 IU/L, $P = 0.0004$; ALT: 106 ± 43 IU/L *vs* 65 ± 32 IU/L, $P = 0.0002$), whereas alkaline phosphatase levels were significantly increased (305 ± 117 IU/L *vs* 337 ± 118 U/L, $P = 0.0020$). There was a tendency toward a decrease in serum type IV collagen 7S levels after treatment with polaprezinc. However, administration of polaprezinc did not affect peripheral blood cell counts, other liver function tests, or HCV-RNA loads. These findings suggest that polaprezinc exerts an anti-inflammatory effect on the liver in patients with HCV-related CLD by reducing iron overload.

Furthermore, Matsuoka *et al*^[36] reported that zinc supplementation improves the outcome of chronic hep-

atitis C and liver cirrhosis. Takahashi *et al*^[37] investigated the effect of oral zinc supplementation on liver fibrosis in patients with advanced chronic liver disease. The serum levels of type IV collagen and the activity of tissue inhibitors of metalloproteinase-1 were significantly reduced. This suggests that oral zinc supplement therapy is safe and may be a novel and useful strategy for antifibrosis therapy in patients with early liver cirrhosis.

Generally, the oxidative stress is high in hepatitis patients, and significant correlations among HCV-RNA. Oxidant stress is a significant feature of hepatitis C infection^[38]. There is evidence that the production of free radicals increases while anti-oxidant defense decreases significantly in all types of liver damage. Alongside the direct effect of the HCV core protein, hepatocellular iron accumulation and the production of reactive oxygen species associated with the immune response are considered to be of crucial significance for the creation of oxidative stress in chronic HCV. Mitochondrial effects may contribute to liver injury and oxidative stress seen in chronic hepatitis C^[39]. Zinc plays an important role in the redox process as a signal molecule and second messenger.

Decrease of supportive nutrients such as zinc have been documented in patients with viral or alcoholic liver disease. These markers may contribute to the monitoring the degree of liver damage, the response to antiviral therapies and to the design of new therapeutic strategies^[40]. Effects of zinc in the treatment of chronic hepatitis C are produced *via* immunological reactions, antiviral defence mechanisms and the role of zinc as an antioxidant^[41]. Furthermore, Yuasa *et al*^[42] have shown that zinc substitution negatively influences HCV replication. Zinc supplementation thus appears to offer a novel approach to the development of future strategies for the treatment of intractable chronic hepatitis C.

TREATMENT FOR CHRONIC HEPATITIS C BY ADMINISTERING ZINC

Leucopenia or thrombocytopenia is often found in elderly patients with chronic hepatitis C with severe fibrosis, and in many such cases, the IFN therapy must be discontinued due to side effects.

According to the results of a study in which zinc was administered in applying IFN and RBV therapy to cases of chronic hepatitis C with a tendency toward zinc deficiency to assess its effects on cytopenia, zinc served to protect against a reduction in white blood cells or platelets and thus effectively inhibited side effects.

The reduction in the number of peripheral blood cells due to IFN therapy seems to be caused by inhibition of the hematopoietic function of bone marrow. When RBV is combined with IFN therapy, hemolytic anemia due to the accumulation of RBV in red blood cells also seems to cause such a reduction. In cases of chronic hepatitis C with severe fibrosis, the number of blood cells tends to be already low before the adminis-

tration of IFN or RBV, and therefore how to prevent the reduction in blood cells as a side effect is important.

Nagamine *et al*^{43]} conducted a basic study on the activity of IFN- α in U937 cells in order to elucidate whether zinc would enhance the action of interferon. They found that zinc chloride and polaprezinc increased IFNAR mRNA by 30% to 40%, whereas monotherapy of L-carnosine had no such effect, suggesting that zinc enhanced the action of interferon and induced the production of anti-viral proteins. Hence, many cases of liver disease are accompanied by complaints of symptoms in the mouth such as dysgeusia, dry mouth and stomatitis when treated with PEG-IFN/RBV. Zinc supplement seems to be effective against oral mucosa disorders in IFN therapy treatment.

A new antiviral drug called DAA became covered by the public health insurance system in Japan. Hence, the treatment of choice for cases with a high quantity of genotype 1 virus is likely to be DAA + PEG-IFN α -2b + RBV combination therapy (the "3-drug therapy"). However, it has been reported that this therapy causes side effects such as severe hemoglobin reduction or severe rash, and many side effects in elderly patients.

Zinc supplementation to reduce such side effects may be the key to developing more effective anti-viral therapies. As this paper has suggested, the administration of zinc in many clinical cases requires further study. Prospective double-blind studies with large sample sizes are necessary.

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Gastroenterostoma after Billroth antrectomy as a premalignant condition

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special interest in Eastern European countries, where surgery for benign gastroduodenal ulcers has remained a practice for a much longer time than in Western Europe, and therefore GSC is found with higher frequency.

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Abstract

Gastric stump carcinoma (GSC) following remote gastric surgery is widely recognized as a separate entity within the group of various types of gastric cancer. Gastrectomy is a well established risk factor for the development of GSC at a long time after the initial surgery. Both exo- as well as endogenous factors appear to be involved in the etiopathogenesis of GSC, such as achlorhydria, hypergastrinemia and biliary reflux, Epstein-Barr virus and *Helicobacter pylori* infection, atrophic gastritis, and also some polymorphisms in interleukin-1 β and maybe cyclo-oxygenase-2. This review summarizes the literature of GSC, with special reference to reliable early diagnostics. In particular, dysplasia can be considered as a dependable morphological marker. Therefore, close endoscopic surveillance with multiple biopsies of the gastroenterostomy is recommended. Screening starting at 15 years after the initial ulcer surgery can detect tumors at a curable stage. This approach can be of

INTRODUCTION

The first partial gastrectomy with gastroduodenostomy was performed by Billroth in 1881 and was followed by the first gastrojejunostomy 3 years later. Both procedures became known as Billroth I and II, respectively^[1]. Although the famous Viennese surgeon Theodor Billroth is credited for the first gastric resection, known as the Billroth I procedure, the less well-known Ludwik Rydygier from Poland, performed and described the procedure several months earlier^[2]. Particularly in Europe, the Billroth II resection became the most popular treatment for peptic ulcer disease until the mid 1970s^[3]. Since then, a significant decrease of peptic ulcer surgery was seen due to the introduction of H₂-receptor antagonists^[4], and later the proton pump inhibitors^[5]. In the early 1980s, the discovery of *Helicobacter pylori* (*H. pylori*)^[6] as the main cause of peptic ulcer disease further diminished the role of surgery in the treatment of this disease^[7-12]. Surgical

treatment for uncomplicated peptic ulcer disease became rare, but operations for complications of peptic ulcer disease such as perforation, bleeding or gastric outlet obstruction are still regularly performed. The rise of the use of nonsteroidal anti-inflammatory drugs explains part of this occurrence^[13-16]. In Eastern Europe, the prevalence of surgery for benign gastroduodenal ulcers remained higher for a longer time than in Western Europe. In Poland for example, several thousand complicated as well as chronic peptic ulcer patients were still operated upon^[17-20], and there, the introduction of antisecretory drugs occurred in the late 1980s^[21]. Nevertheless, complications of peptic ulcer surgery will presumably become less important there as a public health problem^[22].

HISTOPATHOLOGY

Billroth antrectomy and its various modifications remove the part where the ulcer is located and that contains the gastrin-producing antral mucosa responsible for the stimulation of acid production through the oxyntic mucosa. It also induces biliary reflux, felt to be beneficial for healing due to its alkaline contents. The majority of patients with peptic ulcer disease will have an antrum-predominant *H. pylori* gastritis^[23,24]. The biliary reflux creates a microenvironment that is not suitable for *H. pylori* and it will eradicate the microorganisms from the anastomosis after surgery. The microscopy of the anastomosis will therefore change from the chronic active *H. pylori* gastritis picture into that of the typical reflux gastritis. The most important features of reflux gastritis are foveolar hyperplasia, congestion, paucity of inflammatory infiltrate, reactive epithelial change and smooth muscle fiber proliferation. These changes are already apparent shortly after surgery; less so when a Roux-en-Y conversion is carried out to avoid reflux^[25,26]. The picture is therefore directly related to the reflux of bile, as is the eradication of *H. pylori* from the anastomosis.

In the long run, other microscopic features are encountered in the operated stomach^[27]. Loss of parietal cells with the subsequent disappearance of the chief cells introduces an accelerated mucosal atrophy that is caused by the lack of the trophic hormone gastrin and the vagotomy that is mostly done simultaneously. The specialized glandular mucosa is replaced by intestinal metaplasia and pseudopyloric metaplasia^[28-31]. Atrophy of the gastric mucosa may lead to vitamin B12 deficiency. At the site of the anastomosis, the glands often become cystically dilated, and sometimes these cystically dilated glands herniate through the muscularis mucosae. This provides a nodular aspect to the anastomosis and gives rise to a microscopic picture known as gastritis polyposis cystica or gastritis cystica profunda^[32-34]. Erosions may occur as a result of compromised vasculature due to the surgery, but in the case of persistent ulceration after surgery, Zollinger-Ellison-like syndrome or a retained antrum needs consideration and these conditions are accompanied by high gastrin levels. The retained antrum is caused

by resection that is too limited and G-cell hyperplasia in the stretch of antral mucosa left behind^[35,36].

Xanthelasma, also known as gastric xanthomas or gastric lipid islands, are aggregates of foamy macrophages filled with lipids that can be seen in the stomach and more often after partial gastrectomy^[37,38]. At endoscopy, they appear as grossly visible whitish nodules or plaques, well circumscribed, with a size varying from 1 to 10 mm in diameter^[39,40]. They typically occur along the lesser curve^[41], the so-called "magenstrasse" where generally reflux is most severe. It is felt that these aggregates phagocytose remnants of cellular debris after degradation due to chemical injury, and they are harmless. Their importance lies in the fact that these should not be confused with signet ring cell carcinoma, because the microscopy of xanthelasma can resemble signet ring cells. Special stains for mucin or immunohistochemistry for cytokeratins versus histiocytic macrophages makes this differentiation easy^[42,43]. Xanthelasma occur more frequently in stomachs harboring other pathological changes such as chronic gastritis, atrophic gastritis and intestinal metaplasia^[39,44]. The significance of these lesions remains unknown.

PREMALIGNANT CONDITION

The stump of the stomach after remote gastric resection because of benign ulcer disease is a well-defined premalignant condition. Many studies in the past have confirmed that, after remote partial gastrectomy, there is an increased risk for stomach cancer^[3,45-49]. GSC is defined as a malignancy of the stomach occurring > 5 years after initial partial gastrectomy, to confusion with cancer recurrence after initial misdiagnosis. The risk for stump cancer is remarkable because most of these patients suffer from peptic ulcer disease prior to surgery. The relation between peptic ulcer disease and gastric cancer is not fully understood. Gastric cancer and peptic ulcer disease are inversely associated and they are accompanied by distinct patterns of acid secretion^[50]; by contrast, gastric ulcers, non-peptic gastric ulcers, and gastric cancer partly share pathophysiological features^[51-53]. The part of the stomach that is at the highest risk for gastric cancer is removed by surgery. Nevertheless, with an increasing postoperative interval, there is a steadily increasing risk for stomach cancer in the gastric remnant. After more than 15-20 years postoperatively, the risk is higher than can be expected for an age- and sex-matched general population, and it rapidly increases thereafter^[45,48,54]. In line with the increased risk for stomach cancer, the post-gastrectomy stomach also harbors dysplasia relatively frequently^[29,55,56]. The dysplasia is typically encountered around the gastric anastomosis, and similarly the cancers are almost exclusively found there. Both the dysplasia and the cancers can be multifocal and extensive mapping of the mucosa with endoscopic biopsies is warranted. Unlike primary gastric cancer, which is frequently resectable (resectability rate in Poland: 66%), gastric stump carcinoma once detected be-

Table 1 IL-1 β -31T>C genotype in gastric stump cancer and in conventional gastric cancer *n* (%)

Genotype of IL-1 β -31T>C ¹	Gastric stump cancer	Conventional gastric cancer
CC	8/30 (27)	21/96 (22)
TC	19/30 (63)	36/96 (37)
TT	3/30 (10)	39/96 (41)
Presence of C allele	90	59

¹All percentages rounded to the nearest digit. IL: Interleukin.

Table 2 Prevalence of -765G>C COX-2 genotype *n* (%)

COX-2 -765 genotype ¹	Gastric stump cancer	Conventional gastric cancer	Early-onset gastric cancer	Controls
GG	23/30 (77)	73/96 (76)	80/115 (70)	59 (59)
GC	5/30 (17)	19/96 (20)	33/115 (29)	32 (32)
CC	2/30 (7)	4/96 (4)	2/115 (2)	9 (9)
Presence of C allele	23	24	30	41

¹All percentages rounded to the nearest digit.

cause of symptoms is virtually unresectable in the majority of patients. In view of the increased risk, surveillance of post-gastrectomy patients has been advocated and it can indeed detect cancer at an early and operable stage^[57]. Whether or not large-scale screening would be indicated is however questionable. In a screening program carried out in Amsterdam among > 500 patients > 15 years post-operatively, 10 cancers were found; six of which turned out to be early cancers^[56]. Mortality of stomach cancer among these screened individuals was, however, only marginally different from that among the patients who did not participate in the screening program, after an observation period of 10 years. Moreover, a similar difference in mortality was observed in lung cancer, suggestive of selection bias among the screened patients^[58].

ETIOLOGY

It is thought that after partial gastrectomy, an environment is created that is favorable for the development of cancer. Patients enter an accelerated neoplastic process due to the altered microenvironment. The stump carcinomas evolve as the end result of a series of mutagenic cell transformations, leading to stepwise tumor progression from atrophic gastritis with metaplasia *via* dysplasia to invasive carcinoma. During tumor progression an increased proliferation and expansion of the proliferative compartment towards the luminal surface is observed. This is accompanied by an increasing concomitant chance of mutations, because the proliferating cells are particularly vulnerable to mutation. Biliary reflux, achlorhydria, atrophic gastritis and formation of N-nitroso compounds are considered factors that contribute to carcinogenesis in the gastric stump^[3,59,60]. It has been shown that the changes in the quantity of the nitrate-reducing bacteria and in the N-nitrosamine concentration depend on the type of

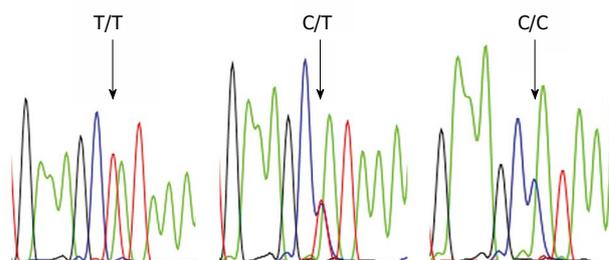


Figure 1 An example of the sequence analysis of the T/T, C/T and C/C interleukin-1 β -polymorphism.

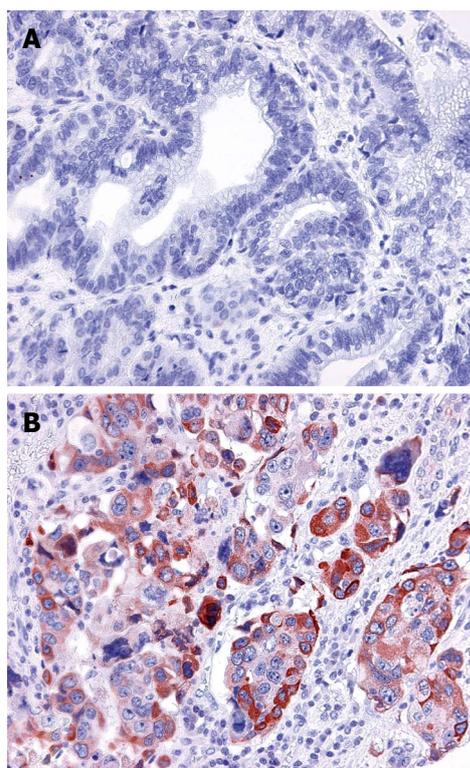


Figure 2 An example of cyclo-oxygenase-2 immunohistochemistry. A: Cyclo-oxygenase (COX)-2 low staining; B: COX-2 high staining.

surgical intervention performed. The highest content of nitrate-reducing bacteria and the N-nitrosamine concentration were found in the gastric juice of patients after Billroth II antrectomy, and the lowest values after highly selective vagotomy^[61].

Epstein-Barr virus (EBV) positivity by RNA *in situ* hybridization is seen more often in carcinomas in the gastric remnant than in the intact stomach^[62]. In contrast, *H. pylori* is less frequently observed^[63]. Interestingly, there is an inverse relationship between positivity for EBV and positive immunohistochemistry for TP53; also loss of heterozygosity of 17p at the locus of TP53 is less frequently observed in EBV-positive stump carcinomas^[64]. It has been reported that the EBV-encoded EBNA-5 protein can form a complex with TP53 and retinoblastoma proteins, and it is conceivable that this may lead to accel-

erated degradation of either one of these proteins^[65].

Endogenous factors may also play a role. The interleukin (IL)-1 β 31T>C polymorphism is associated with higher gastric cancer risk. This allele confers hypoacidity to the oxyntic mucosa of the *H. pylori*-infected intact stomach and it thereby induces a corpus-predominant inflammatory gastritis^[66,67]. The IL-1 β -31T>C polymorphism is also associated with GSC (Table 1 and Figure 1)^[68]. Apparently, the relatively few peptic ulcer disease patients carrying this genotype are at particularly high risk for the development of stomach cancer, after operation. Surgery has a similar effect as pharmacologically induced acid suppression; a fact that is known to lead from an antrum- to a corpus-predominant inflammatory picture in the gastric mucosa.

Cyclo-oxygenase (COX)-2 expression is increased in gastric stump cancer and the 765 G allele of COX-2 is associated with higher gastric cancer risk (Table 2 and Figure 2)^[69]. There is however no direct relationship between expression of COX-2 and the -765 C>G polymorphism in gastric stump cancer. This finding contrasts with observations in the duodenum of familial adenomatous polyposis patients, in whom this polymorphism is associated with increased COX-2 expression in the normal mucosa^[70].

CONCLUSION

Partial gastrectomy for benign peptic ulcer disease is a well-established premalignant condition. With increasing postoperative interval since the initial surgery, the risk steadily increases and after more than 15-20 years postoperatively, gastric cancer risk is higher than that of age and sex-matched controls with intact stomachs. The surgery itself and the resulting biliary reflux seem responsible for the risk. Endogenous factors such as polymorphisms in IL-1 β and COX-2 may contribute. EBV infection is more prevalent than in the intact stomach, and *H. pylori* infection less frequent. EBV may interact with the *TP53* gene. Stump cancer is preceded by well-defined preinvasive precursor lesions, most notably dysplasia. Dysplasia can be considered a dependable morphological marker, amenable for early detection by endoscopic surveillance.

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Mangiferin, a natural xanthone, accelerates gastrointestinal transit in mice involving cholinergic mechanism

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Abstract

AIM: To investigate the effects of mangiferin on gastrointestinal transit (GIT) in normal and constipated mice, together with the possible mechanism.

METHODS: Intragastrically-administered charcoal meal

was used to measure GIT in overnight starved Swiss mice. In the first experiments, mangiferin (3 mg/kg, 10 mg/kg, 30 mg/kg, and 100 mg/kg, *po*) or tegaserod (1 mg/kg, *ip*) were administered 30 min before the charcoal meal to study their effects on normal transit. In the second series, mangiferin (30 mg/kg) was tested on delayed GIT induced by several different pharmacological agonists (morphine, clonidine, capsaicin) or antagonists (ondansetron, verapamil, and atropine) whereas in the third series, mangiferin (30 mg/kg, 100 mg/kg and 300 mg/kg) or tegaserod (1 mg/kg) were tested on 6 h fecal pellets outputted by freely fed mice. The ratio of wet to dry weight was calculated and used as a marker of fecal water content.

RESULTS: Mangiferin administered orally significantly ($P < 0.05$) accelerated GIT at 30 mg/kg and 100 mg/kg (89% and 93%, respectively), similarly to 5-hydroxytryptamine₄ (5-HT₄) agonist tegaserod (81%) when compared to vehicle-treated control (63%). Co-administered mangiferin (30 mg/kg) totally reversed the inhibitory effect of opioid agonist morphine, 5-HT₃-receptor antagonist ondansetron and transient receptor potential vanilloid-1 receptor agonist capsaicin on GIT, but only to a partial extent with the GIT-delay induced by α_2 -adrenoceptor agonist clonidine, and calcium antagonist verapamil. However, co-administered atropine completely blocked the stimulant effect of mangiferin on GIT, suggesting the involvement of muscarinic acetylcholine receptor activation. Although mangiferin significantly enhanced the 6 h fecal output at higher doses (245.5 ± 10.43 mg vs 161.9 ± 10.82 mg and 227.1 ± 20.11 mg vs 161.9 ± 10.82 mg of vehicle-treated control, at 30 and 100 mg/kg, $P < 0.05$, respectively), the effect of tegaserod was more potent (297.4 ± 7.42 mg vs 161.9 ± 10.82 mg of vehicle-treated control, $P < 0.05$). Unlike tegaserod, which showed an enhanced water content in fecal pellets ($59.20\% \pm 1.09\%$ vs $51.44\% \pm 1.19\%$ of control, $P < 0.05$), mangiferin evidenced no such effect, indi-

cating that it has only a motor and not a secretomotor effect.

CONCLUSION: Our data indicate the prokinetic action of mangiferin. It can stimulate the normal GIT and also overcome the drug-induced transit delay, *via* a cholinergic physiological mechanism.

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Key words: Mangiferin; Glucosylxanthone; Gastrointestinal transit; Prokinetic action; Cholinergic mechanism

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INTRODUCTION

Dyspepsia and constipation are common gastrointestinal disorders of ageing populations, irritable bowel syndrome, and in chronic users of narcotic and non-narcotic analgesics^[1-5]. The high prevalence, economic consequences and decrement in health-related quality of life make these disorders a major public issue. Constipation remains a major gastrointestinal ailment with multiple symptoms and a diverse etiology. To get relief from chronic constipation, conventional treatments include the use of fiber supplements and laxatives, but the clinical data do not support the efficacy of these therapies^[6]. Even recently introduced drugs such as tegaserod [a 5-hydroxytryptamine₄ (5-HT₄) partial agonist] and lubiprostone (a chloride channel activator approved by the Food and Drug Administration) were not found to be clinically useful, due to intolerable side effects^[7,8]. Erythromycin, a non-peptide motilin receptor agonist, has been shown to induce phase 3 of the migrating motor complex in the antro-duodenum and cause an improvement in clinical symptoms of constipation^[9], but there was a report that it lacks colon prokinetic effect in children with chronic constipation^[10]. Although advances have been made in understanding gastrointestinal motility, visceral pain, mucosal inflammation, and tissue repair, the major gastrointestinal disorders like constipation remain significant therapeutic challenges^[11]. Therefore, there is a need for intense research to develop newer, well-tolerated and effective drugs to cure or to alleviate the symptoms of chronic constipation.

Dyspepsia is a highly prevalent condition characterized by symptoms originating in the gastroduodenal

region. Patients experience postprandial fullness, early satiation, epigastric pain, or burning in the absence of causative structural disease. These symptoms may coexist with irritable bowel syndrome, in which patients frequently complain of occasional bowel movement disorders, associated with abdominal pain or discomfort, but they are rarely due to an underlying organ involvement^[12]. Treatment options that may be beneficial for functional dyspepsia include histamine H₂ blockers, proton pump inhibitors, and prokinetic agents. However, most of the available treatments have only limited efficacy^[13,14]. Recent reports described the prokinetic and laxative effects of *Lepidium sativum* (garden cress) and *Aquilaria sinensis* (agarwood) in mice, which were partially mediated through a cholinergic pathway^[15,16]. Developing drugs from natural sources (plant extracts or plant-derived substances) may be a treatment option to combat symptoms associated with dyspepsia and constipation.

Mangiferin is a naturally occurring glucosylxanthone commonly encountered in several traditionally used medicinal plants^[17] that has been shown to exhibit multiple pharmacological effects that include antioxidant, anti-inflammatory^[18-20], and immunomodulatory activities^[21]. Diminutions in glutamate-induced neurotoxicity and memory enhancement effects of mangiferin have also been reported^[22,23]. Mango fruit is rich in mangiferin^[24] and, according to Nadkarni^[25], the ripe fruit is very wholesome, nourishing, and useful in nervous and atonic dyspepsia and constipation. We previously demonstrated that mangiferin affords gastroprotection against absolute ethanol or indomethacin-induced gastric ulceration through an antioxidant mechanism^[26]. Mangiferin also attenuated acidified ethanol-induced gastric damage in mice and this gastroprotective effect was accompanied by enhanced gastric emptying (unpublished observations from our laboratory) suggesting a likely prokinetic effect. In the light of these observations, the present study was aimed to verify a possible prokinetic effect of mangiferin on normal and delayed gastrointestinal transit, evoked by several different pharmacological agents in mice, and to analyze the underlying mechanism.

MATERIALS AND METHODS

Plant material and isolation of mangiferin

Mangiferin (Figure 1) used in this study was extracted and isolated from the bark of *Mangifera indica* L. (Anacardiaceae) as per procedures reported earlier^[26]. A voucher specimen (No. 32628) of the plant material authenticated by Dr. Francisco Edson de Paula has been deposited at the Herbário Prisco Bezerra of the Federal University of Ceará. The isolated mangiferin (MGF) was approximately of 95% purity^[20] having the molecular weight 422.5 and melting point (mp) 27 °C.

Animals and animal procedures

Swiss albino male mice (20-25 g) obtained from the Central Animal House of the Federal University of Ceará

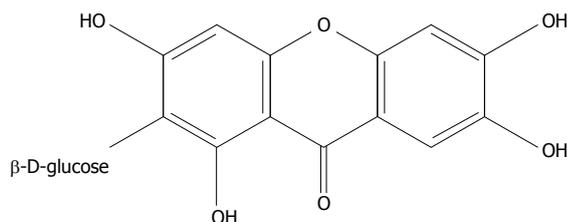


Figure 1 Chemical structure of mangiferin.

were used. They were housed in environmentally-controlled conditions ($23\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$, 12-h light-dark cycle), with free access to a standard diet (Purina Chow) and water *ad libitum*. They were kept in wire-mesh cages to prevent coprophagy. Mice were fasted for 15 h prior to the experiments, but allowed free access to water.

The experimental protocols were approved by the Animal Care and Use Committee of the Federal University of Ceará in accordance with the ethical guidelines of the International Association for the Study of Pain.

Chemicals

Capsazepine (Calbiochem, San Diego, California, United States), indomethacin, capsaicin, clonidine hydrochloride (Sigma Aldrich Co., St. Louis, MO, United States), and morphine (Cristália, Brazil) were used. All other chemicals used were of analytical grade. MGF was dissolved in 2% dimethylsulfoxide (DMSO) and further dilutions were made in distilled water. Drug concentrations were adjusted for treatment to give a volume of 10 mL/kg.

Gastrointestinal transit in mice

Gastrointestinal transit was measured using the charcoal propulsion test^[27]. In the first series of experiments, overnight fasted mice were used to establish the dose-response of mangiferin on gastrointestinal transit (GIT). These animals were distributed into 6 groups (eight in each): group 1 received 10 mL/kg vehicle (the diluent of mangiferin, 2% DMSO in distilled water) whereas groups 2, 3, 4 and 5 were treated orally with mangiferin in doses of 3 mg/kg, 10 mg/kg, 30 mg/kg or 100 mg/kg. The 6th group served as the positive control and was treated with tegaserod (1 mg/kg, *ip*). Thirty minutes following the treatments, each mouse was orally given 0.1 mL of charcoal meal (5% activated charcoal suspended in 10% aqueous gum Arabic). The animals were killed 20 min later by cervical dislocation, and the intestines were removed from the pylorus through the ileocecal junction. The extent of charcoal propulsion in the small intestine was measured (distance travelled by the charcoal (from pylorus to the most distal part of the small intestine) and expressed as follows:

Gastrointestinal transit (%) = distance travelled by the charcoal/total length of the small intestine \times 100.

In the second series of experiments, mice in groups (eight/group) were used to study the effect of mangiferin (30 mg/kg, *po*) on gastrointestinal transit delay caused by the opioid agonist morphine (2.5 mg/kg, *sc*), 5-HT₃-

receptor antagonist ondansetron (3 mg/kg, *ip*), transient receptor potential vanilloid 1 (TRPV1) agonist capsaicin (0.3 mg/kg, *po*), α_2 -adrenoceptor agonist clonidine (0.1 mg/kg, *ip*), calcium antagonist verapamil (5 mg/kg, *ip*), and cholinergic muscarinic antagonist atropine (3 mg/kg, *sc*). The effects of these drugs on GIT (administered 30 min before the charcoal meal test) alone, or their co-administration with mangiferin were established. To verify the specificity of the above drugs that cause transit delay, the effects of their corresponding agonists (serotonin 3 mg/kg, *sc*; calcium chloride 50 mg/kg, *ip*; and bethanechol 3 mg/kg, *ip*) or antagonists (naloxone 1 mg/kg, *ip*; yohimbine 2 mg/kg, *sc*; and capsazepine 5 mg/kg, *ip*) alone or in their combination (administered 15 min before) were observed on GIT. The dose selection of test drugs was based on our pilot studies and literature reports.

Fecal pellets output and water content

The third series of experiments were performed to determine whether the prokinetic action of mangiferin or 5-HT₄ agonist tegaserod was capable of propagating a prokinetic signal along the entire length of the gastrointestinal tract. With this aim, the treatment effects of mangiferin or tegaserod were verified on 6 h fecal pellets output and fluidity (water content) as per the method described earlier, with little modification^[28]. Briefly, mice in groups (eight in each) were treated with vehicle, mangiferin at oral doses of 30 mg/kg, 100 mg/kg and 300 mg/kg or tegaserod (1 mg/kg, *ip*) and then transferred to individual cages (19 cm \times 31 cm, lined with bright non-absorbent white paper) and monitored constantly over six hours. To prevent water absorption, no bedding was included in the observation cages; also, to minimize the risk of water evaporation and coprophagia, fecal pellets were collected at 1 h intervals. Fecal pellets were then weighed (wet weight, in mg), desiccated in an oven (50 $^{\circ}\text{C}$, 6 h), and weighed again (dry weight, in mg). Fecal water content was calculated according to the equation:

$$\text{Water content (\%)} = 100 (\text{wet weight} - \text{dry weight}) / \text{wet weight}.$$

Statistical analysis

Statistical analysis was performed by analysis of variance followed by Student Newman Keuls as *post-hoc* tests using GraphPadPrisma 4.0 (GraphPad Software, San Diego, CA, United States). The parametric data was expressed as mean \pm SEM. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Effect of mangiferin on gastrointestinal transit

Mangiferin significantly ($P < 0.05$) accelerated GIT at oral doses of 30 mg/kg and 100 mg/kg by 89% and 93%, respectively, compared with the vehicle control which showed 63% GIT (Figure 2). However, mangiferin response was not dose-related. On the other hand, tegas-

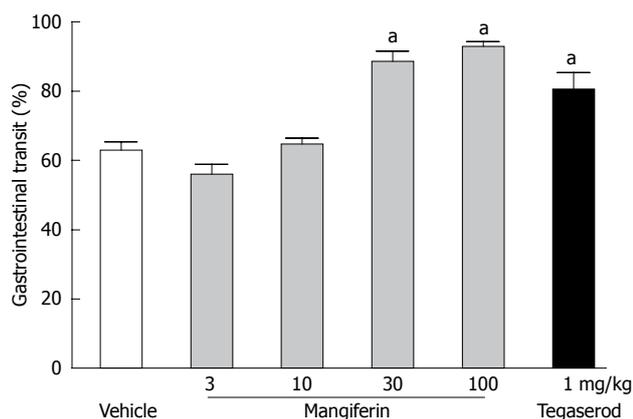


Figure 2 Effects of mangiferin and tegaserod on normal gastrointestinal transit. Each column represents mean \pm SEM ($n = 8$). ^a $P < 0.05$ vs vehicle control group.

erod, a known prokinetic included in the study as a positive control, stimulated GIT by 81%. We observed from pilot experiments that the vehicle (2% DMSO, 10 mL/kg, *po*) itself does not influence the GIT. Therefore, test drug responses on GIT were compared with the vehicle-treated control.

Effect of mangiferin on drug-induced gastrointestinal transit-delay

The opioid agonist morphine (2.5 mg/kg, *sc*), α_2 -adrenoceptor agonist clonidine (0.1 mg/kg, *ip*), 5-HT₃-receptor antagonist ondansetron (3 mg/kg, *ip*), calcium antagonist verapamil (5 mg/kg, *ip*), TRPV1 agonist capsaicin (0.3 mg/kg, *po*), and cholinergic muscarinic antagonist atropine (3 mg/kg, *sc*), all significantly ($P < 0.05$) delayed GIT by 42.4%, 63.2%, 32.9%, 50.7%, 28.7%, and 31%, respectively, when compared to corresponding vehicle-treated control transit values (Figures 3 and 4). These delayed transits were found to be effectively reversed in mice pretreated with respective antagonists (naloxone 1 mg/kg, *ip*; yohimbine 2 mg/kg, *sc*; and capsazepine 5 mg/kg, *ip*) or agonists (serotonin 3 mg/kg, *sc*; calcium chloride 50 mg/kg, *ip*; and bethanechol 3 mg/kg, *ip*). While co-administered mangiferin totally reversed the inhibitory effects of morphine, ondansetron and capsaicin on GIT, the transit delays caused by clonidine and verapamil were only partially reversed. However, co-administered atropine completely blocked the stimulant effect of mangiferin on GIT (Figure 5), suggesting the involvement of muscarinic acetylcholine receptor activation.

Effect of mangiferin on 6 h fecal pellets weight and water content

Table 1 shows the 6 h fecal pellets output and water content from freely fed mice treated with vehicle, mangiferin (30 mg/kg, 100 mg/kg and 300 mg/kg) or tegaserod (1 mg/kg). Six hours cumulative measurement of fecal mass output and water content in the vehicle-treated group was not significantly different from the normal control group.

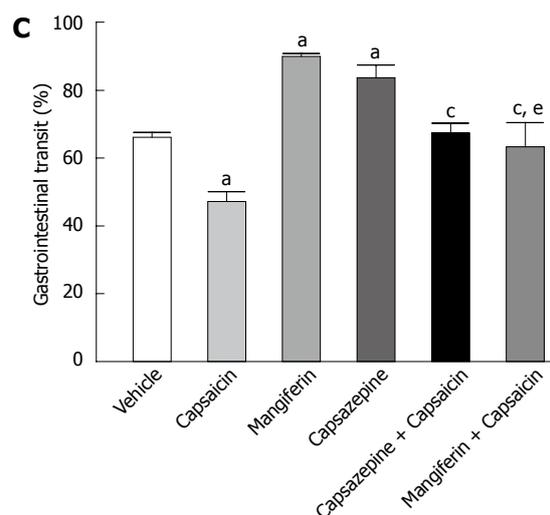
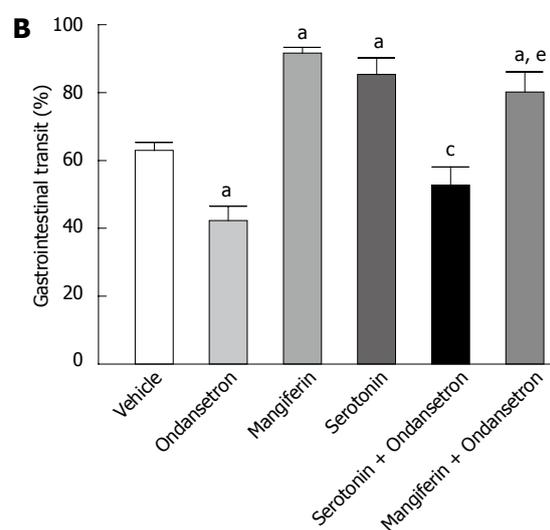
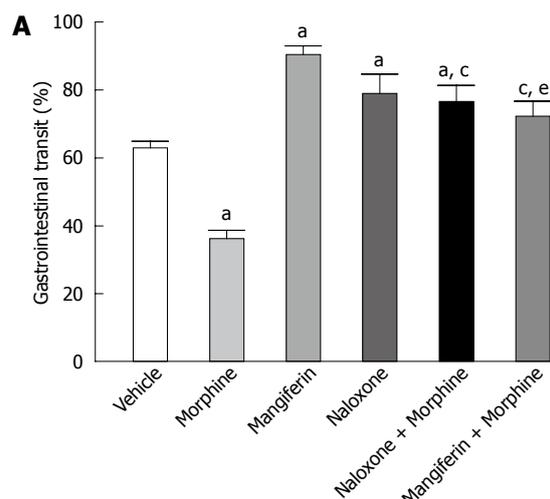


Figure 3 Effect of co-administered mangiferin on delayed gastrointestinal transit induced by morphine, ondansetron, and capsaicin in mice. A: Morphine (2.5 mg/kg, *sc*); B: Ondansetron (3 mg/kg, *ip*); C: Capsaicin (0.3 mg/kg, *po*) induced gastrointestinal transit-delay. Each column represents mean \pm SEM ($n = 8$). ^a $P < 0.05$ vs vehicle control group; ^c $P < 0.05$ vs respective morphine/ondansetron/capsaicin; ^e $P < 0.05$ vs mangiferin alone group.

However, mangiferin at doses of 100 mg/kg and 300

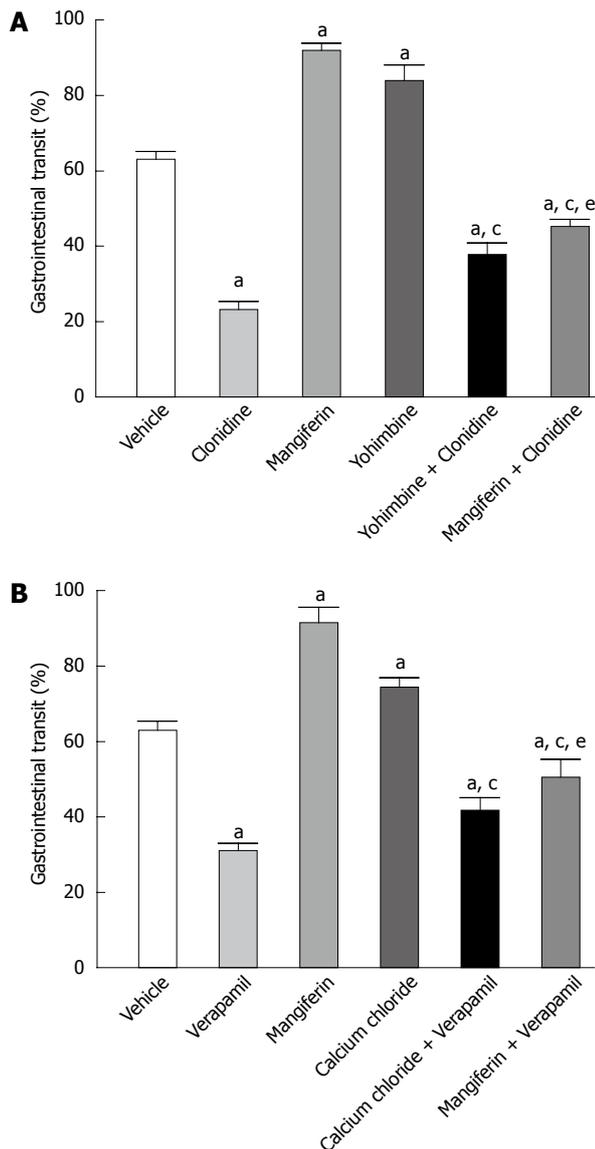


Figure 4 Effect of co-administered mangiferin on delayed gastrointestinal transit induced by clonidine and verapamil in mice. A: Clonidine (0.1 mg/kg, ip); B: Verapamil (5 mg/kg, ip) induced gastrointestinal transit-delay. Each column represents mean \pm SEM ($n = 8$). ^a $P < 0.05$ vs vehicle control group; ^c $P < 0.05$ vs respective clonidine/verapamil group; ^e $P < 0.05$ vs mangiferin alone group.

mg/kg, and tegaserod at 1 mg/kg significantly ($P < 0.05$) enhanced the fecal pellets output by 52%, 40%, and 80%, respectively, when compared to fecal output in vehicle-treated mice (161.9 mg). Fecal mass analysis from different treatment groups revealed that only the tegaserod-treated group evidenced significantly ($P < 0.05$) elevated water content (59%) relative to the vehicle-treated control (51%), suggesting that, unlike mangiferin, tegaserod possibly promotes ionic secretion.

DISCUSSION

In this study, we demonstrated the stimulant effect of mangiferin, a glucosylxanthone on GIT in mice using the charcoal meal test, a suitable preclinical model for quanti-

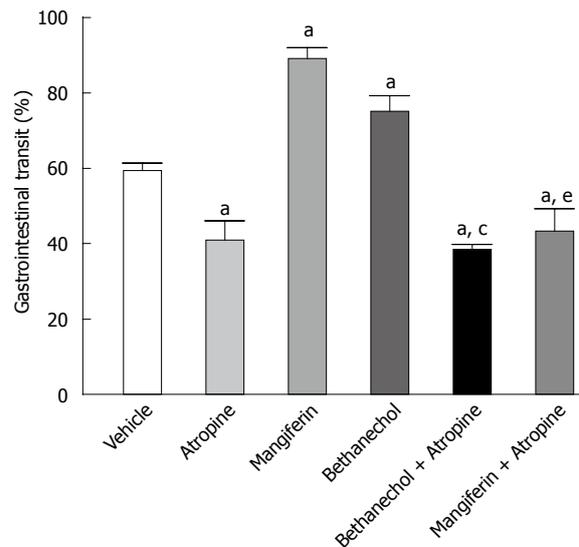


Figure 5 Effect of co-administered mangiferin on atropine-induced delayed gastrointestinal transit in mice. Each column represents mean \pm SEM ($n = 8$). ^a $P < 0.05$ vs vehicle control group; ^c $P < 0.05$ vs bethanechol alone group; ^e $P < 0.05$ vs mangiferin alone group.

Table 1 Effects of mangiferin and tegaserod on 6 h cumulative fecal pellet wet weight and water content in freely fed mice

Group	Dose	Fecal pellets wet weight (mg)	Water content (%)
Control (normal)	-	156.8 \pm 20.98	50.46 \pm 2.28
Control (vehicle)	-	161.9 \pm 10.82	51.44 \pm 1.19
Mangiferin	30 mg/kg, po	205.7 \pm 12.69	50.31 \pm 1.61
	100 mg/kg, po	245.5 \pm 10.43 ^{a,c}	49.76 \pm 3.25
	300 mg/kg, po	227.1 \pm 20.11 ^{a,c}	49.11 \pm 3.01
Tegaserod	1 mg/kg, ip	297.4 \pm 7.42 ^{a,c}	59.20 \pm 1.09 ^{a,c}

The results are expressed as mean \pm SEM of 8 animals per group. Statistical comparison was performed using analysis of variance followed by Student Newman Keuls test. ^a $P < 0.05$ vs control (normal) group; ^c $P < 0.05$ vs control (vehicle) group.

fication of changes in GIT and to analyze the effects of test drugs on gastro-intestinal motility^[28,29]. However, the present experiment is not completely suitable to demonstrate the effect on constipation, because the charcoal test measures gastric emptying and small intestinal transit, rather than the colonic transit, that is important for constipation. Mangiferin accelerated normal GIT and showed an oral efficacy at doses of 30 mg/kg and 100 mg/kg. Our principal aim in this study was to verify whether mangiferin can exert a prokinetic action that could help to alleviate constipation, a major gastrointestinal functional disorder affecting 12%-17% of the general population in North America and elsewhere^[30,31]. Prokinetic drugs share the common characteristic of accelerating GI motility and they appear to be more effective than a placebo in the treatment of functional dyspepsia^[32], wherein constipation is a common feature.

Prokinetics exert their physiological actions through effects on a variety of neurotransmitter receptors in-

cluding acetylcholine, dopamine, motilin and serotonin. Therefore, to characterize mangiferin as a prokinetic, we tested its ability to revert the delayed GIT induced by several pharmacological agents which act by different action mechanisms. The results show that mangiferin accelerates normal GIT as well as the delayed GIT promoted by different pharmacological agents, indicating its prokinetic action *in vivo*. Co-administered mangiferin (30 mg/kg) effectively suppressed the GIT-delay induced by opioid receptor agonist morphine, 5-HT₃-receptor antagonist ondansetron, or TRPV1-receptor agonist capsaicin. This implies that mangiferin's action is non-specific and likely to combat constipation associated with therapeutic or surgical interventions. Patients with chronic pain during daily opioid therapy are frequently burdened with symptoms of constipation^[5]. It is estimated that up to 81% of patients still report constipation despite regular use of laxatives. Studies by Iwata *et al*^[33] reveal that morphine inhibits small intestinal transit, causing tonic contraction of the ileal circular muscle *via* inhibition of the nitrenergic pathway in mice in a naloxone reversible manner. Also, mangiferin administered orally at a similar dose (30 mg/kg) significantly prevented morphine-induced delay in gastric emptying in Wistar rats (unpublished observations from our laboratory).

In the present study we found that systemic administration of clonidine in mice inhibits GIT in mice, consistent with earlier reports^[34,35]. This inhibitory effect of clonidine was significantly inhibited by pre-administration of yohimbine, an α_2 -adrenoceptor antagonist, or by co-administration of mangiferin. However, the reversal of clonidine's effect on GIT delay was incomplete and did not attain the levels of normal transit. Clonidine by its central sedative effect might have contributed to greater depression of GIT.

Prokinetic drugs either directly or indirectly stimulate smooth muscle function, activating intrinsic primary afferent neurons (IPANs) located in the myenteric plexus that play important roles in controlling gastric emptying and small- and large-intestinal transit^[36]. The release of acetylcholine from these neurons is amplified by presynaptic serotonin 5-HT₄ receptor activation or blockade of 5-HT₃ receptors^[37]. Thus, prokinetic drugs like cisapride, mosapride and tegaserod, which have dual action (i.e., induce both 5-HT₄ receptor agonistic and 5-HT₃ receptor blockade effects), have gained clinical importance for the treatment of functional dyspepsia and chronic constipation^[38]. Although the greatest interest has been focused on the development of serotonergic drugs as prokinetics^[39], conflicting data currently exists on the efficacy of mixed 5-HT₄ agonist and 5-HT₃ antagonist drugs, like cisapride, mosapride and tegaserod, for the treatment of constipation^[7], attention has been now drawn to discover alternatives for the treatment of constipation. Interestingly, mangiferin was able to overcome the delayed GIT induced by several pharmacological agents suggesting an action similar to mosapride, a commonly used prokinetic drug and recently described prokinetic,

and the laxative effects of lepidium sativum in mice, which act *via* a mechanism that facilitates cholinergic neurotransmission^[40].

In rodents, the cholinergic stimulant bethanechol acts not only in the enterocytes, but also in submucosal nerves^[41], and since the effects on ion transport were nerve-dependent we decided to investigate the GIT stimulating effect of mangiferin in comparison with bethanechol in atropinized animals. The results show that atropine, the cholinergic antagonist, blocks the stimulatory effects of both mangiferin and bethanechol suggesting that these drugs, by specifically activating cholinergic muscarinic receptors, could induce Ca²⁺ mobilization in the submucosal neurons, thereby stimulating gut motility and secretion. The part played by calcium in mangiferin's effect is further supported by our results with verapamil, a calcium channel blocker that could also produce an effective blockade. In this study, while atropine totally blocked the prokinetic action of mangiferin, only a partial blockade was observed with clonidine, an α_2 -adrenoceptor agonist, and verapamil, a voltage dependent calcium channel blocker, suggesting that participation of muscarinic cholinergic receptors could play a major role in the stimulant effect of mangiferin on GIT.

Interestingly, mangiferin, besides accelerating GIT in normal as well as in constipated mice, could enhance the fecal output (but not the water content) in fecal mass. In contrast, fecal pellets produced by tegaserod-treated mice had higher water content than vehicle- or mangiferin-treated mice, presumably due to its greater secretomotor action on the colon. The increase in water content is indicative of augmented secretory activity, which might be the reason for its diarrhea-inducing side effect when used to control constipation^[42]. Thus mangiferin appears to have a safer prokinetic profile than tegaserod, since it has no diarrhea inducing effect.

In conclusion, this study has shown that mangiferin, a glucosyl xanthone ameliorate, delayed gastrointestinal transit induced by several pharmacologic agents *via* cholinergic mechanism, an action similar to that of some prokinetic drugs. Our study suggests that mangiferin could be an alternative to available prokinetic drugs for the treatment of functional gastrointestinal disturbances such as dyspepsia and postoperative ileus.

ACKNOWLEDGMENTS

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COMMENTS

Background

Dyspepsia and constipation are functional gastrointestinal ailments with diverse etiology and symptoms which adversely influence the health-related quality of life. Treatment modalities include the use of fiber supplements, laxatives and prokinetic agents, but the clinical data do not support the efficacy of these therapies. Therefore, there is a need for intense research to develop newer well-tolerated and effective drugs for a cure or symptom-alleviation for dyspepsia

and constipation.

Research frontiers

Mangiferin is a natural polyphenolic compound found in several traditional medicinal plants, including *Mangifera indica* (mango). The mango fruit contains mangiferin and is very wholesome, nourishing and useful in nervous and atonic dyspepsia and constipation. Mangiferin may be a treatment option to combat symptoms associated with dyspepsia and mild to moderate constipation.

Innovations and breakthroughs

In the present study, the authors showed that mangiferin is remarkably effective as a prokinetic agent *via* a cholinergic mechanism in the upper gastrointestinal tract. Mangiferin could enhance gastrointestinal transit and also increase fecal output in normal and physiological situations.

Applications

The study results suggest that mangiferin is a promising lead compound for the treatment of functional gastrointestinal disturbances such as dyspepsia and light to moderate constipation.

Terminology

Dyspepsia: a functional gastrointestinal disorder wherein patients experience postprandial fullness, early satiation, epigastric pain, or burning in the absence of causative structural disease; Constipation: a functional problem of the gastrointestinal tract, with symptoms including hard stools, straining during defecation, and a sense of incomplete evacuation. Treatment options include use of fiber supplements, and prokinetics/laxatives to relieve these functional disorders; Prokinetics: an important class of medicinal products for the treatment of all clinical forms of dyspepsia/moderate constipation that promote gastric emptying and intestinal transit; Mangiferin: a naturally-occurring xanthone glucoside present in many medicinal plants, which has antioxidant and anti-inflammatory actions.

Peer review

The authors performed an experimental study aimed at evaluating the effects of mangiferin in comparison with tegaserod on normal and pharmacologically constipated mice, along with investigating the possible mechanism, and found that mangiferin has a prokinetic action *via* a cholinergic mechanism. This paper is sufficiently well done and most interesting.

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Predictive value of ^{18}F -fluorodeoxyglucose PET/CT for transarterial chemolipiodolization of hepatocellular carcinoma

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Abstract

AIM: To investigate the correlation of ^{18}F -fluorodeoxyglucose (^{18}F -FDG) positron emission tomography (PET) with clinical features and the prediction of treatment response.

METHODS: A total of 83 hepatocellular carcinoma (HCC) patients undergoing ^{18}F -FDG PET before transarterial chemolipiodolization with systemic chemo-infusion between October, 2006 and May, 2009 were retrospec-

tively enrolled. The patients included 68 men and 15 women (mean age, 60 ± 10.7 years). The effect of ^{18}F -FDG-monitored PET uptake on clinical features and on the evaluated treatment response was ascertained with modified Response Evaluation Criteria in Solid Tumors. The PET parameters of maximal standardized uptake value of the tumor (T_{SUVmax}), the ratio of the tumor maximal standardized uptake value (SUV) to the liver maximal SUV ($T_{\text{SUVmax}}/L_{\text{SUVmax}}$) and the ratio of tumor maximal SUV to the liver mean SUV ($T_{\text{SUVmax}}/L_{\text{SUVmean}}$) were tested as predictive factors.

RESULTS: Among the 3 SUV parameters, the $T_{\text{SUVmax}}/L_{\text{SUVmean}}$ ratio (cutoff value of 1.90) was significantly associated with tumor burden including tumor size, tumor number, α -fetoprotein levels and tumor stage ($P < 0.001$, $P = 0.008$, $P = 0.011$, $P < 0.001$, respectively). The objective response rates in patients with a high SUV ratio (≥ 1.90) were significantly better than those with a low SUV ratio (< 1.90) ($P = 0.020$). The overall survival rates of patients exhibiting a low $T_{\text{SUVmax}}/L_{\text{SUVmean}}$ ratio (< 1.90) and those with a high SUV ratio (≥ 1.90) was 38.2 and 10.3 mo, respectively ($P < 0.01$). However, the time to progression showed no significant difference between the groups ($P = 0.15$).

CONCLUSION: ^{18}F -FDG PET can be an important predictor of HCC treatment. In particular, the $T_{\text{SUVmax}}/L_{\text{SUVmean}}$ ratio (cutoff value of 1.90) can provide useful information in treatment prognosis for HCC patients treated with locoregional therapy.

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Key words: ^{18}F -fluorodeoxyglucose positron emission tomography; Transarterial chemolipiodolization with systemic chemo-infusion; Treatment response; Predictive factor; Overall survival

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy, with an increasing incidence worldwide^[1], and the third most common cause of cancer related death^[2]. Surveillance programs have been implemented for cirrhotic patients. However, curative therapies such as resection or transplantation can be applied to fewer than 30% of HCC patients^[3], because most are diagnosed at an intermediate-to-advanced stage of the disease. The prognosis of HCC patients remains poor, and life expectancy is difficult to predict because of variable factors that include tumor burden and liver reserve function^[4]. Thus, it is important to assess the aggressive nature and metabolic change in HCC because this information is valuable in predicting the treatment response and in aiding in the selection of treatment modalities. One approach used to assess the biological activity of a tumor is positron emission tomography (PET).

^{18}F -fluorodeoxyglucose (FDG) PET is an imaging modality that can gauge the glucose metabolism of tumors, which has been established as a useful diagnostic tool for evaluating extrahepatic metastasis^[5]. However, ^{18}F -FDG PET has limitations in its ability to detect primary HCC because of variable F-FDG uptake in HCC^[6]. Nonetheless, PET monitored FDG uptake has the potential to be an additional tool for assessing biological behavior in HCC^[7,8]. The difference in uptake between a tumor and the liver can be expressed as a standardized uptake value (SUV) ratio ($\Gamma/\text{L}_{\text{SUV}}$), which is associated with prognostic aspects of tumor aggressiveness such as differentiation grade, tumor size/number and tumor recurrence in liver transplantation^[8]. It is feasible that FDG uptake, similar to tumor size/number and vascular invasion, may be of value in the prognosis of the treatment response in HCC^[9,10]. However, the usefulness of ^{18}F -FDG PET has not been investigated in the prediction of treatment response for HCC by transarterial chemolipiodolization (TACL) with systemic chemo-infusion.

The present study was undertaken to evaluate whether FDG uptake in HCC correlated with tumor characteristics and to determine, which PET/computed tomography (CT) parameter is especially important as a

predictor of treatment response. Finally, the probability of prognostic ability of ^{18}F -FDG PET for the prediction of treatment response in HCC was assessed.

MATERIALS AND METHODS

Patients

A total of 83 HCC patients, who were aged > 18 years were retrospectively selected and analyzed. Patients included 68 men and 15 women (mean age, 60 ± 10.7 years). All patients had undergone ^{18}F -FDG PET within 3 d before treatment between October, 2006 and May, 2009. The median duration of follow-up was 10.3 mo (range, 1.8-35.7 mo). The eligibility criteria were as follows: no previous transarterial chemo embolization, chemotherapy or radiotherapy; a confirmed diagnosis of HCC according to the American Association for the Study of Liver Disease criteria; an Eastern Cooperative Oncology Group performance status of 0 or 1; and preserved liver function (Child-Pugh class A or B). Patients with potentially resectable or ablative lesions who were high risk for surgery and radiofrequency ablation were also enrolled. Exclusion criteria included any extrahepatic metastasis, another primary tumor, advanced liver disease (bilirubin levels > 3 mg/dL, and a level of aspartate aminotransferase (AST) or alanine transaminase (ALT) > 5 \times the upper limit of normal). The study was approved by the institutional Ethics Review Board and was in compliance with the Declaration of Helsinki.

Treatment methods

The treatment regimen was a combination of intra-arterial epirubicin (50 mg/m^2) and/or cisplatin (60 mg/m^2) in a mixture of lipiodol (5-10 mL) without gelform embolization, and received an additional systemic infusion of 5-fluorouracil (200 mg/m^2) after completing the transarterial chemolipiodolization^[11,12]. The dose or treatment interval was modified whenever any treatment-related toxicity was encountered. Based on our previous report, the following formula for dosage modification was derived: administration dosage = $D \times \text{body surface area (BSA)} \times M$ where D is initial dosage of each agent, BSA is body surface area, and M is the modification rate = $(\text{white blood cell count}/4000) \times [1 - (\text{age} - 45)/100] \times [1 - (\text{Child-Pugh score} - 5)/10]$. According to this formula, the administration dosage of each chemotherapeutic agent was modified. Only the Child-Pugh score was calculated using this formula if the patient's white blood cell count was > $4000/\text{mm}^3$ or the patient's age was < 45 years^[13].

Follow-up imaging and laboratory tests including α -fetoprotein, albumin, bilirubin, AST, ALT and prothrombin time, were performed 4 wk after treatment. Repeat treatment was scheduled within 4 wk after follow-up imaging if there was residual viable tumor.

All CTs were performed with a 64-slice multidetector CT (Siemen Somatom Sensation 64, Munich, Germany). CT examination was performed using a 4-phase protocol

including a non-enhanced acquisition. Arterial phase (delay 20-30 s), portal venous phase (delay 60 s) and delayed venous phase (delay 80 s) were obtained using 120 mL of contrast (Iopromide 300 mg I/L, Schering, Germany) at a rate of 4 mL/s. The images were acquired with a slice thickness of 5 mm.

Treatment response

Treatment response was evaluated at 1 mo after receiving 3 sessions of TACL by modified Response Evaluation Criteria in Solid Tumors^[14]. In the modified criteria of the tumor response for HCC, complete response (CR) is the disappearance of any intratumoral arterial enhancement in all lesions; partial response (PR) is at least a 30% decrease in the sum of the diameters of viable (contrast enhancement in the arterial phase) lesions; progressive disease (PD) is an increase of at least 20% in the sum of the diameters of viable lesions; stable disease (SD) denotes any cases that do not qualify for either PR or PD^[15]. Objective response (OR) included CR and PR.

Evaluation of tumor characteristics according to treatment response

For the analysis of treatment response, all images were retrospectively assessed based on consensus by two attending radiologists. The imaging parameters including tumor size and number, portal vein thrombosis and Barcelona Liver Clinic Cancer (BCLC) stage was also evaluated. According to OR, HCC patients were categorized into an objective response group and a non-response group. The clinical features including the $T_{suvmax}/L_{suvmean}$ ratio were evaluated in both groups. Treatment response according to SUV ratio was analyzed based on stage.

¹⁸F-fluorodeoxyglucose- PET/CT

All patients fasted for at least 6 h prior to the PET/CT study. ¹⁸F-FDG (370-555 MBq) was injected intravenously, and scanning began 60 min later. None of the patients had blood glucose levels > 130 mg/dL before the injection. No intravenous contrast agent was administered. Data were acquired using a combined PET/CT in-line system, Biograph Turepoint (Siemens Medical Solutions, Knoxville, TN). The acquisition time was 2-3 min per bed position during PET/CT scanning. Precontrast CT began at the orbitomeatal line and progressed to the proximal thigh (130 kVp, 80 mAs, and 5 mm slice thickness; 120 kVp, 50 mAs, and 5 mm slice thickness). The PET scan followed immediately over the same body region. The CT data were used for attenuation correction, and images were reconstructed using a standard ordered-subset expectation maximization algorithm. The axial spatial resolution was 4.5 mm or 6.5 mm at the center of the field of view.

To evaluate ¹⁸F-FDG uptake, the region of interest (ROI) was drawn for each tumor, and the normal liver and measured standardized uptake value in each ROI were determined. The ROI was drawn to encircle the highest activity of each tumor. For normal liver regions, two cir-

cular 1.5 cm-diameter ROIs were drawn in both lobes. All tumor and non-tumor regions were defined by correlation with diagnostic CT undergone within 3 d. The maximum SUV (SUV_{max}) was measured in each ROI, and mean SUV (SUV_{mean}) was measured in each normal-liver ROI.

Statistical analysis

To evaluate the usefulness of ¹⁸F-FDG PET, calculated parameters included the following: the SUV_{max} of tumor (T_{suvmax}), the ratio of tumor SUV_{max} to liver maximal SUV (T_{suvmax}/L_{suvmax}), and the ratio of tumor SUV_{max} to liver mean SUV ($T_{suvmax}/L_{suvmean}$). The predictive value of each factor for the treatment response was analyzed based on analysis of the area under the receiver-operating-characteristic curve. After determination of the most effective ¹⁸F-FDG PET predictive factor, this parameter was compared with other prognostic factors, including tumor size and number, portal vein thrombosis, serum α -fetoprotein (AFP) and BCLC stage. The significance of the prognostic value was analyzed with Mann-Whitney and Fisher's exact tests in a univariate analysis and by logistic regression testing in a multivariate analysis. A value of $P < 0.05$ was considered significant (SPSS 16, IL, Chicago).

RESULTS

Clinical characteristics of the HCC patients

The patients included 68 men and 15 women. The average age was 60 ± 10.7 years and hepatitis B virus infected patients were 78%. 85.5% of the patients had Child-Pugh class A liver function. The mean values of tumor number and size were 2.2 ± 1.6 cm and 7.5 ± 5.0 cm, respectively. The BCLC stage of the patients consisted of stage A ($n = 26, 31.3\%$), B ($n = 20, 24.0\%$) and C ($n = 37, 44.5\%$).

Predictive values of ¹⁸F-fluorodeoxyglucose-PET parameters of objective response

The median values of T_{suvmax} , T_{suvmax}/L_{suvmax} and $T_{suvmax}/L_{suvmean}$ were 4.03 (1.5-20.8), 1.36 (0.77-7.64) and 1.82 (0.96-10.79), respectively. The area under the curve of $T_{suvmax}/L_{suvmean}$ was the highest on the receiver-operating-characteristic curve (Figure 1). The cutoff value of T_{suvmax} , T_{suvmax}/L_{suvmax} and $T_{suvmax}/L_{suvmean}$ was 4.0, 1.45 and 1.90, respectively. The cutoff level of $T_{suvmax}/L_{suvmean}$ was used as the effective parameter of ¹⁸F-FDG PET in the prediction of an objective response to HCC treatment. The tumor characteristics of the 83 patients according to the cutoff value of the $T_{suvmax}/L_{suvmean}$ ratio are summarized in Table 1. Forty patients displayed a $T_{suvmax}/L_{suvmean} \geq 1.90$, and the other 43 patients showed a $T_{suvmax}/L_{suvmean} < 1.90$. Two examples of FDG uptake according to $T_{suvmax}/L_{suvmean}$ ratio are shown in Figure 2. The number and size of the tumors, portal vein thrombosis, serum AFP, and BCLC stage in patients with $T_{suvmax}/L_{suvmean} (\geq 1.90)$ indicated significantly more poor prognostic characteristics than in the other patient group.

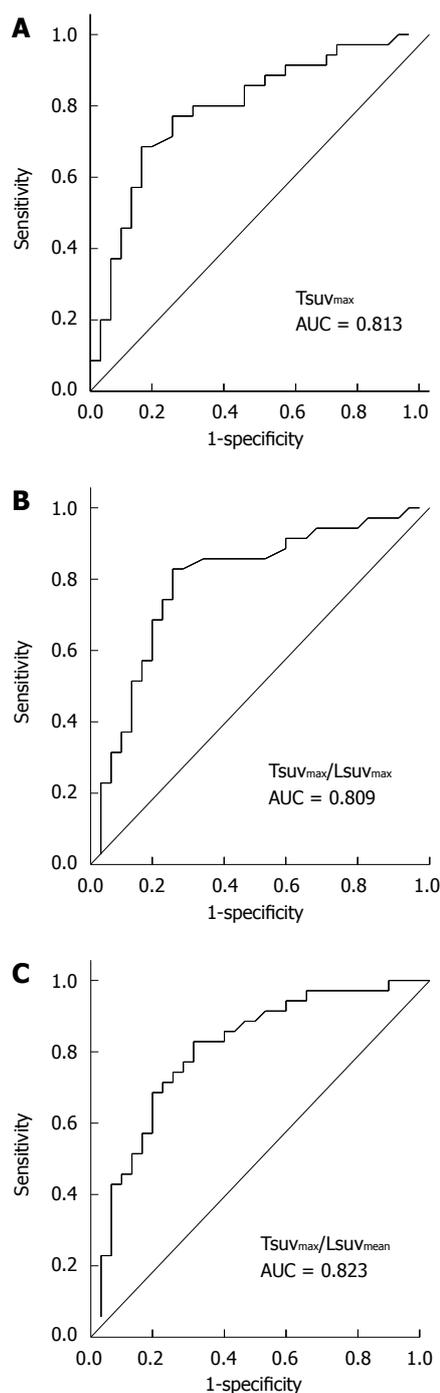


Figure 1 Predictive value of parameters on ¹⁸F-fluorodeoxyglucose positron emission tomography. $TsuV_{max}/LsuV_{mean}$ (C) demonstrated the highest area under curve based on the receiver-operating-characteristic curve analysis compared to $TsuV_{max}$ (A), and $TsuV_{max}/LsuV_{max}$ (B). AUC: Area under curve.

Tumor characteristics according to objective response

CR was observed in 29 (34.9%) of the 83 patients; PR, SD and PD was observed in 16 (19.3%), 20 (24.1%) and 18 (21.7%) patients, respectively. Objective response rates were different above (77.7%) and below (23.6%) the 1.90 cutoff value of $TsuV_{max}/LsuV_{mean}$ ($P < 0.001$).

According to treatment response, HCC patients were categorized into an objective response group and a non-response group. The clinical features associated with ob-

Table 1 Tumor characteristics according to level of $TsuV_{max}/LsuV_{mean}$			
	$TsuV_{max}/LsuV_{mean} < 1.90$ (n = 43)	$TsuV_{max}/LsuV_{mean} \geq 1.90$ (n = 40)	P value
Mean age \pm SD (yr)	60 \pm 11.7	59.9 \pm 9.7	0.276
Sex (male:female)	34:9	34:6	0.574
Etiology			0.802
HBV/HCV/ alcohol/others	35/4/1/3	34/5/0/1	
Tumor number	1.8 \pm 1.3	2.7 \pm 1.8	0.008
Single/multiple			0.008
Tumor size (cm)	5.2 \pm 3.2	10 \pm 5.5	0.000
< 3 cm	14	1	0.000
3-5 cm	11	8	
> 5 cm	18	31	
Portal vein thrombosis			0.000
Absent/present	39/4	18/22	
Child-Pugh class			0.758
A/B	36/7	35/5	
Serum AFP (ng/dL)	2928.5 \pm 11573.6	35275.2 \pm 103428	0.011
< 400/> 400	30/13	18/22	0.028
BCLC stage			0.000
A/B/C	21/13/9	7/28/2005	

HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFP: Serum α -fetoprotein; BCLC: Barcelona clinic liver cancer score.

jective response are shown in Table 2. Tumor size, portal vein thrombosis, BCLC stage and $TsuV_{max}/LsuV_{mean}$ ratio showed significant differences and were also determined as prognostic factors in univariate analysis. However, in the multivariate analysis, $TsuV_{max}/LsuV_{mean}$ was the only significant factor for objective response (Table 3).

The treatment response according to the $TsuV_{max}/LsuV_{mean}$ ratio was analyzed based on BCLC stage (Table 4). Treatment response showed a significant difference on BCLC stage B and C ($P = 0.048$ and $P < 0.001$, respectively). These data implicated the $TsuV_{max}/LsuV_{mean}$ ratio as being associated with HCC in more than the intermediate stage.

Overall survival rates and $TsuV_{max}/LsuV_{mean}$ ratio

During follow-up, 31 of the 83 patients (37.3%) died. The median survival time was 416 d (range, 55-1221 d). The overall survival rates of patients exhibiting a low $TsuV_{max}/LsuV_{mean}$ ratio (< 1.90) and those with a high SUV ratio (≥ 1.90) was 38.2 mo and 10.3 mo, respectively. The survival curve is shown in Figure 3. The cumulative survival rates at 6 mo, 12 mo, and 24 mo were 91%, 88%, and 75% in patients with low SUV ratio and 63%, 42%, and 33% in those with high SUV ratio. The patients exhibiting a low SUV ratio (< 1.90) survived significantly longer than those with a high SUV ratio (≥ 1.90). In addition, tumor size ($P = 0.006$), number ($P < 0.001$), portal vein thrombosis ($P < 0.001$), AFP ($P = 0.016$) and BCLC stage ($P < 0.001$) were related to overall survival rates. In the multivariate analysis, the tumor-to-liver ratio of SUV significantly increased survival rate (Table 5). These results suggest that this ¹⁸F-FDG PET parameter was associated with survival in HCC patients.

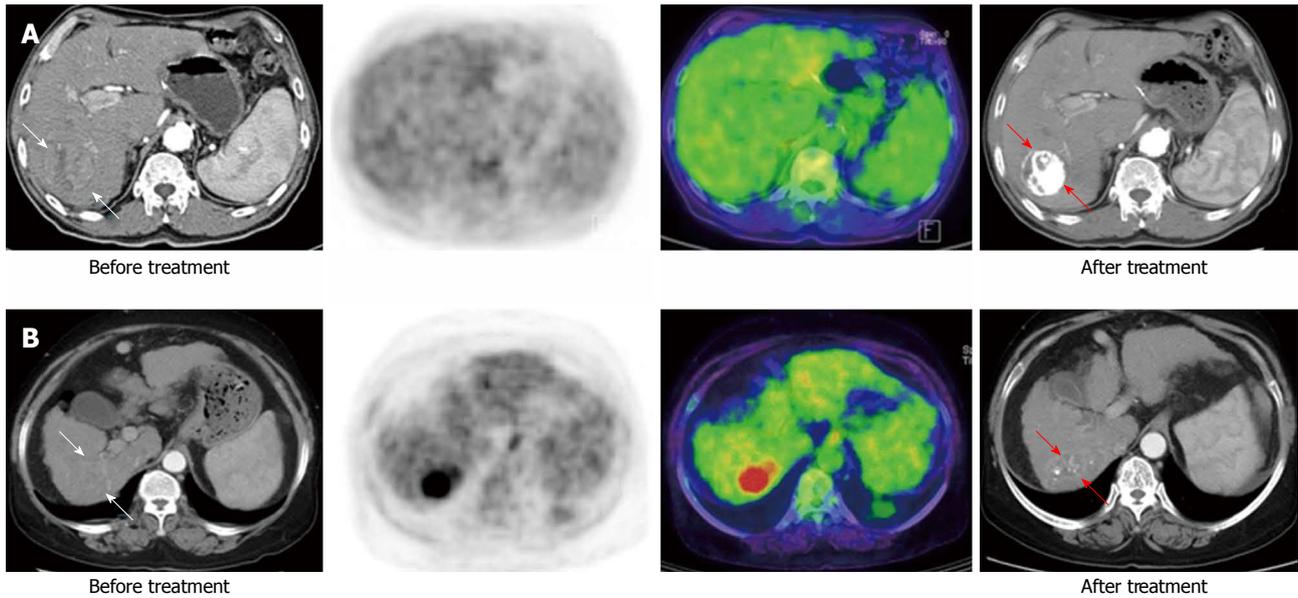


Figure 2 Baseline and follow-up images after treatment of fluorodeoxyglucose uptake according to the $Tsuv_{max}/Lsuv_{mean}$ ratio. A: Patient with nodular hepatocellular carcinoma (HCC) (white arrow) based on liver dynamic computed tomography (CT) showed fluorodeoxyglucose (FDG) uptake with a low $Tsuv_{max}/Lsuv_{mean}$ ratio (< 1.90) in ^{18}F -FDG positron emission tomography/CT. After transarterial chemo embolization, this patient showed compact lipiodol uptake (red arrow) on follow-up liver CT; B: Patient with infiltrative type HCC (white arrow) in liver dynamic CT showed FDG uptake with a high $Tsuv_{max}/Lsuv_{mean}$ ratio (≥ 1.90). This patient showed faint lipiodol uptake (red arrow) in a follow-up liver CT. The patient with a low standardized uptake value (SUV) ratio showed improved treatment response and survival over the patient with a high SUV ratio (complete response vs stable disease, 782 d vs 167 d, respectively).

Table 2 Tumor characteristics according to objective response

	Objective response group (n = 45)	Non-objective response group (n = 38)	P value
Mean age \pm SD (yr)	60.8 \pm 11.5	59.0 \pm 9.7	0.372
Sex (male:female)	37:8	31:7	0.940
Etiology			0.555
HBV/HCV/alcohol/others	36/5/1/3	33/4/0/1	
Tumor number			0.077
Single/multiple	25/20	13/25	
Tumor size (cm)	4.4 \pm 2.3	11.2 \pm 4.9	0.000
Portal vein thrombosis			0.000
Absent/present	42/3	15/23	
Child-Pugh class			0.756
A/B	38/7	33/5	
Serum AFP	3795 \pm 12054	35951 \pm 106137	0.054
BCLC stage			0.000
A/B/C	26/12/7	0/8/30	
$Tsuv_{max}/Lsuv_{mean}$			0.000
1.90 $</\geq$ 1.90	34/11	9/29	

HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFP: Serum α -fetoprotein; BCLC: Barcelona clinic liver cancer score.

Time to progression and $Tsuv_{max}/Lsuv_{mean}$ ratio

Progression of HCC after objective response was observed in 13 of 39 patients (32.5%) with a low $Tsuv_{max}/Lsuv_{mean}$ ratio (< 1.90) and 13 of 24 patients (56.5%) with a high SUV ratio (≥ 1.90). Although progression of low ratio patients was slower than that of high ratio patients, no significant difference was found between both groups (Figure 3B, $P = 0.15$).

Table 3 Univariate and multivariate analysis for the factors that influence objective response

Factors	P value	
	Univariate	Multivariate
Tumor number single/multiple	0.077	0.357
Tumor size (cm)	0.000	0.530
Portal vein thrombosis	0.000	0.386
Absent/present		
BCLC stage	0.000	0.408
A/B/C		
$Tsuv_{max}/Lsuv_{mean}$	0.000	0.020
1.90 $</\geq$ 1.90		

BCLC: Barcelona clinic liver cancer score.

DISCUSSION

^{18}F -FDG PET is an imaging modality that can be used to assess glucose metabolism of tumors. PET detects high ^{18}F -FDG uptake in rapidly growing tumors in which the rate of glycolysis increases^[8,16]. PET CT has been widely utilized for detection of extrahepatic metastasis from HCC^[5,17]. Recent quantitative studies of glucose utilization in liver tumors have shown that PET uptake is useful for tumor characterization and assessment of therapeutic response^[18-21]. ^{18}F -FDG uptake in HCC depends on the difference in the activity of glucose-6-phosphatase, which is responsible for the conversion of FDG-G-phosphate to FDG^[20]. Increased uptake of ^{18}F -FDG is associated with poorly differentiated HCC and poor outcome indi-

Table 4 Analysis of treatment response according to standardized uptake value ratio by barcelona clinic liver cancer score staging

BCLC stage	TsuV _{max} /LsuV _{mean}	Treatment response					P value
		CR	PR	SD	PD	Total	
A	< 1.90	18	3	0	0	21	1.0
(n = 26)	≥ 1.90	4	1	0	0	5	
B	< 1.90	5	3	2	3	13	0.048
(n = 20)	≥ 1.90	0	4	3	0	7	
C	< 1.90	2	4	4	0	10	0.000
(n = 37)	≥ 1.90	0	1	11	15	27	
Total		29	16	20	18	83	

CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; BCLC: Barcelona clinic liver cancer score.

Table 5 Multivariate analysis for the factors that influence overall survival rates

Factors	P value	Exp (B)	95% CI
Age			
< 60 yr/≥ 60 yr	0.135	0.814	0.832-3.955
Tumor size			
< 3 cm	0.520		
3-5 cm	0.456	2.420	0.269-20.861
> 5 cm	0.519	0.689	0.237-2.320
Tumor number			
Single/multiple	0.079	0.413	0.154-1.107
Portal vein thrombosis			
Absent/present	0.348	0.636	0.247-1.638
BCLC stage			
A	0.032		
B	0.034	0.082	0.008-0.824
C	0.053	0.314	0.097-1.017
TsuV _{max} /LsuV _{mean}			
1.90 </≥ 1.90	0.036	0.337	0.122-0.932

BCLC: Barcelona clinic liver cancer score; CI: Confidence interval.

cating that ¹⁸F-FDG uptake in HCC is closely related to tumor progression and prognosis^[7].

The present study demonstrates that ¹⁸F-FDG PET is a feasible tool for assessing biological behavior in HCC. The increase of FDG uptake in HCC was significantly associated with tumor burdens such as size, number of tumors and portal vein thrombosis. Because these tumor burdens are regarded as predictive factors of the aggressiveness of HCC, glucose metabolism on ¹⁸F-FDG PET may be a factor that is related to the aggressive character of tumors. FDG uptake is increased in more advanced HCC stages. The results suggest that PET might be useful as a modality for assessing biologic activity in HCC.

This study focused on the predictive value of FDG PET uptake for evaluating the treatment response in HCC patients. Transarterial chemoembolization is the main treatment that is indicated for unresectable HCC in intermediate stage based on the BCLC guideline^[22,23], but there are limited data on managing these patients^[24-27]. Still, the homogeneity of the treatment modality was important for more reliable analysis. We previously reported promising results with combination treatment

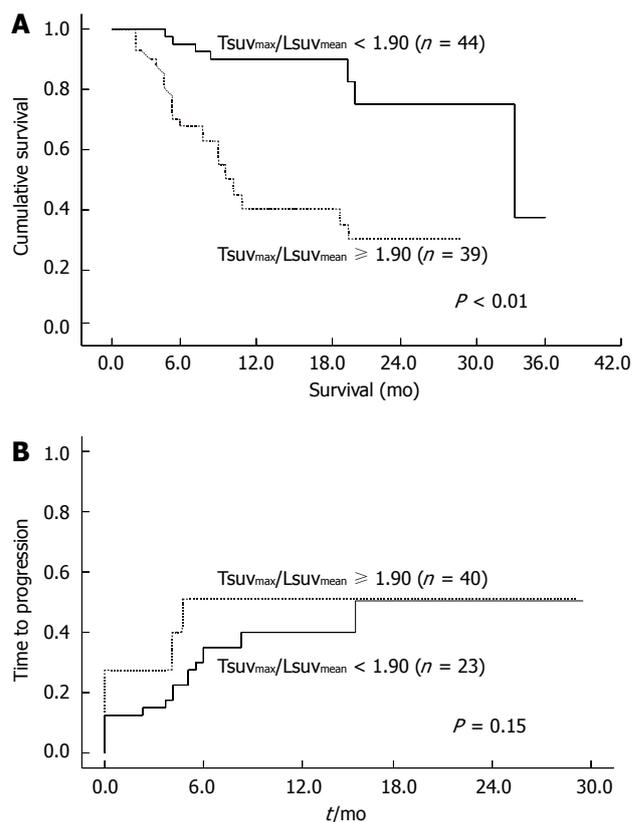


Figure 3 Overall survival and time to progression rates according to TsuV_{max}/LsuV_{mean} level. A: Patients exhibiting low TsuV_{max}/LsuV_{mean} (< 1.90) survived longer than those with high TsuV_{max}/LsuV_{mean} (≥ 1.90); B: No significant difference of progression was evident between patients exhibiting low TsuV_{max}/LsuV_{mean} and those with high TsuV_{max}/LsuV_{mean}. SUV: Standardized uptake value.

using TACL and systemic chemo-infusion therapy for advanced HCC with portal vein invasion^[12]. This combination therapy could be applied for treating advanced HCC. At this point, the present study determined the predictive factors for treatment response of the combination therapy in deciding on a management strategy for HCC. Tumor burdens such as tumor size and the reserved liver function have been previously reported to be predictive factors for treatment response^[24]. However, these factors are unable to exactly predict the degree of tumor malignancy, and there is a need for considering PET CT in assessing the biological activity of HCC as an additional predictive factor.

¹⁸F-FDG uptake showed the potential to predict the TACL with systemic chemo-infusion treatment response for HCC, with a cutoff TsuV_{max}/LsuV_{mean} value of 1.90. Objective response rates were significantly different above (77.7%) and below (23.6%) the cutoff value (P < 0.001). According to stage, the prediction of treatment response after three cycles of TACL was significantly better in the B and C stage than in the A stage (P = 0.048, P = 0.000, respectively). These facts demonstrate the predictive value for ¹⁸F-FDG uptake in HCC.

Presently, the SUV ratio correlated with treatment response suggesting that this ratio may be a useful index of HCC aggressiveness^[28-30]. Previous reports have dem-

onstrated comparative SUV ratio between tumors and non-tumors is a more useful parameter than the SUV of tumors^[28,31,32]. Because ¹⁸F-FDG uptake is affected by underlying liver cirrhosis, the ratio reflects more the underlying variation of glucose metabolism in the liver than SUV of tumor itself^[33,34]. This SUV ratio correlates with tumor volume doubling time and the differentiation of HCC^[5]. The tumor-to-non tumor ratio of SUV may be an effective parameter of progression or aggressiveness in HCC.

Furthermore, our principal finding is that the SUV ratio in FDG PET diagnosis before treatment is an independent predictor of survival in unresectable HCC patients. This finding is compatible with previous studies performed in other treatments such as LT and liver resection^[28,35]. In the univariate analysis, tumor size, portal vein thrombosis and BCLC stage were determined to be as significant as the $T_{suvmax}/L_{suvmean}$ ratio. The increase in FDG uptake ratio in HCC was significantly associated with the aggressive character of the tumor burden such as size, number of tumors and portal vein thrombosis. However, in the multivariate analysis, $T_{suvmax}/L_{suvmean}$ was a significant prognostic factor with BCLC stage. This result suggests the ratio of ¹⁸F-FDG uptake might provide additional information to staging system.

The BCLC staging system has been used to predict outcome and inform decision about treatment strategy in HCC^[36]. Although this system includes variables such as tumor burden and liver function reserve, according to Child-Pugh class and performance status, this stage does not adequately consider the biological activity of HCC. FDG-PET permits the evaluation of glucose metabolism in HCC and the detection of extrahepatic metastasis^[37]. The determination of the SUV ratio might contribute to the clinical management of HCC patients and compensate for the drawbacks of this staging system for the prediction of treatment response and prognosis in HCC.

This study has some limitations inherent to a retrospective study. First, the number of HCC cases for each stage was relatively small, although PET/CT was performed to detect extrahepatic metastasis and to evaluate the treatment response. Second, sorafenib is considered to be the standard treatment in advanced HCC. Sorafenib was not applicable in the advanced HCC cases in our study. However, we showed beneficial results with combination treatment using TACL and systemic chemo-infusion therapy for advanced HCC and the homogeneity of treatment would rather allow a more reliable analysis. We intended to analyze the correlation of tumor characteristics and treatment response according to ¹⁸F-FDG uptake. Prospective studies are needed to confirm these results in the future.

In summary, this study shows that ¹⁸F-FDG PET is a significant predictor of treatment response with TACL and systemic chemo-infusion therapy in HCC. The $T_{suvmax}/L_{suvmean}$, can be a significant way of distinguishing, overall survival. Therefore, ¹⁸F-FDG PET could provide effective information on the prognosis of the treatment response in the evaluation of HCC cases.

COMMENTS

Background

¹⁸F-fluorodeoxyglucose positron emission tomography (¹⁸F-FDG PET) is an imaging modality that can assess the glucose metabolism of tumors. PET monitoring of FDG uptake may be an additional tool to assess biological behavior of hepatocellular carcinoma (HCC). The present study evaluated the correlation of ¹⁸F-FDG PET with clinical features and prediction of treatment response.

Research frontiers

This study showed that the standardized uptake value ratio of FDG uptake could be used to predict the treatment response and overall survival of HCC patients treated with transarterial chemo embolization (TACE).

Innovations and breakthroughs

¹⁸F-FDG PET is a significant predictor of treatment response with transarterial chemolipiodolization (TACL) and systemic chemo-infusion therapy in HCC. The $T_{suvmax}/L_{suvmean}$ (cutoff value of 1.90) was significantly associated with overall survival of HCC patients.

Applications

The study results suggest that the FDG PET is a potential modality that could be used in predicting treatment prognosis for HCC patients treated with locoregional therapy.

Terminology

TACL refers to transarterial treatment with chemotherapeutic agents and lipiodol without embolic materials such as gelatin or polyvinyl alcohol particles. Systemic chemo-infusion is a type of systemic chemotherapy that is administered after TACE.

Peer review

In this study, the authors have studied the role of FDG PET in the evaluation of treatment response in patient undergoing the TACL procedure for treatment of HCC. Based on a retrospective review of 83 patients, the authors find that FDG PET can be used to predict the treatment response to HCC. This study is interesting and well performed, and the authors need to be lauded for their efforts. The findings of this study will definitely contribute to the scientific literature and improve our understanding of the biological behavior of HCC.

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Effect of biologically active fraction of *Nardostachys jatamansi* on cerulein-induced acute pancreatitis

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Abstract

AIM: To determine if the fraction of *Nardostachys jatamansi* (NJ) has the potential to ameliorate the severity of acute pancreatitis (AP).

METHODS: Mice were administered the biologically active fraction of NJ, i.e., the 4th fraction (NJ4), intraperitoneally, and then injected with the stable cholecystokinin analogue cerulein hourly for 6 h. Six hours after the last cerulein injection, the pancreas, lung, and blood were harvested for morphological examination,

measurement of cytokine expression, and examination of neutrophil infiltration.

RESULTS: NJ4 administration attenuated the severity of AP and lung injury associated with AP. It also reduced cytokine production and neutrophil infiltration and resulted in the *in vivo* up-regulation of heme oxygenase-1 (HO-1). Furthermore, NJ4 and its biologically active fraction, NJ4-2 inhibited the cerulein-induced death of acinar cells by inducing HO-1 in isolated pancreatic acinar cells.

CONCLUSION: These results suggest that NJ4 may be a candidate fraction offering protection in AP and NJ4 might ameliorate the severity of pancreatitis by inducing HO-1 expression.

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Key words: *Nardostachys jatamansi*; Acute pancreatitis; Cytokines; Heme oxygenase-1

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INTRODUCTION

Acute pancreatitis (AP) refers to inflammation of the pancreas and is caused by an imbalance in the factors that maintain cellular homeostasis^[1]. AP could lead to distant

organ damage and multiple organ dysfunction, the primary cause of morbidity and mortality in this condition^[2]. The AP cascade is complex. An unknown trigger within the pancreas converts digestive pro-enzymes into their active form, initiating auto-digestion of the gland, causing necrosis, edema, and destruction of the pancreatic parenchyma^[2].

Nardostachys jatamansi (NJ) is widely used in several Asian countries to treat mental disorders, insomnia, and disorders of the circulatory system^[3]. It has protective effects against diabetes and sepsis^[4,5]. We have previously reported that NJ protects against cerulein-induced AP^[6]. Further, several studies have reported on the protective effects afforded by compounds from NJ^[3,7] such as jatamansic acid and nardosinone. However, the compound in NJ that protects against AP remains to be identified.

This study aimed to identify the candidate fraction of NJ that protects against cerulein-induced AP in a mouse model. To achieve this, we fractionated NJ by using RP C-18 column chromatography and the 4th fraction (NJ4) showed more potent effects than the aqueous extract of NJ. Our results suggest that NJ4 may be a candidate fraction for reducing the severity of AP.

MATERIALS AND METHODS

Materials

Avidin peroxidase and 3,3',5,5'-tetramethylbenzidine (TMB), cerulein, Tris-HCl, NaCl, Triton X-100, curcumin, ZnPP, and hexadecyltrimethyl ammonium bromide were purchased from Sigma-Aldrich (St. Louis, MO). Anti-mouse interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α antibodies and recombinant IL-1 β , IL-6, and TNF- α were purchased from R and D Systems (Minneapolis). Phosphospecific mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinases (ERK)1/2, c-Jun NH₂-terminal kinases (JNK), and p38 were purchased from Cell Signaling Technology (Beverly, MA). ERK1/2, JNK, p38, inhibitory kappa-B α heme oxygenase-1 (HO-1), and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Plant materials

The roots of NJ were purchased from a standard commercial source (Omni Herb, Seoul, South Korea). The herb's identity was confirmed at Wonkwang University. Voucher specimens were deposited at the College of Oriental Medicine Herbarium of Wonkwang University. The NJ roots were prepared by decocting the dried prescription of herbs (100 g) with boiling distilled water (1 L). The decoction time was approximately 2 h. The water extract was frozen at -80 °C and then freeze-dried to be powdered (7.35 g, 7.35 w/w%).

Preparation of NJ4 fraction

The water extract (3.8 g) was subjected to octadecyl functionalized silica gel flash column (5 cm \times 20 cm; 63-200 μ m particle size) chromatography (Figure 1). The

column was eluted with a stepwise gradient with 500 mL aliquots of MeOH in H₂O (starting from 10% and followed by 20%, up to 100% at 20% increments), affording 6 fractions (NJ1: 1.24 g; NJ2: 79.1 mg; NJ3: 490.7 mg; NJ4: 416.2 mg; NJ5: 273.1 mg; NJ6: 67.8 mg). NJ4 (60% MeOH) in saline was used as the main fraction (Figure 1).

Preparation of NJ4 fraction

A portion (9.0 mg) of the fraction eluted with 60% MeOH in H₂O (NJ4-2) was then purified by semi-preparative reversed-phase high-performance liquid chromatography (HPLC) [Shiseido Capcell Pak C₁₈ column (10 mm \times 250 mm; 5 μ m particle size); 2 mL/min; detection at 254 nm] eluting with a gradient from 40% to 70% MeOH in H₂O (0.1% formic acid) over 30 min to yield NJ4-2 (3.0 mg, *t_R* = 28.0 min) (Figure 1).

HPLC sample preparation and HPLC conditions

For HPLC analysis, the chromatographic system consisted of a pump (3000 HPLC pump; Dionex Association, United States), a ultraviolet detector (Photodiode array detector; Dionex Association), and an autosampler (Waters Association, United States). A hydrosphere C₁₈ column (4.6 mm \times 250 mm, 5 μ m) was used. Water-methanol glacial (50:50) was used as the mobile phase. Detection of the peaks was made at 254 nm and the sensitivity was set at 0.5 absorbance units full scales. The injection volume was 10 μ L and the flow rate was 1.0 mL/min. A standard solution was prepared by dissolving in distilled methanol (10 μ g/10 mL). The solution was filtered through a 0.45 μ m membrane filter and applied to HPLC (Figure 2).

Animals

Protocols approved by the Animal Care Committee of Wonkwang University were used for all experiments. Female 6- to 8-wk-old C57BL/6 mice weighing 15-20 g were purchased from Orient Bio (Sungnam, KyungKiDo, South Korea). All animals were bred and housed in standard shoebox cages in a climate-controlled room with an ambient temperature of 23 \pm 2 °C under a 12-h light-dark cycle for 7 d. The animals were fed standard laboratory chow, allowed water *ad libitum*, and randomly assigned to control or experimental groups. The mice were fasted for 18 h before AP was induced.

Experimental design

AP was induced by intraperitoneal injections of supra-maximal concentrations of the stable cholecystokinin analogue cerulein (50 μ g/kg) or saline, hourly for 6 h^[6]. Prior to injecting the NJ4 treatment group with cerulein, NJ4 (1 μ g/kg, 5 μ g/kg, or 10 μ g/kg, *n* = 6) or saline (control group, *n* = 6) were intraperitoneally administered (1 h before the first cerulein injection). Mice were killed 6 h after the last cerulein injection was administered. Blood samples were taken to determine serum amylase, lipase, and cytokine levels. For histological examination and scoring, the entire pancreas and lungs were rapidly re-

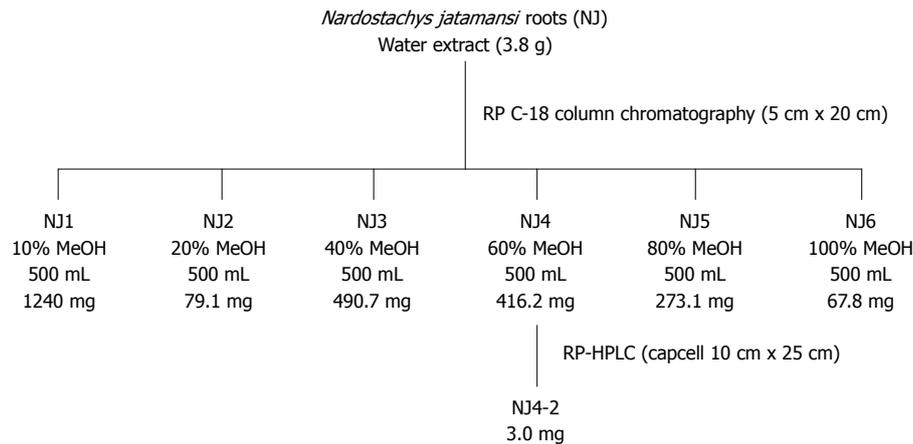


Figure 1 Fractionation of the aqueous extract of *Nardostachys jatamansi*. NJ: *Nardostachys jatamansi*; HPLC: High-performance liquid chromatography.

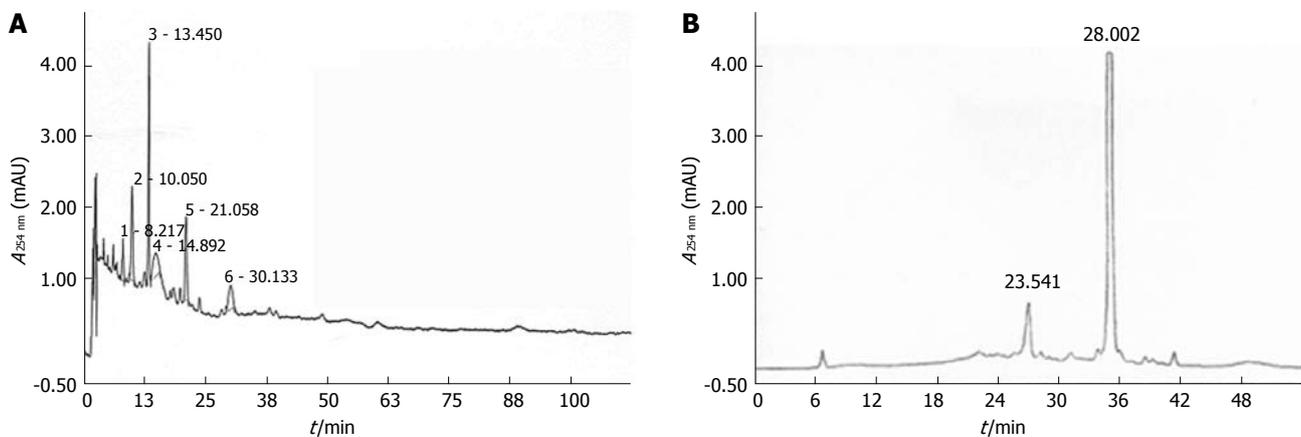


Figure 2 High-performance liquid chromatography findings of the aqueous extract of *Nardostachys jatamansi* (A) and the 4th fraction of *Nardostachys jatamansi* (B).

moved from each mouse and fixed in formalin. To measure tissue myeloperoxidase (MPO) activity, an indicator of neutrophil sequestration, and for real-time reverse transcriptase polymerase chain reaction (RT-PCR) studies, the pancreas and lungs were stored at -80°C .

Histological analysis

The pancreases from each treatment group were examined and semi-quantitatively described in terms of necrosis, vacuolization, inflammation, and edema. A tissue section representing a minimum of 100 fields was examined for each sample and scored on a scale of 0-3 (0 being normal and 3 being severe disease) on the basis of the number of necrotic acinar cells and the presence of interstitial edema and interstitial inflammation.

Measurement of serum amylase and lipase

Blood samples to determine serum amylase and lipase were obtained 6 h after inducing pancreatitis. Mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (4 mg/kg). After anesthetization, blood was withdrawn from the heart of each mouse into a syringe. Serum amylase and lipase were measured using an assay kit from BioAssay Systems (CA).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) for IL-1 β , IL-6, and TNF- α were carried out in duplicate in 96-well plates coated with 100 μL aliquots of anti-mouse IL-1 β , IL-6, and TNF- α monoclonal antibodies in phosphate buffered saline (PBS) at pH 7.4 during an overnight incubation at 4°C . The plates were washed in PBS containing 0.05% Tween-20 and blocked with PBS containing 10% FBS for 2 h. After additional washes, standards and samples were added and incubated at room temperature for 2 h. Subsequently, the wells were washed, and biotinylated anti-mouse IL-1 β , IL-6, and TNF- α were added and incubated at room temperature for 1 h. The wells were washed, avidin-peroxidase was added, and the plates were incubated for 30 min at room temperature before washing again and adding the TMB substrate. Color development was measured at 450 nm by using an automated microplate ELISA reader. Standard samples were run on each assay plate using serial dilutions of recombinant IL-1 β , IL-6, and TNF- α .

Messenger RNA expression

Transcription of target cytokines in mouse pancreatic tissues and acini was analyzed using RT-PCR. Total RNA was isolated from the mouse pancreas using TriZol (In-

vitrogen, Carlsbad, CA) and subjected to reverse transcription using SuperScript II RT (Invitrogen). TaqMan quantitative RT-PCR using the LightCycler 2.0 detection system was performed according to the manufacturer's instructions (Roche, Basel, Switzerland). For each sample, triplicate test reactions and a control reaction lacking reverse transcriptase were analyzed for the expression of the gene of interest, and the results were normalized to those of "housekeeping" hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA. Arbitrary expression units were calculated by dividing the expression of the gene of interest by ribosomal protein HPRT mRNA expression. The sequences of forward, reverse, and probe oligonucleotide primers for multiplex real-time TaqMan PCR were as follows: for mouse IL-1 β (forward, 5'-TTG ACG GAC CCC AAA AGA T-3'; reverse, 5'-GAA GCT GGA TGC TCT CAT CTG-3'; universal probe, M15131.1, Roche Applied Science), for mouse IL-6 (forward, 5'-TTC ATT CTC TTT GCT CTT GAA TTA GA-3'; reverse, 5'-GTC TGA CCT TTA GCT TCA AAT CCT-3'; universal probe, M20572.1, Roche Applied Science), and for mouse TNF- α (forward, 5'-TCT CTT CAA GGG ACA AGG CTG-3'; reverse, 5'-ATA GCA AAT CGG CTG ACG GT-3'; probe, 5'-CCC GAC TAC GTG CTC CTC ACC CA-3'). For mouse HO-1, we purchased a custom primer from Applied Biosystems (CA).

MPO estimation

Neutrophil sequestration in the pancreas was quantified by measuring tissue MPO activity. Tissue samples were thawed, homogenized in 20 mmol/L phosphate buffer (pH 7.4), and centrifuged (13 000 rpm, 10 min, 4 °C). The pellet was resuspended in 50 mmol/L phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich). The suspension was subjected to 4 cycles of freezing and thawing and further disrupted by sonication for 40 s. The sample was then centrifuged (13 000 rpm, 5 min, 4 °C), and the supernatant was used for the MPO assay. The reaction mixture consisted of the supernatant, 1.6 mmol/L TMB, 80 mmol/L sodium phosphate buffer (pH 5.4), and 0.3 mmol/L hydrogen peroxide. This mixture was incubated at 37 °C for 110 s, the reaction was terminated with 2 mol/L H₂SO₄, and the absorbance was measured at 450 nm.

Western blotting

Pancreatic tissues and pancreatic acini were homogenized, following which the lysates were boiled in a sample buffer (62.5 mmol/L Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol, and 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated using 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Then, the membrane was blocked with 5% skim milk in PBS-Tween-20 for 2 h at RT and then incubated with primary antibodies overnight. After washing 3 times, each blot was incubated with peroxidase-conjugated secondary antibody for 1 h, and antibody-specific proteins were visualized using an

enhanced chemiluminescence detection system (Amersham, Piscataway, NJ) according to the manufacturer's recommended protocol.

Acinar cell isolation

Pancreatic acini were isolated from C57BL/6 mice using collagenase digestion. All experiments were performed according to protocols approved by the Animal Care Committee of Wonkwang University. Briefly, pancreatic tissue was minced with scissors and digested for 15 min in solution Q (120 mmol/L NaCl, 20 mmol/L HEPES, 5 mmol/L KCl, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, 10 mmol/L sodium pyruvate, 10 mmol/L ascorbate, 10 mmol/L glucose, 0.1% BSA, 0.01% soybean trypsinogen inhibitor, and 150 units of collagenase/mL). Cells were continuously shaken and gassed with 100% O₂ in a 37 °C water bath and subsequently washed in fresh isolation medium. After collagenase digestion, the tissue was gently pipetted. Dispersed acini were filtered through a 150- μ m nylon mesh, centrifuged 3 times (each for 90 s at 720 rpm), resuspended in Waymouth medium (Invitrogen, Gibco, CA) and incubated with 95% O₂ and 5% CO₂ for 4 h.

Cell viability assay

Cell viability was assayed using a modified colorimetric technique that is based on the ability of live cells to convert the tetrazolium compound 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) into purple formazan crystals. MTT (5 mg/mL) was dissolved in Krebs-Henseleit buffer (115 mmol/L NaCl, 3.6 mmol/L KCl, 1.3 mmol/L KH₂PO₄, 25 mmol/L NaHCO₃, 1 mol/L CaCl₂, and 1 mol/L MgCl₂), and 50 μ L was added to each well. After incubating for 30 min at 37 °C, the suspension was removed, and the formazan crystals formed were dissolved in 200 μ L dimethyl sulfoxide. Aliquots from each well were seeded in the wells of a 96-well plate in duplicate and assayed at 540 nm using a microplate ELISA reader. The number of viable cells was expressed as a percentage of the control.

Statistical analysis

Results were expressed as means \pm SE. The significance of differences was evaluated using a two-way analysis of variance (ANOVA) with time and dose parameters. Differences between the experimental groups were evaluated using ANOVA. *P* values < 0.05 were considered statistically significant.

RESULTS

Effect of NJ4 on cerulein-induced AP

To examine the effect of NJ4 on the development and severity of AP, mice pretreated with saline or NJ4 were injected intraperitoneally with a supramaximal dose (50 μ g/kg) of cerulein. Intraperitoneal injection of cerulein resulted in significant pancreatic parenchyma destruction, inflammation, edema, and necrosis. However, NJ4-treated

Table 1 Semi-quantitative analysis of pancreas in acute pancreatitis

Severity score	Inflammation	Edema	Necrosis
Saline	0.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.2
Saline + AP	2.5 ± 0.5 ^a	2.5 ± 0.5 ^a	2.5 ± 0.5 ^a
NJ4 1 + AP	1.5 ± 0.3 ^{a,c}	2.0 ± 0.3 ^a	2.0 ± 0.3 ^a
NJ4 5 + AP	1.5 ± 0.2 ^{a,c}	1.5 ± 0.3 ^{a,c}	1.5 ± 0.2 ^{a,c}
NJ4 10 + AP	1.1 ± 0.1 ^{a,c}	1.0 ± 0.2 ^{a,c}	1.2 ± 0.15 ^{a,c}

Histological sections of the pancreas were scored from 0 (normal) to 3 (severe) for inflammation, edema, and necrosis. ^a*P* < 0.05 vs the saline treatment; ^c*P* < 0.05 vs cerulein treatment alone. AP: Acute pancreatitis; NJ: *Nardostachys jatamansi*.

Table 2 Semi-quantitative analysis of lung in acute pancreatitis

Severity score	Inflammation	Edema
Saline	0.5 ± 0.2	0.5 ± 0.2
Saline + AP	2.5 ± 0.5 ^a	2.5 ± 0.5 ^a
NJ4 1 + AP	1.3 ± 0.4 ^{a,c}	1.8 ± 0.3 ^a
NJ4 5 + AP	1.3 ± 0.3 ^{a,c}	1.6 ± 0.3 ^{a,c}
NJ4 10 + AP	1.1 ± 0.2 ^{a,c}	1.2 ± 0.1 ^{a,c}

Histological sections of the lung were scored from 0 (normal) to 3 (severe) for inflammation, and edema. ^a*P* < 0.05 vs the saline treatment; ^c*P* < 0.05 vs cerulein treatment alone. AP: Acute pancreatitis; NJ: *Nardostachys jatamansi*.

mice showed reductions in damage of the pancreas and severity of AP (Table 1 and Figure 3A). As an additional assessment of the severity of the inflammatory response, we measured MPO activity, which is an indicator of neutrophil infiltration in the pancreas. Cerulein-induced MPO activity in the pancreas of NJ-treated mice was lower than that in the pancreas of saline-injected mice (Figure 3B).

Effect of NJ4 on lung injury associated with AP

Acute lung injury in AP still represents a substantial problem, with a mortality rate in the range of 30%-40%^[8]. The cytokines and inflammatory mediators from pancreatic acini regulate the migration and pulmonary infiltration of neutrophils to the interstitial tissue, where they cause injury and breakdown of pulmonary parenchyma^[9]. As shown in Figure 4, cerulein injection resulted in lung injury, which is characterized by destruction of the lung parenchyma, edema, and inflammatory cell infiltration. However, NJ4 treatment reduced lung damage, inflammation, and edema and inhibited MPO activity in the lung in cerulein-induced AP (Table 2 and Figure 4A and B).

Effect of NJ4 on pancreatic weight/body weight ratio and serum digestive enzymes in cerulein-induced AP

In order to assess the effect of NJ4 on pancreatic edema, the pancreatic weight/body weight (PW/BW) was measured. As shown in Figure 5A, NJ4 administration inhibited the PW/BW ratio in a dose-dependent manner during cerulein-induced AP. When acinar cells are challenged with inflammatory responses, pro-enzymes in the pan-

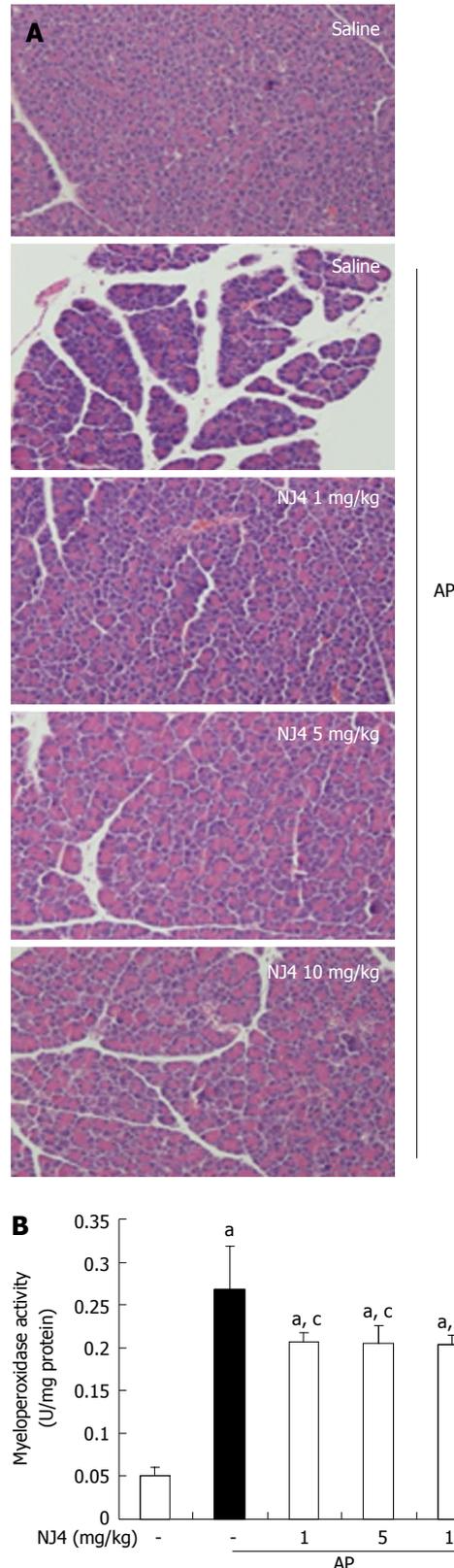


Figure 3 Effects of the 4th fraction of *Nardostachys jatamansi* on inflammatory events in the pancreas after pancreatitis. A: Representative hematoxylin-eosin stained sections of the pancreas in the control mice not administered cerulein, in mice given cerulein, and in mice given *Nardostachys jatamansi* (NJ) (1 mg/kg, 5 mg/kg, or 10 mg/kg) 1 h before the first cerulein injection; B: Neutrophil infiltration was assessed on the basis of myeloperoxidase activity. This figure shows representative images of 1 experiment that involved 6 mice. The results were similar in 3 additional experiments. ^a*P* < 0.05 vs the saline treatment; ^c*P* < 0.05 vs cerulein treatment alone. Original magnification: × 100. AP: Acute pancreatitis.

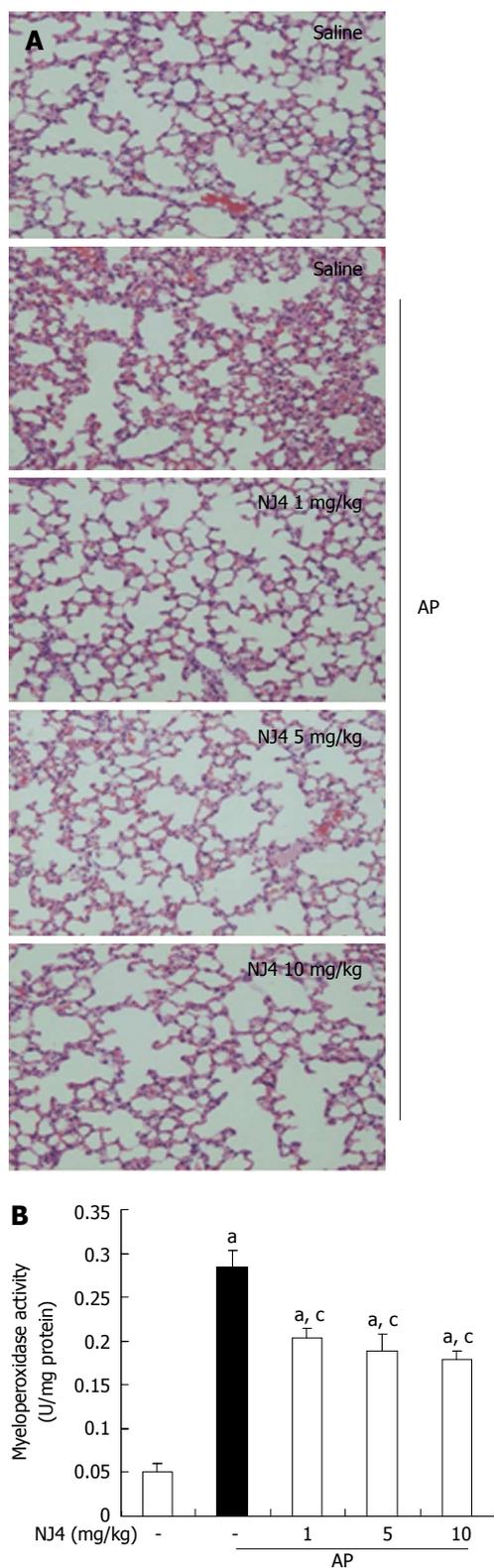


Figure 4 Effects of the 4th fraction of *Nardostachys jatamansi* on acute pancreatitis-associated lung injury. **A**: Representative hematoxylin-eosin stained sections of the lung in the control mice not given cerulein, in mice given cerulein, and in mice given *Nardostachys jatamansi* (NJ) (1 mg/kg, 5 mg/kg, or 10 mg/kg) 1 h before the first cerulein injection; **B**: Neutrophil infiltration was assessed on the basis of myeloperoxidase activity. This figure shows representative images from 1 experiment that involved 6 mice. The results were similar in 3 additional experiments. ^a*P* < 0.05 vs the saline treatment; ^c*P* < 0.05 vs cerulein treatment alone. Original magnification: × 100. AP: Acute pancreatitis.

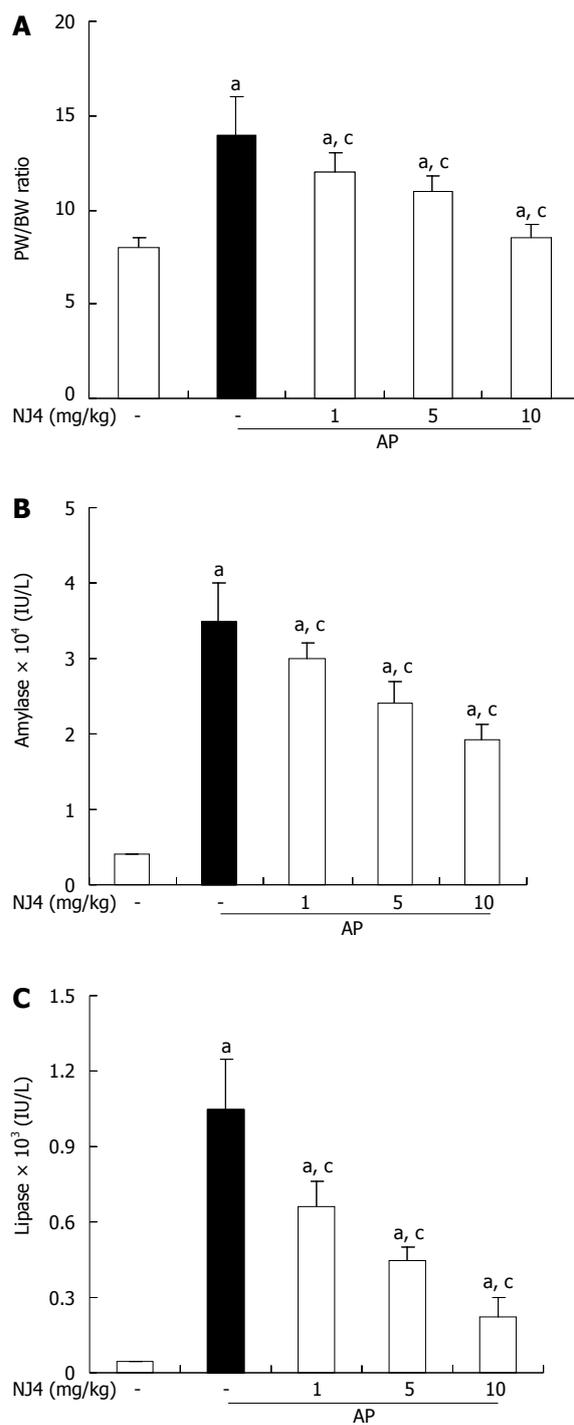


Figure 5 Effects of the 4th fraction of *Nardostachys jatamansi* pretreatment on pancreatic weight/body weight (A), serum amylase activity (B), and serum lipase activity (C) during cerulein-induced acute pancreatitis. Mice pretreated with *Nardostachys jatamansi* (NJ) were challenged with intraperitoneal injections of cerulein (50 μg/kg). Mice were killed 6 h after the last cerulein injection. Their serum and pancreas were harvested and (A) pancreatic weight (PW)/body weight (BW) ratio and the levels of digestive enzymes such as (B) amylase and (C) lipase were measured as indicated in the experimental protocol. Data show the mean ± SE for 6 mice in 1 group. The results were similar in 3 additional experiments. ^a*P* < 0.05 vs the saline treatment; ^c*P* < 0.05 vs cerulein treatment alone. AP: Acute pancreatitis.

creatic zymogen are converted to active enzymes such as amylase and lipase. Therefore, serum amylase and lipase

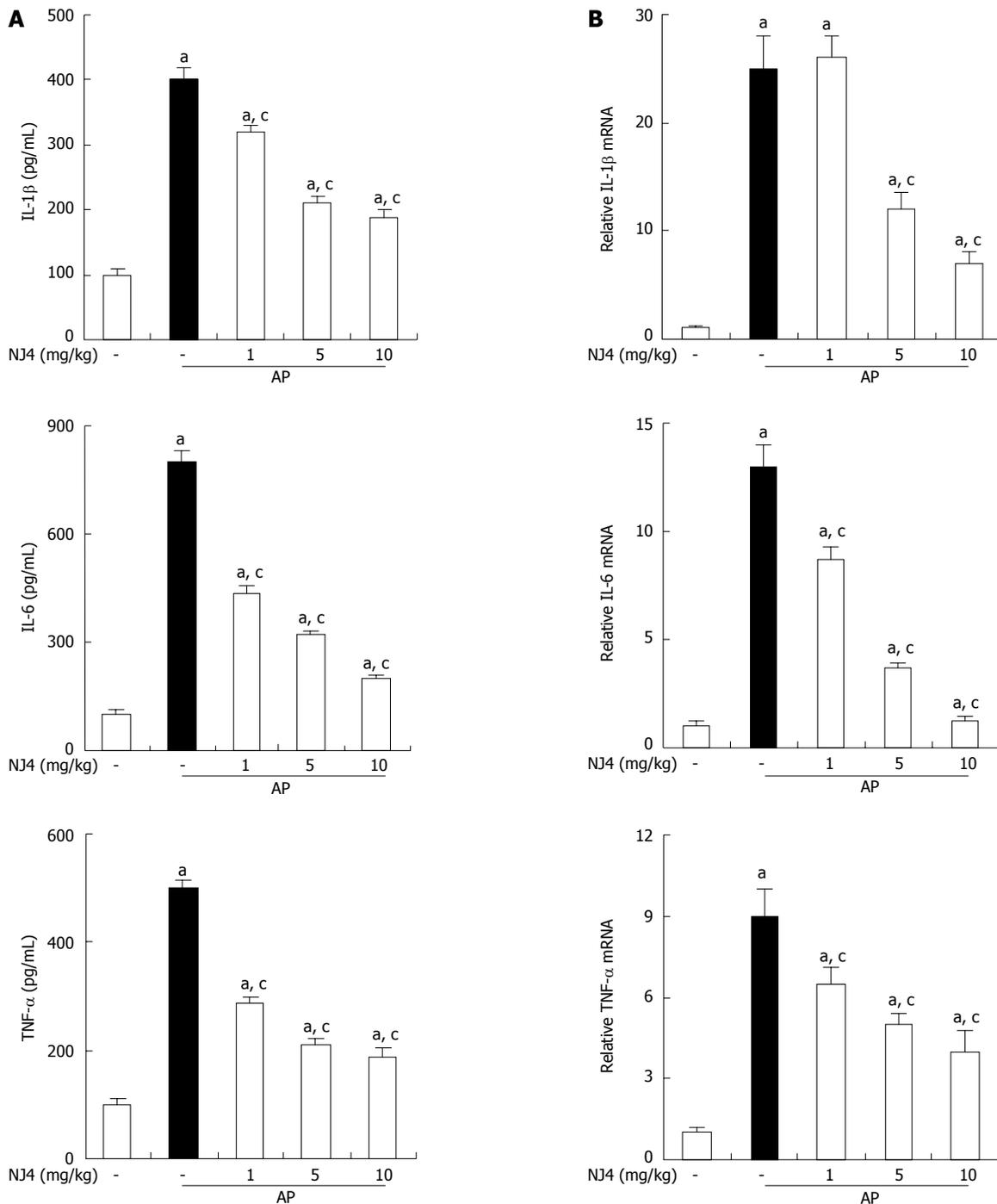


Figure 6 Effects of the 4th fraction of *Nardostachys jatamansi* on interleukin 1, interleukin 6, and tumor necrosis factor during cerulein-induced pancreatitis. Mice pretreated with *Nardostachys jatamansi* (NJ) were challenged with intraperitoneal injections of cerulein at a supramaximal dose (50 μg/kg). Mice were killed at 6 h after the last cerulein injection. Levels of serum cytokines were measured by enzyme-linked immunosorbent assay (A). Levels of pancreatic mRNA for interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α were quantified using real-time reverse transcriptase polymerase chain reaction (B). Data show the mean ± SE for 6 mice in 1 group. The results were similar in 3 additional experiments. ^a*P* < 0.05 vs the saline treatment; ^c*P* < 0.05 vs cerulein treatment alone. AP: Acute pancreatitis.

activity were used to assess the severity of AP. The activities of amylase and lipase were up-regulated by cerulein injection. However, these activities were inhibited by NJ4 treatment (Figure 5B and C).

Effect of NJ4 on pro-inflammatory cytokine production

Several inflammatory mediators such as ILs and TNFs have been shown to increase in AP^[10]. Therefore, the levels of cytokines were examined in the serum and pan-

creas. Substantial increases in IL-1β, IL-6, and TNF-α were found in the serum and pancreas at 6 h after the final administration of cerulein. However, the levels of IL-1β, IL-6, and TNF-α in the serum and pancreas were significantly reduced with NJ4 treatment (Figure 6A and B).

Effect of NJ4 on in vivo HO-1 induction

To evaluate the protective mechanisms of NJ4, we determined the levels of HO-1, which exhibits certain bio-

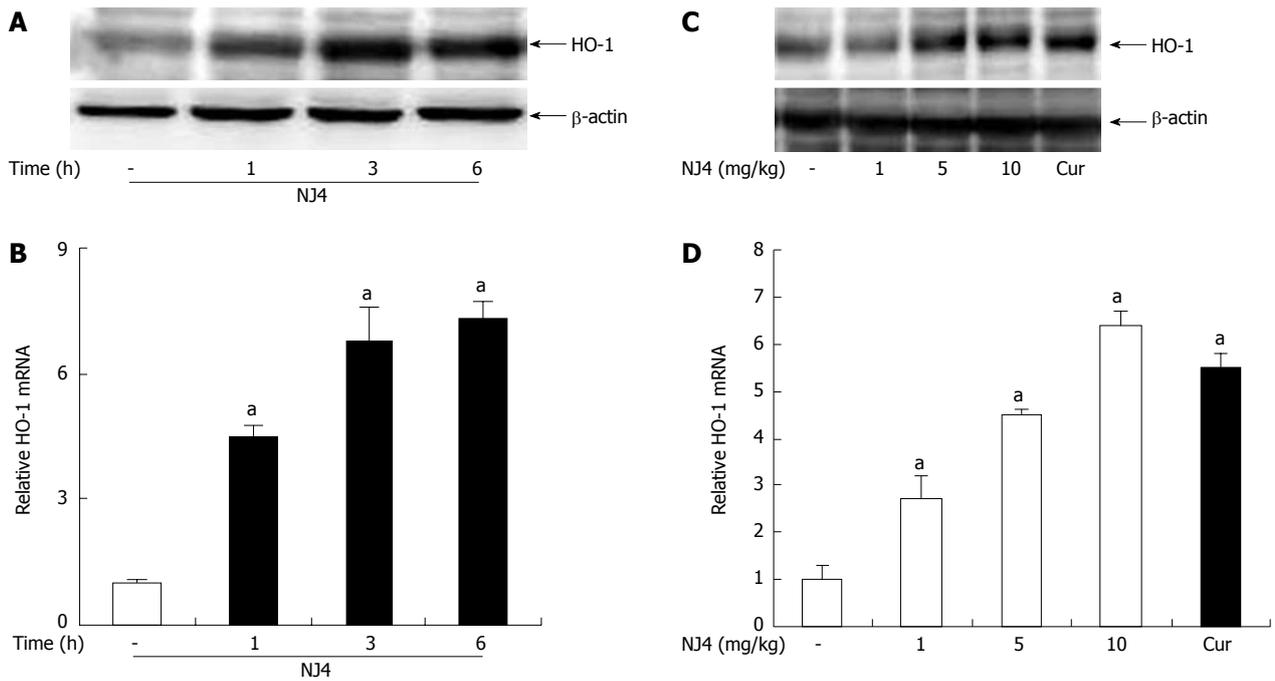


Figure 7 Effects of the 4th fraction of *Nardostachys jatamansi* on heme oxygenase-1 expression in the pancreas. Mice were treated with saline, or *Nardostachys jatamansi* (NJ) (10 mg/kg). Then, the pancreas was harvested at the indicated time. A: The protein level of heme oxygenase-1 (HO-1) in the pancreas was measured using Western blotting. β -actin was used as the loading control; B: The mRNA expression of HO-1 was measured using real-time reverse transcriptase polymerase chain reaction (RT-PCR). Mice were treated with the indicated dose of NJ4. At 6 h after NJ4 injection, the pancreas was harvested; C: The protein level of HO-1 in the pancreas was measured using Western blotting. β -actin was used as the loading control; D: The mRNA expression of HO-1 was measured using real-time RT-PCR. The results were similar in 3 additional experiments. ^a $P < 0.05$ vs the saline treatment. Cur: Curcumin.

logical properties, including anti-inflammatory^[11,12] and antioxidant^[13,14] properties. Therefore, NJ4 (10 mg/kg) was administered intraperitoneally at the indicated time point. Administration of NJ4 was followed by significant up-regulation of pancreatic HO-1 after 1 h (Figure 7A and B). Pancreatic HO-1 induction also occurred in a dose-dependent manner at 6 h after the injection of NJ4 (Figure 7C). Curcumin, an HO-1 inducer, was loaded as the positive control. The pancreatic mRNA levels of HO-1 were correlated with the pancreatic protein levels of HO-1 (Figure 7D).

Effect of NJ4 on in vitro HO-1 induction and acinar cell death

To examine the effect of NJ4 on HO-1 expression in isolated pancreatic acinar cells, HO-1 expression was measured. As shown in Figure 8A and B, NJ4 treatment increased the HO-1 expression after 1 h, but significantly at 3 h. The expression was increased in time dependant manner. HO-1 induction in pancreatic acinar cells occurred in a dose-dependent manner at 6 h after NJ4 treatment (Figure 8C and D).

AP is characterized by acinar cell death in the pancreas. Therefore, we examined the effect of NJ4 on pancreatic acinar cell death. The cells were pretreated with NJ4 for 1 h and then stimulated with cerulein for 6 h. NJ4 significantly increased the viability of pancreatic acinar cells in a dose-dependent manner (Figure 8E). We also investigated whether NJ4 could regulate cerulein-induced acinar cell death through the induction of HO-1. The HO-1

inhibitor ZnPP inhibited the effect of NJ4 on cerulein-induced acinar cells (Figure 8F). Furthermore, the HO-1 inducer curcumin also inhibited cerulein-induced acinar cell death (Figure 8F).

Effect of NJ4-2 on in vitro acinar cell death

To examine the effect of NJ4-2 on HO-1 expression in isolated pancreatic acinar cells, HO-1 expression was measured. As shown in Figure 9A and B, NJ4-2 treatment increased the HO-1 expression after 1 h. The expression was increased in time dependant manner. HO-1 induction in pancreatic acinar cells occurred in a dose-dependent manner at 6 h after NJ4-2 treatment (Figure 9C and D).

The cells were pretreated with NJ4-2 for 1 h and then stimulated with cerulein for 6 h. NJ4-2 significantly increased the viability of pancreatic acinar cells in a dose-dependent manner (Figure 9E).

DISCUSSION

Various studies have investigated candidates that could treat AP^[6,15-18]. However, specific and effective candidates to treat AP remain unknown. Our previous report showed that the total extract of NJ reduces the severity of mild acute pancreatitis^[6]. In this study, we report that the biological main fraction of NJ, i.e., NJ4, has a protective effect against cerulein-induced AP. To identify the fraction responsible for the main effect of NJ, we fractionated NJ and obtained 6 fractions. The effects of the 6

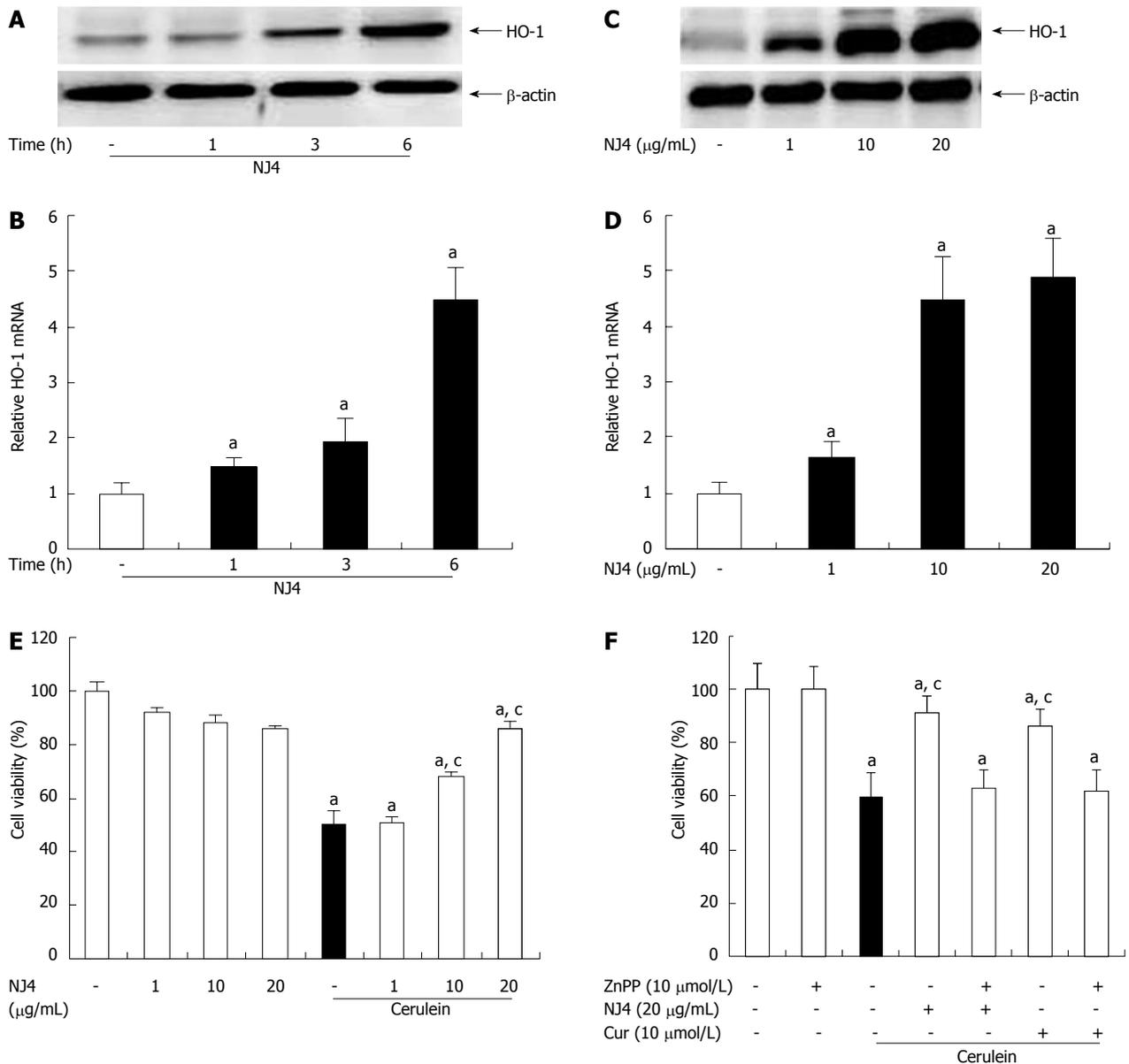


Figure 8 Effects of the 4th fraction of *Nardostachys jatamansi* on heme oxygenase-1 expression in acinar cells and cerulein-induced acinar cell death. Pancreatic acinar cells were pretreated with *Nardostachys jatamansi* (NJ) (20 $\mu\text{g/mL}$), and then the cells were harvested at the indicated time. A, B: The protein level (A) and mRNA level (B) of heme oxygenase-1 (HO-1) in pancreatic acini were measured. Pancreatic acinar cells were pretreated with the indicated dose of NJ4. Then, the cells were harvested at 6 h; C, D: The protein level (C) and mRNA level (D) of HO-1 in the pancreatic acini were detected. The pancreatic acinar cells were pretreated with the indicated dose of NJ4 and then stimulated with cerulein (10 nmol/L); E: After 6 h of cerulein stimulation, cell viability was measured as described in the experimental protocol. Pancreatic acinar cells were pretreated with ZnPP (10 $\mu\text{mol/L}$), an HO-1 inhibitor, for 1 h and then treated with NJ4 (20 $\mu\text{g/mL}$), curcumin (10 $\mu\text{mol/L}$); F: At 1 h after treatment, cerulein (10 nmol/L) was added; After 6 h of cerulein stimulation, cell viability was measured. The results were similar in 3 additional experiments. ^a $P < 0.05$ vs the saline treatment; ^c $P < 0.05$ vs cerulein treatment alone. Cur: Curcumin.

fractions on lipopolysaccharide (LPS)-induced inflammatory responses were examined in peritoneal macrophages. NJ4 significantly inhibited the LPS-induced production of inflammatory mediators such as nitric oxide (NO), IL-1, IL-6, and TNF- α (unpublished data). Therefore, NJ4 was chosen to examine its effect on pancreatitis. Also among the 3 fractions from NJ4, NJ4-2 inhibited the inflammatory mediators significantly. Thus, we also choose NJ4-2 as NJ4's candidate. NJ4 ameliorated the severity of AP and AP associated with lung injury and inhibited neutrophil infiltration, digestive enzymes, and cytokines. NJ4 also increased cell viability against cerulein

challenge. In the present study, we showed that the effect of NJ4 on pancreatitis was comparable with the effect of the total aqueous extract of NJ.

AP remains a challenging clinical problem in which mortality is significantly increased depending on its severity^[19]. Despite a few clinical trials with pharmacological agents, no effective treatment exists for AP. AP is commonly caused by excessive alcohol consumption, biliary tract disease, hereditary factors, certain medications, and invasive procedures of the biliary and pancreatic ducts^[20-23]. In this experiment, we used a well-established murine model. Mice with AP showed destruction in acinar

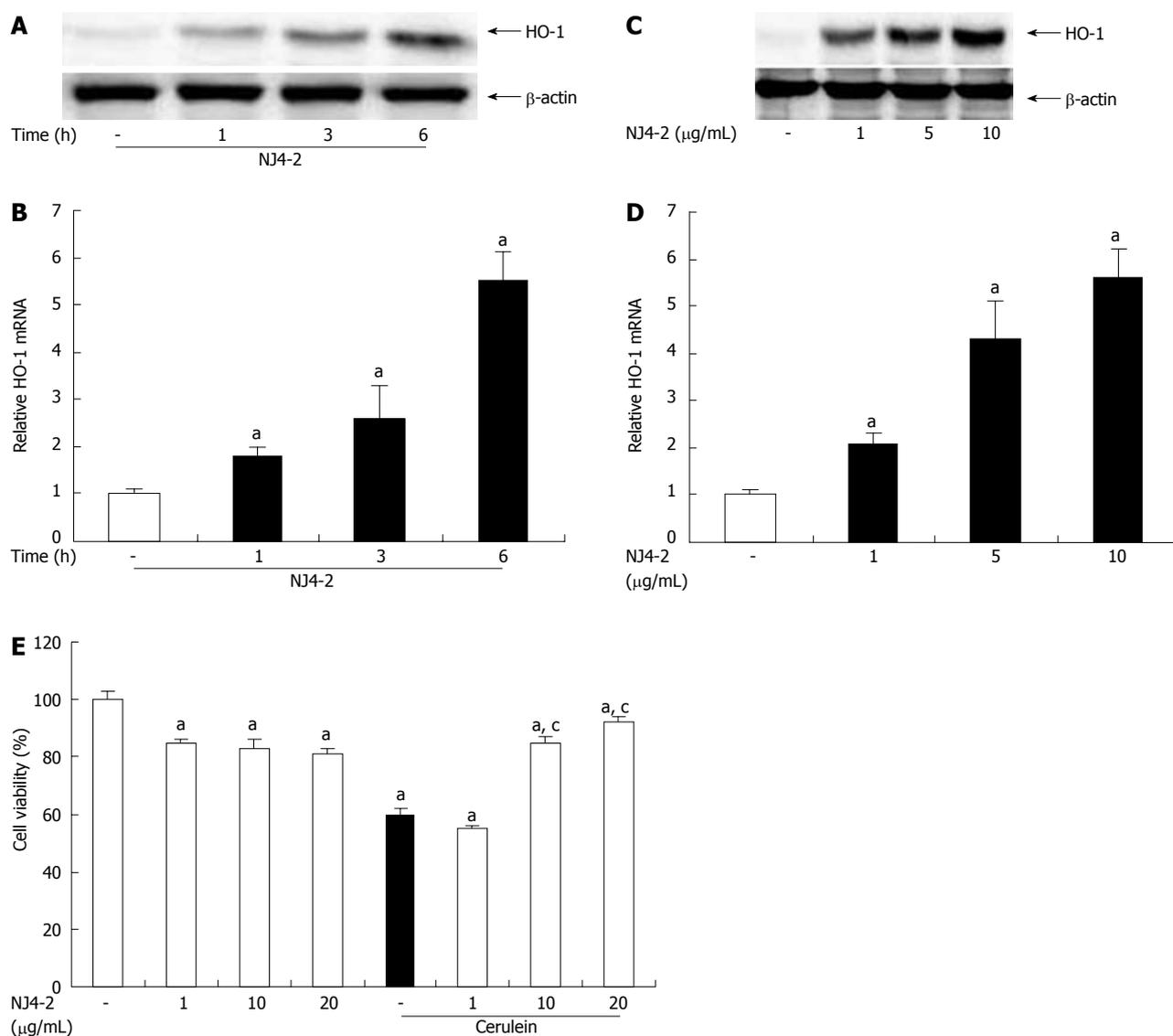


Figure 9 Effects of *Nardostachys jatamansi*-2 on heme oxygenase-1 expression in acinar cells and cerulein-induced acinar cell death. Pancreatic acinar cells were pretreated with *Nardostachys jatamansi* (NJ4)-2 (10 μg/mL), and then the cells were harvested at the indicated time. A, B: The protein level (A) and mRNA level (B) of heme oxygenase-1 (HO-1) in pancreatic acini were measured. Pancreatic acinar cells were pretreated with the indicated dose of NJ4-2; C, D: Then, the cells were harvested at 6 h, the protein level (C) and mRNA level (D) of HO-1 in the pancreatic acini were detected. The pancreatic acinar cells were pretreated with the indicated dose of NJ4 and then stimulated with cerulein (10 nmol/L); E: After 6 h of cerulein stimulation, cell viability was measured as described in the experimental protocol. The results were similar in 3 additional experiments. ^a*P* < 0.05 vs the saline treatment; ^c*P* < 0.05 vs cerulein treatment alone.

cell structure and increased neutrophil infiltration. The NJ4-pretreated mice with AP showed reduced acinar cell destruction and neutrophil infiltration (Figure 3).

The mortality rate in AP is approximately 20%-30%, and the major cause of AP-induced death is acute lung injury, referred to as acute respiratory distress syndrome. After cytokines, or the digestive enzymes from the pancreas, attack the lung alveolar cells, inflammation and vascular injury occurs in the lung^[8]. Cells that have infiltrated the pulmonary tissues cause breakdown of the pulmonary parenchyma^[24]. This lung attack is particularly damaging in the elderly, and if they survive an attack, their quality of life is impaired^[25-27]. Therefore, it is important to protect against lung injury caused by AP. In the present study, we showed that NJ4 treatment reduces lung injury and inflammation in AP (Figure 4).

Experimental pancreatitis models and pancreatitis patients showed increased IL and TNF-α levels^[28-30]. IL-1β, IL-6, and TNF-α play a pivotal role in AP, acting early in the disease course in addition to transitioning the acute inflammatory response to a chronic response^[31,32]. We have previously reported that the total aqueous extract of NJ could inhibit cytokine production in mice with AP and LPS-induced cytokine production in macrophages^[5,6]. Similar to the total aqueous extract of NJ, NJ4 inhibited serum and pancreatic cytokine production in mice with AP.

HO-1 is a stress-inducible enzyme, whereas its isoform HO-2 is constitutively expressed^[33]. HO-1 catabolizes heme into a free iron, carbon monoxide, and bilirubin/biliverdin^[34]. Studies have shown that HO-1 has protective and anti-inflammatory effects on AP^[35,36]. In the above

experiment, the administration of HO-1 inducers resulted in significant induction of HO-1 in a specific target organ, the pancreas, and reduction in pro-inflammatory cytokines, a major factor in AP regulation. We have already reported that the protective mechanism of the total water extract of NJ on AP involves the inhibition of MAPKs. Similarly, NJ4 inhibited the activation of MAPKs, but the inhibition was not significant (data not shown). Therefore, we hypothesize that the main protective mechanism of NJ4 is *via* HO-1 upregulation. HO-1 was up-regulated 1 h after NJ4 injection, and the upregulation was dose-dependent. The HO-1 up-regulation by NJ4 was comparable with that observed with curcumin. Furthermore, NJ4 induced expression of HO-1 in isolated pancreatic acinar cells at 3 h significantly; this finding is different from that of the *in vivo* experiment. In the *in vivo* experiment, NJ4 induced pancreatic HO-1 expression at 1 h significantly; however, in the *in vitro* experiment, NJ4 treatment led to the significant up-regulation of HO-1 at 3 h. We speculate that differences in metabolism between tissues and cells might be the reason for the difference in the expression time of HO-1 in the *in vivo* and *in vitro* experiments.

In this study, we suggest and evaluate the possible protective effect of NJ4 against AP. NJ4 treatment attenuated the severity of AP and lung injury associated with AP *via* the up-regulation of HO-1. The biological fraction of the total aqueous extract of NJ (NJ4) showed similar effects to those obtained with the total extract, which means that NJ4 might contain a compound useful for treating AP. Further fractionation and analyses are needed to identify this novel compound from NJ4.

COMMENTS

Background

Acute pancreatitis (AP) is a serious, unpredictable clinical problem, whose pathophysiology remains poorly understood. Therefore, drugs and therapies need to be developed.

Research frontiers

The paper previously reported that the total water extract of *Nardostachys jatamansi* (NJ) could protect against AP. This study aimed to determine if the fraction of NJ has the potential to ameliorate the severity of AP.

Innovations and breakthroughs

Nowadays, the treatment of acute pancreatitis is limited to protease inhibitors, and the pathogenesis is not well-understood. In this paper, the authors studied a possible candidate to develop as drug treatment for acute pancreatitis, in line with the previous report. Also the authors provided the regulating mechanisms in AP. This finding could strengthen up further studies of acute pancreatitis.

Applications

The papers, here, firstly provided the possible candidate of NJ. These results could provide the clinical basis for development of a drug or compound to treat acute pancreatitis.

Terminology

Acute pancreatitis: a sudden inflammation of the pancreas. It can have severe complications and high mortality despite treatment. While mild cases are often successfully treated with conservative measures and aggressive intravenous fluid rehydration, severe cases may require admission to the intensive care unit or even surgery to deal with complications of the disease process.

Peer review

This submission shows interesting results.

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Effect of Yiguanjian decoction on cell differentiation and proliferation in CCl₄-treated mice

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Abstract

AIM: To investigate the cellular mechanisms of action of Yiguanjian (YGJ) decoction in treatment of chronic hepatic injury.

METHODS: One group of mice was irradiated, and received enhanced green fluorescent protein (EGFP)-positive bone marrow transplants followed by 13 wk of CCl₄ injection and 6 wk of oral YGJ administration. A second group of Institute for Cancer Research mice was treated with 13 wk of CCl₄ injection and 6 wk of oral YGJ

administration. Liver function, histological changes in the liver, and Hyp content were analyzed. The expression of α -smooth muscle actin (α -SMA), F4/80, albumin (Alb), EGFP, mitogen-activated protein kinase-2 (PKM2), Ki-67, α fetoprotein (AFP), monocyte chemotaxis protein-1 and CC chemokine receptor 2 were assayed.

RESULTS: As hepatic damage progressed, EGFP-positive marrow cells migrated into the liver and were mainly distributed along the fibrous septa. They showed a conspicuous coexpression of EGFP with α -SMA and F4/80 but no coexpression with Alb. Moreover, the expression of PKM2, AFP and Ki-67 was enhanced dynamically and steadily over the course of liver injury. YGJ abrogated the increases in the number of bone marrow-derived fibrogenic cells in the liver, inhibited expression of both progenitor and mature hepatocyte markers, and reduced fibrogenesis.

CONCLUSION: YGJ decoction improves liver fibrosis by inhibiting the migration of bone marrow cells into the liver as well as inhibiting their differentiation and suppressing the proliferation of both progenitors and hepatocytes in the injured liver.

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Key words: Yiguanjian decoction; Bone marrow transplantation; Hepatic progenitors; Hepatocytes; Hepatic injury

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INTRODUCTION

Yiguanjian (YGJ) decoction, containing 6 herbs: *radices glehniae*, *radices ophiopogonis*, *radix angelicae sinensis*, *dried rehmannia root*, *lycium barbarum L* and *fructus meliae toosendan*, was first described in an ancient book which was printed in about the 18th century. The decoction has been used clinically for almost 3 centuries for the treatment of liver diseases in China. It is reported that the decoction is effective for various hepatic diseases of different etiologies^[1-3]. We previously reported that YGJ exerts significant therapeutic effects on CCl₄-induced chronic liver injury in rats, through mechanisms which inhibit hepatocyte apoptosis and activation of hepatic stellate cells, as well as regulating the function of Kupffer cells^[4]. Modified YGJ could also induce apoptosis in hepatic stellate cells, preventing liver fibrosis^[5]. However, little is known about the molecular and cellular mechanisms of YGJ which are responsible for chronic hepatic injury. This has also limited the use of YGJ in the clinic. In this study, we aimed to investigate the cellular and molecular mechanisms of action of this decoction in suppression of hepatic fibrosis, using a CCl₄-induced enhanced green fluorescent protein (EGFP) transgenic mouse model of chronic liver injury.

Liver fibrosis is a repair reaction to chronic liver injuries of varying etiologies. Many investigations have shown that bone marrow (BM) plays an important role in the progression of liver fibrosis. However it remains controversial whether the BM contributes to the promotion^[6] or regression of liver fibrosis^[7]. The cells in the liver derived from BM are also varied, and both parenchymal^[8] and nonparenchymal cells^[9-12] have been reported.

Upon liver injury, activated hepatic stellate cells (HSCs) expressing their marker protein α -smooth muscle actin (α -SMA) play a key role in the progression of hepatic fibrosis. However, the origins of HSCs are still obscure. In many studies, BM cells have been confirmed to differentiate into myofibroblasts both *in vitro* and *in vivo*. Besides liver, BM cells can contribute to the procession of many organs such as the lung, skin and kidney by virtue of differentiating into myofibroblasts in response to injury^[13]. Hepatocytes are the most abundant cells in liver and are always the initial target attacked by any injury factor which contributes to their loss in number. Some reports have shown that BM cells can differentiate into hepatocytes^[14] while others have shown that bone marrow-derived mesenchymal stromal cells support hepatocyte growth and proliferation^[15]. Kupffer cells in liver have diverse activities, and their role in the pathogenesis of liver injury remains controversial, as it has been demonstrated that these cells display pro-toxicant and hepato-protective functions. Two major classes of Kupffer cells have been identified, but only the BM-derived class is recruited into inflammatory foci^[12]. In the present study, we investigated the possibility of BM derivation of these cells in liver and the influence of YGJ on this process.

MATERIALS AND METHODS

Preparation of YGJ

All six herbs were chosen according to the Pharmacopoeia of the People's Republic of China (2000), and authenticated by a pharmacologist. The YGJ was prepared by the Shanghai Shuguang Hospital affiliated to the Shanghai University of Traditional Chinese Medicine. Briefly, YGJ herbs, including 2000 g *radices glehniae*, 2000 g *radices ophiopogonis*, 2000 g *radix angelicae sinensis*, 3600 g *dried rehmannia root*, 2400 g *lycium barbarum L* and 900 g *fructus meliae toosendan* were ground into a powder, and then decocted with boiling water. The filtrate was concentrated with a rotary vacuum evaporator, and freeze-dried to 4270 g, and stored at 4 °C. Before use, the filtrate was diluted with normal saline to a final concentration of 0.2682 g/mL.

Animals

Fifty-two male 5- to 6-wk-old Institute for Cancer Research (ICR) mice and 26 EGFP⁺ transgenic ICR mice (license number: SCXK, 2003-0003, Shanghai, China) were purchased from the Shanghai Animal Center of the Chinese Academy of Sciences. All mice were housed in the animal center of Shanghai University of Traditional Chinese Medicine and fed a standard pelleted diet and water *ad libitum*.

Bone marrow transplantation plus hepatic fibrosis model

EGFP transgenic ICR 5- to 6-wk-old mice were killed by dislocation of the cervical vertebrae and used as BM donors. Approximately 0.5×10^7 whole BM cells were isolated by flushing the bones of all four limbs of EGFP donors with a gauge needle containing Roswell Park Memorial Institute 1640 medium with 2% fetal bovine serum. Normal male 5- to 6-wk-old ICR mice served as recipients, after receiving whole body irradiation of 6.2 Gy in a divided dose 2 h apart. The recipient mice received a tail vein injection of BM cells immediately after the second session of radiation.

Five weeks after transplantation, peripheral blood samples were collected and analyzed to verify successful engraftment and reconstitution of BM in transplanted mice. Twenty-four recipient mice were then injected with CCl₄ as above and randomly divided into three groups: control group ($n = 9$), CCl₄ only ($n = 9$) and CCl₄ plus YGJ gavages for 6 wk ($n = 6$).

Mouse hepatic fibrosis model

Hepatic fibrosis was induced in 92 mice by subcutaneously injecting a 1:1 solution of CCl₄ and olive oil (CCl₄ and oil, supplied by the Chemical Agent Company of Shanghai, Shanghai, China) three times a week for 13 wk. At the end of the 7th week, CCl₄-injected mice were divided into two groups: CCl₄ injection only and CCl₄ injection plus YGJ gavages at a dose of 0.2682 g/100 g body weight. Some mice were sacrificed at the end of the 7th, 8th, 9th,

10th, and 13th week of the experiment to dynamically observe the damage. Twelve mice served as normal controls. Blood was collected from postcaval veins for measurement of serum alanine aminotransferase (ALT) activity and albumin (Alb) content. After weighing the entire livers, liver tissue specimens were taken from the right lobe of the liver and fixed in 10% phosphate-buffered formaldehyde, then routinely processed and embedded in paraffin for histopathology, while another two samples from each mouse were embedded in optimum cutting temperature compound and snap-frozen in liquid nitrogen for immunofluorescence. The rest of the livers were stored at -80°C .

Morphological analysis

Paraffin sections ($4\ \mu\text{m}$) were hydrated and stained for 20 min in Sirius red to identify interstitial collagen. Immunohistochemical staining was performed on liver tissue sections. After deparaffinizing in xylene and dehydrating through serial alcohol solutions, we processed the sections by microwave antigen retrieval and then incubated them with monoclonal primary antibodies against α -SMA (Sigma A2547), mitogen-activated protein kinase-2 (PKM2) (Cell signaling No. 3198) and Ki-67 (Abcam ab15580) at 4°C overnight. After washing, peroxidase-conjugated secondary antibody was added and incubated for 30 min. As a negative control, the primary antibody was replaced with phosphate buffered saline (PBS). All sections were stained with diaminobenzidine.

Immunofluorescence

After fixation in ice-cold acetone for 10 min and blocking with 10% goat serum (for Alb without serum block) for 30 min at room temperature (RT), $8\ \mu\text{m}$ cryostat sections were incubated for 1.5 h at RT with a polyclonal primary antibody against green fluorescence protein (GFP) (Cell Signaling, No. 2555). After rinsing with PBS, the secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG (H + L) (Invitrogen A11008) was added and sections were incubated for 1 h at 37°C .

For detection of α -SMA, the GFP-conjugated sections were blocked with 10% goat serum and incubated with monoclonal α -SMA (sigma A2547) at a dilution of 1:200 overnight at 4°C . The secondary antibody Alexa Fluor 633 goat anti-mouse (Invitrogen A21050) was used at a dilution of 1:200 for 1 h at 37°C .

For detection of Alb, $8\ \mu\text{m}$ cryostat sections were fixed in ice-cold acetone for 10 min, then incubated with the primary antibody, a goat polyclonal antibody (Santa Cruz Biotechnology, INC sc-46293), at 1:50 overnight at 4°C . After rinsing, the secondary antibody, donkey anti-goat 633 (Invitrogen A21082) was added at 1:200 for 1 h at 37°C . Following blocking with 5% BSA, the antibody to GFP and the Alexa Fluor 488-labeled secondary antibody described above were used as primary and secondary antibodies, respectively.

All the slides were washed 3 times for 5 min each in PBS between primary and secondary antibodies. The

slides were mounted in a mixture of PBS and glycerol.

Microscopy and image capture

For light microscopy, an Olympus IX70 microscope was used with Image-Pro Plus 6.1 image analysis software. For fluorescent microscopy, slides were visualized under an Olympus CKX41 fluorescent microscope. For confocal microscopy, a Leica laser confocal microscope equipped with a triple bandpass filter was used. The images were analyzed with ScnImage and quantified using the control group as the baseline.

Liver function and hydroxyproline

Liver Hyp content, serum ALT activity and Alb content were determined according to the protocols provided with the kits, which were supplied by the Institute of Biological Products Nanjing Jiancheng (Nanjing, China).

RNA isolation and real-time polymerase chain reaction

Total RNA was isolated with Trizol Reagent (Invitrogen lot: 1401902) and the quality was checked by spectroscopy. The A260/A280 ratio was between 1.9 and 2.1 for all samples.

For the reverse transcription reaction, cDNA was produced using a Revert Aid™ First Strand cDNA Synthesis Kit (Ferments Life Sciences, No. K1 621). Real-time polymerase chain reaction (PCR) was performed using a real-time PCR machine (Rotor-Gene RG-3000, Corbett Research) in quantitative mode. Initial denaturation was performed at 95°C for 10 s, followed by 40 cycles of 95°C denaturing for 5 s, primer annealing, and extension at 62°C for 20 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. The templates and primers were as follows: mouse monocyte chemotaxis protein (MCP)-1 (Forward: 5'-TCAGCCAGATGCAGTTAACGC-3' and Reverse: 5'-TCTGGACCCATTCCTTCTTGG-3', 185 bp); CC chemokine receptor 2 (CCR2) (Forward: 5'-CACGAAGTATCCAAGAGCTT-3', and Reverse: 5'-CATGCTCTTCAGCTTTTAC-3', 199 bp) and they were normalized to GAPDH (Forward: 5'-GAGCGAGACCCACTAACAT-3' and Reverse: 5'-TCTCCATGGTGGTGAAGACA-3', 86 bp). The SYBR Green fluorescence intensity of the specific double-strand reflecting the amount of amplicon formed was read after each elongation step at an additional acquisition temperature using Rotor-Gene Analysis Software V6.0. To verify the specificity of the amplification reaction, melting curve analysis was performed. For quantification analysis, all samples were analyzed in triplicate, and cycle threshold values for target genes and the housekeeping gene were determined for each sample. Relative gene expression was presented using the $2^{-\Delta\Delta\text{CT}}$ method.

Western blotting

Liver samples were homogenized and the supernatants were collected after centrifugation at $12\ 000 \times g$ at 4°C for 10 min. Protein concentrations were determined us-

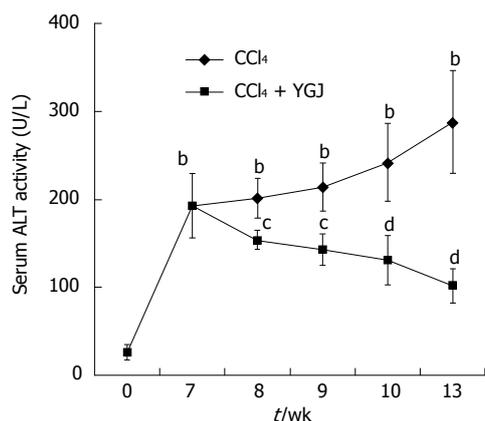


Figure 1 Serum alanine aminotransferase activity in mice. ALT activities in CCl₄ groups increased continuously but declined in YGJ + CCl₄ groups over the entire period. ^b*P* < 0.01 vs the week 0 control group; ^c*P* < 0.05, ^d*P* < 0.01 vs the same time-point CCl₄ group. Results are presented as mean ± SD. ALT: Alanine aminotransferase; CCl₄: Carbon tetrachloride; YGJ: Yiguanjian.

ing a protein assay kit II (BIO-RAD, No. 500-0 122), and 50 µg proteins were loaded and separated on 12% sulfate dodecyl sodium-polyacrylamide gels. The proteins were electrotransferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, United States) in transfer buffer at 4 °C for 1 h. Nonspecific binding to the membrane was blocked for 1 h at room temperature with 5% nonfat milk in Tween-80 Tris-buffered saline. The membranes were then incubated overnight at 4 °C with various primary antibodies in blocking buffer containing 5% nonfat milk, followed by incubation with horseradish peroxidase-conjugated secondary antibody (Chemicon Co) for 1 h in 5% nonfat milk dissolved in Tris-buffered saline. Membranes were then washed with Tris-buffered saline, signals were visualized using the enhanced chemiluminescence system (Amersham Biosciences) and the intensity of the bands was determined by scanning video-densitometry and normalized to the control group.

Statistical analysis

All results were expressed as mean ± SD. Data were analyzed using one-way analysis of variance. Student's *t* test was employed for the comparison of parameters and correlation analysis, respectively.

RESULTS

YGJ decoction dynamically protects against carbone tetrachloride-induced chronic liver fibrosis

Changes in serum ALT activity in the mice receiving CCl₄ which developed chronic liver injury were evaluated at several distinct time points. From week 7 to week 13, serum ALT activity in the CCl₄-injected mice increased continually and significantly compared with that in the control group (*P* < 0.01), but YGJ markedly reduced serum ALT activity even after just one week of administration (*P* < 0.05), and ALT was reduced to 50% of that in the control CCl₄-injected group (*P* < 0.01) after 6 wk of

YGJ administration (Figure 1).

The results of Sirius red staining showed that only a little collagen was present in the area of the portal and central veins in normal mice, while over the course of the study, bridging collagen connecting the central veins and neighboring portal areas increased continually, deposition of collagen fibers was steadily enhanced in livers throughout the entire experimental period, and pseudonodules were formed by the end of the study. In contrast, in the YGJ-treated group, collagen deposition in the livers was significantly attenuated. Semi-quantification of the collagen staining showed that collagen levels increased sharply after CCl₄ injection, reaching 3.5-fold above basal values by week 7, and peaking 6.5-fold above basal values by week 13. YGJ treatment maintained and significantly reduced the increase in collagen. For example, after 6 wk of YGJ treatment, the level of collagen was about 50% lower in YGJ-treated mice than in controls at the same time point (Figure 2A and B).

The results of Sirius red staining also showed that Hyp concentration in livers was significantly increased from week 8 and increased steadily up to week 13. However, in the YGJ-treated group, the content of Hyp in livers significantly decreased as compared with that at the same time-point in the CCl₄-injected group (Figure 2C). These findings support our previous results showing that YGJ protected hepatic function and attenuated collagen deposition during chronic liver injury in rats^[4].

YGJ decoction continuously inhibits α-smooth muscle actin (+) cells in chronically-injured liver

During development of chronic liver injury, accumulation of myofibroblastic cells and increased production of extracellular matrix were the main characteristics. It is clear that α-SMA-positive activated HSCs, the main source of extracellular matrix, play a key role in this pathological process. Our studies of immunohistochemical staining showed that the accumulation in livers of the myofibroblast marker α-SMA increased steadily and then remained at this level until the end of the study, peaking at the 13th week when it was distributed along fibrotic areas (Figure 3A and B). This change was consistent with the expression of α-SMA protein analyzed by Western blotting, which also increased after CCl₄ injection and peaked at the end of the study (Figure 3C and D). The progressive expression of α-SMA in this study is consistent with earlier reports which demonstrated that early increases in the number of activated HSCs, followed by enhanced collagen synthesis and decreased collagen degradation, combined to cause accumulation of collagen matrix following liver injury. YGJ treatment significantly attenuated the induction of α-SMA, even after only 1 wk of YGJ administration (*P* < 0.01) and it continually declined up to 6 wk of YGJ gavages (*P* < 0.01) (Figure 3A and B), a result which was similar to that obtained from the Western blotting of α-SMA in livers (*P* < 0.01) (Figure 3C and D).

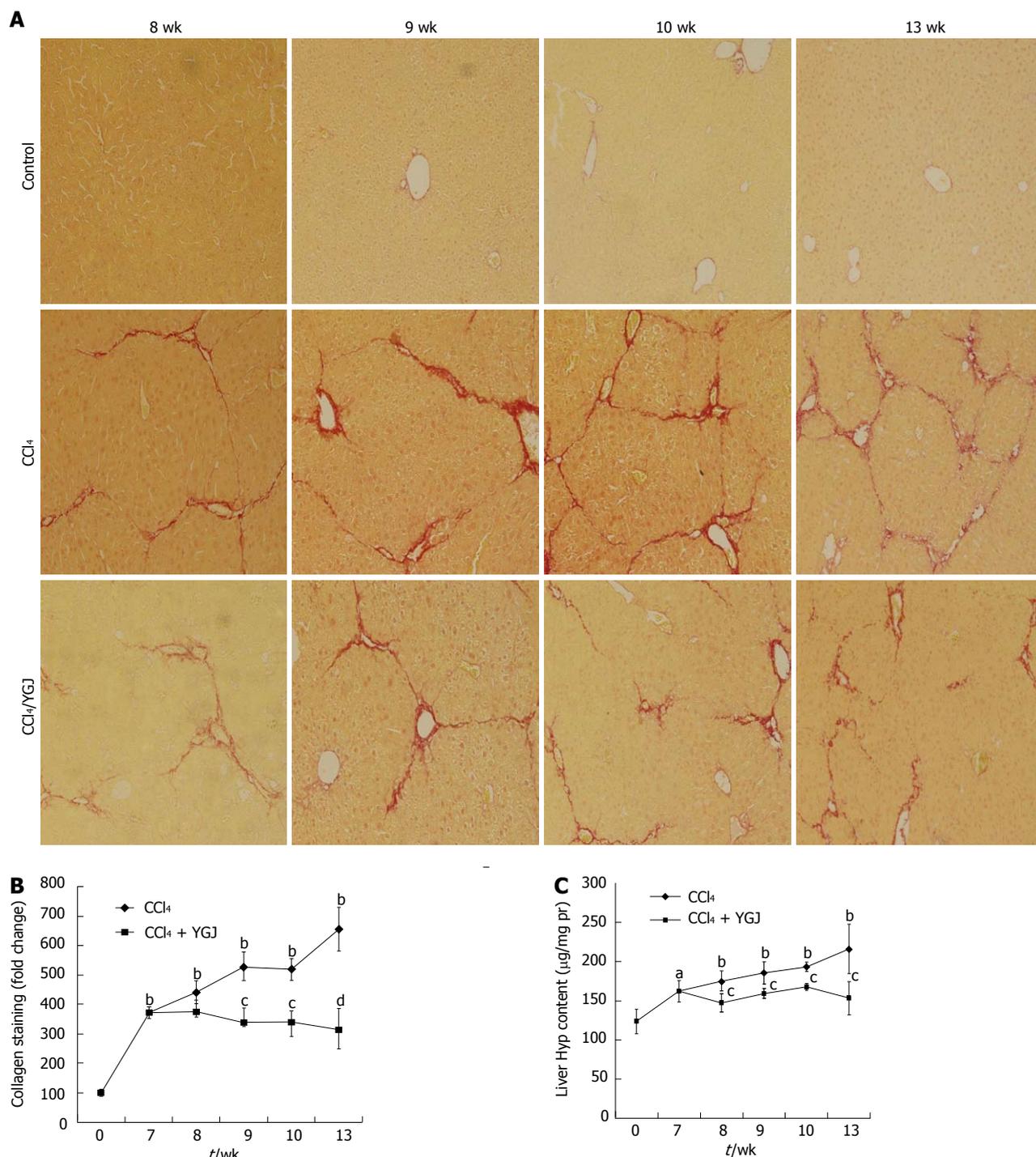


Figure 2 Collagen staining with Sirius red and Hyp content. A: Collagen staining. Week 0 control mice have limited collagen in the portal area, but fibrous septa bridging the portal tracts were found in the livers of CCl₄-treated mice, and this was significantly attenuated in YGJ-treated mice, × 200; B: Semi-quantification of collagen staining, the week 0 control group level was set as the basal level; C: Hyp content in the liver. Results of collagen staining and Hyp content show that they both continuously increased in the CCl₄ group but declined in the YGJ + CCl₄ group over the entire period. ^aP < 0.05, ^bP < 0.01 vs week 0 control group; ^cP < 0.05, ^dP < 0.01 vs the same time-point CCl₄ group. Results are presented as mean ± SD. CCl₄: Carbone tetrachloride; YGJ: Yiguanjian; Hyp: Hydroxyproline.

YGJ decoction inhibits the migration of bone marrow cells into chronically injured liver

It has been reported that bone marrow cells or their progeny can circulate into various damaged organs and differentiate into myofibroblasts or fibrocytes^[13]. In the liver, several studies have suggested that bone marrow contributes to scar forming cells of various types, such as the HSCs^[10].

Our results further confirmed these reports. Following CCl₄ injection into the mice which received bone marrow transplantation, the number of EGFP⁺ cells also increased steadily in injured livers, which were mainly distributed in the fibrotic areas. In contrast, only a few EGFP⁺ cells were found in the vehicle-treated control mice, who received only olive oil but no CCl₄ injection after bone marrow re-

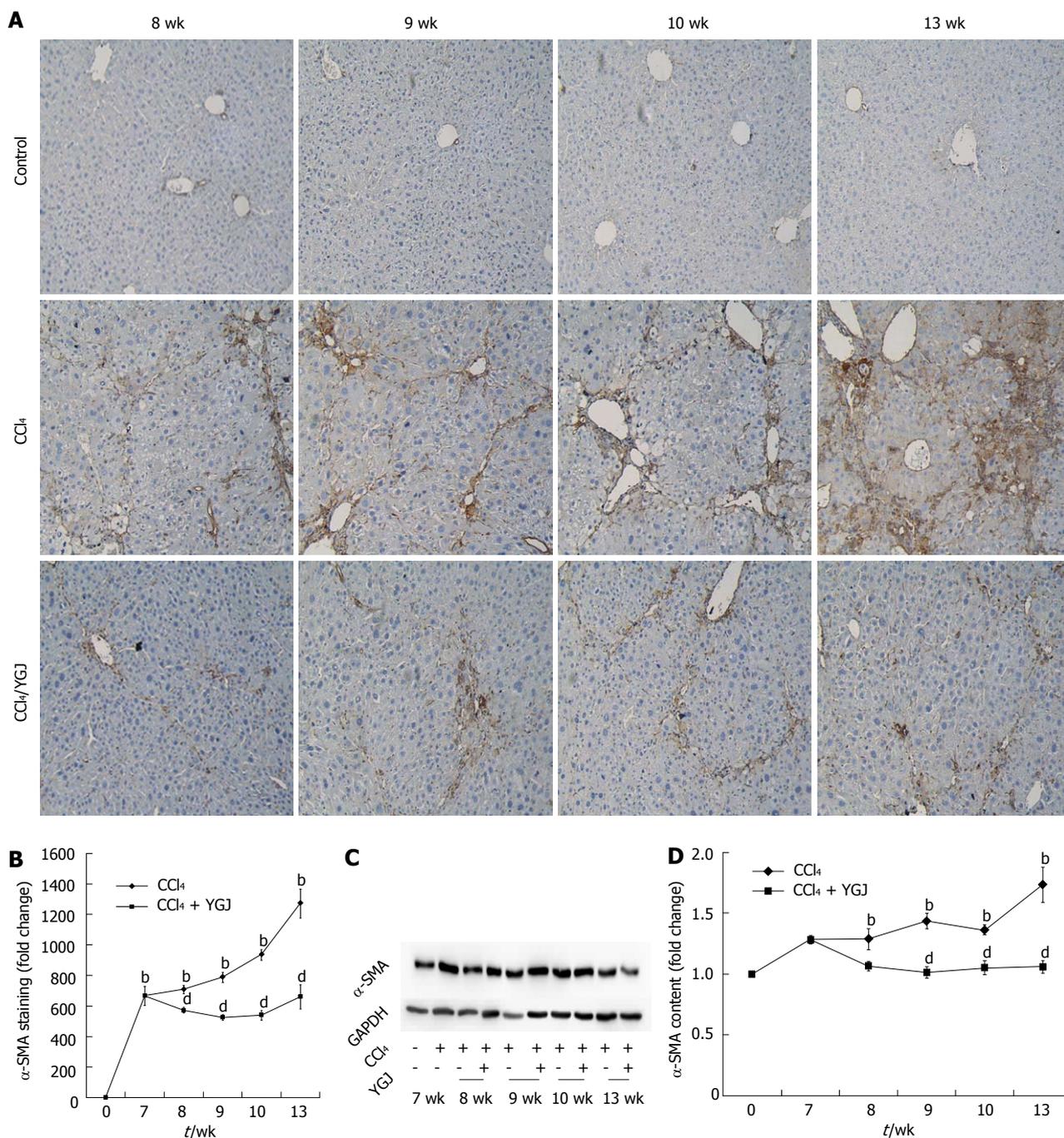


Figure 3 Immunostaining and Western blot analysis of α -smooth muscle actin in liver tissues. A: α -smooth muscle actin (α -SMA) immunostaining, $\times 200$; B: Semi-quantification of α -SMA positive area, with the level in the week 0 control group set as the basal level; C: Western blotting of α -SMA; D: Semi-quantification of the α -SMA Western blot results, with the week 0 control group level set as the basal level. ^b $P < 0.01$ vs week 0 control group; ^d $P < 0.01$ vs the same time-point CCl₄ group. Results are mean \pm SD. CCl₄: Carbone tetrachloride; YGJ: Yiguanjian; GAPDH: Glycerlaldehydes-3-phosphate dehydrogenase.

constitution. In YGJ-treated mice, the number of EGFP⁺ cells decreased markedly as compared with the CCl₄-injected mice at the same time point. Moreover, unlike the distribution of EGFP⁺ cells which were mainly found in the fibrotic area in CCl₄-injected mice, the EGFP⁺ cells were distributed not only in mesenchymal but also in parenchymal areas in YGJ-treated mice (Figures 4-6).

YGJ decoction-mediated bone marrow-derived cell differentiation in chronically injured liver

In accordance with the results of the dynamic CCl₄ injec-

tion model without BM transplantation, both the number and distribution of α -SMA⁺ cells increased constantly over the course of liver injury in mice with bone marrow reconstitution. Treatment with YGJ reduced the number of α -SMA⁺ cells in fibrotic livers. The results of EGFP/ α -SMA double staining showed that there were almost no double positive cells in the vehicle control mice. In contrast, the number of the EGFP⁺/ α -SMA⁺ cells increased over time in all mice after receiving CCl₄ injections for 7 wk ($P < 0.01$) and remained high to the final time-point ($P < 0.01$); moreover, the EGFP⁺/ α -SMA⁺ cells were mainly

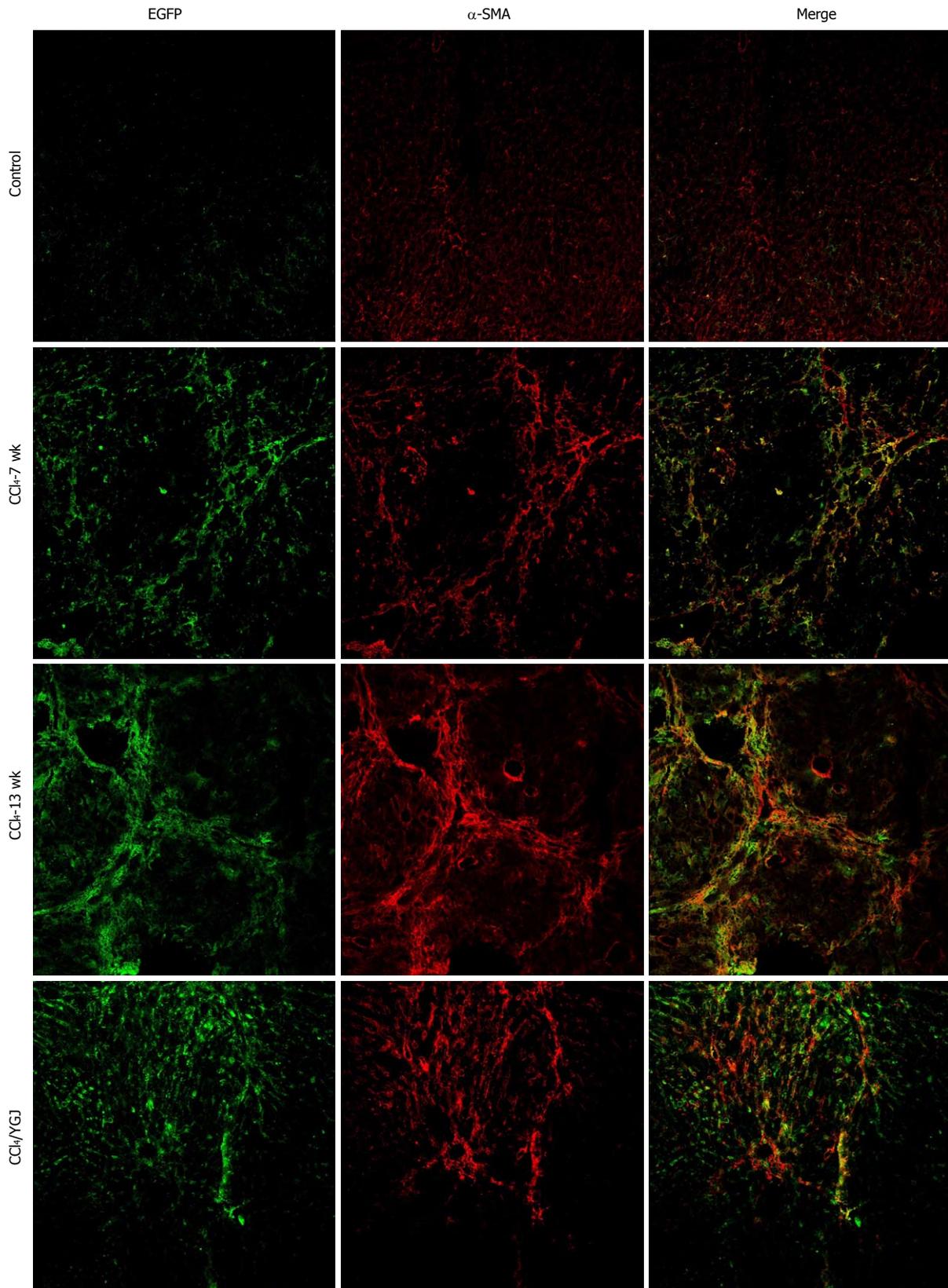


Figure 4 Immunofluorescent colocalization of enhanced green fluorescent protein and α -smooth muscle actin in CCl_4 -induced chronic liver injury in bone marrow chimera mice, $\times 200$. EGFP is shown in green and α -smooth muscle actin (α -SMA) in red. A yellow color confirms colocalization in the merged images. The number of EGFP/ α -SMA double positive cells was found to increase in all mice after receiving CCl_4 injection and they were mainly found in the areas of scarring. In contrast, the number of EGFP⁺/ α -SMA⁺ cells decreased in the YGJ treatment group. EGFP: Enhanced green fluorescent protein; YGJ: Yiguanjian.

found in the areas of scarring. However, YGJ treatment significantly decreased the number of EGFP⁺/ α -SMA⁺

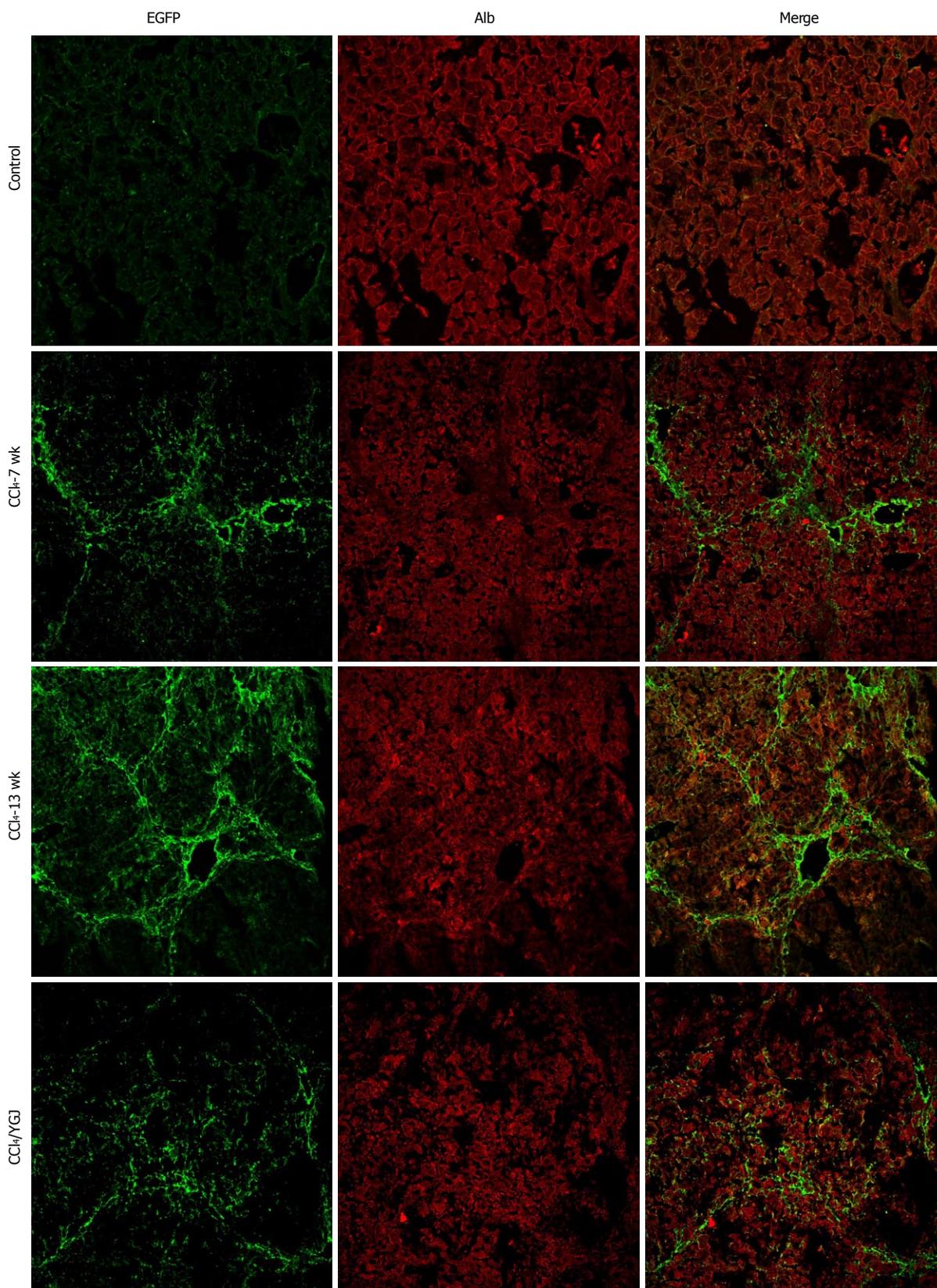


Figure 5 Immunofluorescent colocalization of enhanced green fluorescent protein and albumin in CCl₄-induced chronic liver injured bone marrow chimera mice, × 200. EGFP is shown in green and Alb in red. No yellow color is visible in the merged images in control, CCl₄ or CCl₄ + YGJ groups. EGFP: Enhanced green fluorescent protein; YGJ: Yiguanjian; Alb: Albumin.

cells as compared with the CCl₄-injected controls at the same time-points ($P < 0.01$) (Figure 4).

Because of the apparent increase in the number of EGFP⁺ cells in parenchymal areas in YGJ-treated mice

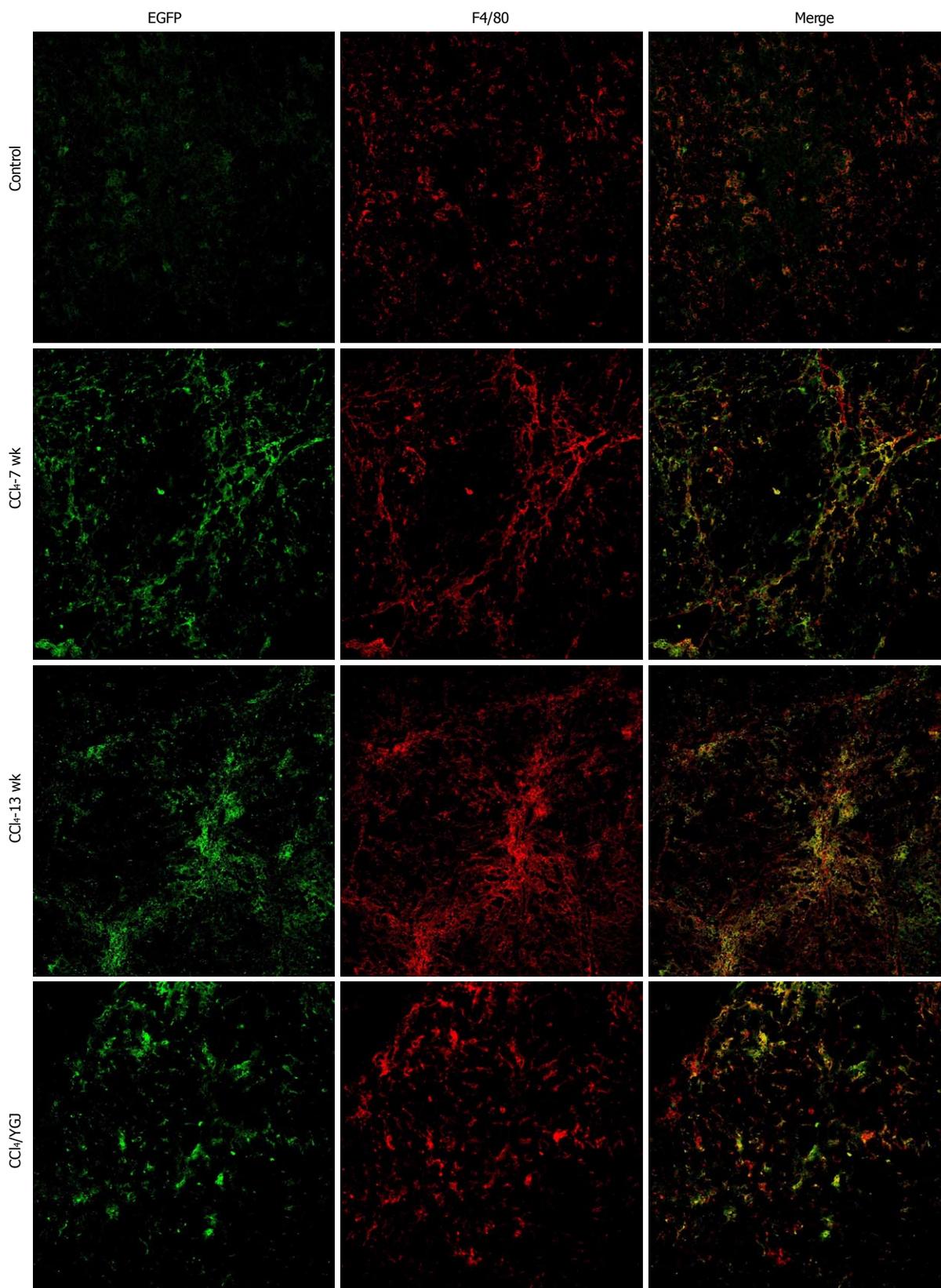


Figure 6 Immunofluorescent colocalization of enhanced green fluorescent protein and F4/80 in CCl₄-induced chronic liver injured bone marrow chimera mice, × 200. EGFP is shown in green and F4/80 in red. A yellow color confirms colocalization in the merged images. The number of EGFP/F4/80 double positive cells was found to increase in all mice after receiving CCl₄ injection. In contrast, the number of EGFP⁺/F4/80⁺ cells decreased in the YGJ treatment group. EGFP: Enhanced green fluorescent protein; YGJ: Yiguanjian.

compared with CCl₄-injected mice, we hypothesized that YGJ could stimulate the EGFP⁺ cells from bone

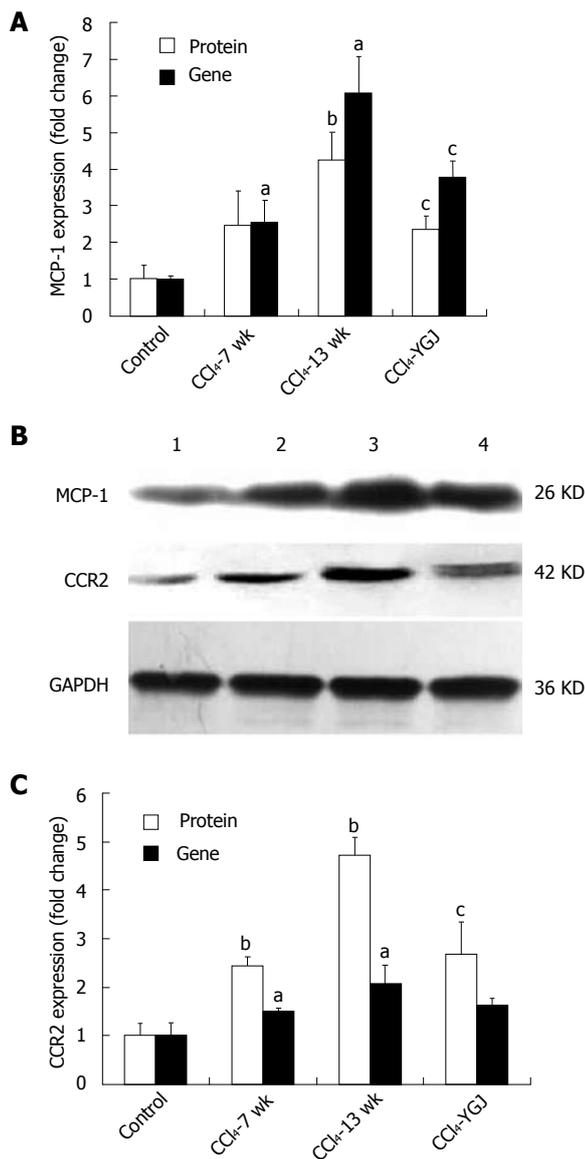


Figure 7 Gene expression analyzed by real-time polymerase chain reaction and protein expression analyzed by Western blotting of monocyte chemotaxis protein-1 and CC chemokine receptor 2 in CCl₄-induced chronic liver injured bone marrow chimera mice. **A:** MCP-1 gene and protein expression. For both genes the relative expression is quantified using the control group as the basal level; **B:** MCP-1 and CCR2 bands by Western blotting. Band 1, control group; band 2, CCl₄ 7 wk group; band 3, CCl₄ 13 wk group; and band 4, CCl₄ + Yiguanjian (YGJ) group; **C:** CCR2 gene and protein expression. For both genes the relative expression is quantified using the control group as the basal level. MCP-1: Monocyte chemotaxis protein-1; CCR2: CC chemokine receptor 2; GAPDH: Glyceraldehydes-3- phosphate dehydrogenase. ^a*P* < 0.05, ^b*P* < 0.01 vs week 0 control group; ^c*P* < 0.05 vs the same time-point CCl₄ group.

marrow to differentiate into hepatocytes. To assess the possibility of YGJ stimulating bone marrow cells to differentiate into hepatocytes, we investigated the presence of EGFP⁺/Alb⁺ cells by double staining. In the CCl₄-injected control mice, the number of EGFP⁺ cells increased steadily over time and the cells were distributed mainly in mesenchymal areas, but Alb⁺ cells were mainly distributed in parenchymal areas, and there was no

overlap between EGFP⁺ cells and Alb⁺ cells, indicating that no EGFP⁺/Alb⁺ cells were present in chronically injured liver. No EGFP⁺/Alb⁺ cells were present in the vehicle control mice or in the YGJ-treated mice (Figure 5). These results seemingly suggest that no hepatocytes were derived from bone marrow in our study.

To further investigate the mechanisms of YGJ mediation of bone marrow cell differentiation in chronic liver injury, we explored the possibility that BM cells differentiated into other main types of cells in the liver, such as Kupffer cells. The EGFP⁺/F4/80⁺ cells represented BM-derived Kupffer cells in our experiments. In the vehicle control mice, despite of a little F4/80 expression, virtually no EGFP⁺/F4/80⁺ cells were present in the liver. However, after 7 wk of CCl₄ injection, coexpression of EGFP and F4/80 increased significantly (*P* < 0.01) and remained at a high level up to 13 wk. These results indicated that some Kupffer cells differentiated from BM cells as the hepatic injury progressed. With YGJ treatment, both EGFP and F4/80 were expressed in parenchymal areas, and some of the areas were overlapped (Figure 6). However, there were fewer EGFP⁺/F4/80⁺ cells in the YGJ-treated mice than in the CCl₄-injected mice (*P* < 0.01).

YGJ decoction suppresses monocyte chemotaxis protein-1 and CCR2 expression in fibrotic liver

MCP-1 was identified as a factor likely to be responsible for stem/progenitor cell recruitment. Therefore, we analyzed the expression of MCP-1 during CCl₄-induced fibrogenesis. For the gene and protein expression, results were normalized to expression in the liver at the time of CCl₄ injection (0 wk), for example the control group mRNA level of MCP-1 reached 2.8-fold after 7 wk (*P* < 0.05) and 6.7-fold after 13 wk of CCl₄ injection (*P* < 0.05) (Figure 7A). Western blotting showed that MCP-1 protein was increased markedly (approximately 4 fold) after 13 wk of CCl₄ injection (Figure 7A and B). YGJ treatment significantly reduced the expression of both MCP-1 gene and protein (Figure 7A and B). CCR2 is the specific receptor of MCP-1, and both Kupffer cells and HSCs, but not hepatocytes, have been shown to express CCR2 in liver^[16]. In our study, the gene expression of CCR2 continuously increased over the course of the experiment (*P* < 0.05), which was in accordance with its protein expression (*P* < 0.01), and treatment with YGJ only suppressed expression at the protein level (*P* < 0.05) but not at the gene level (Figure 7B and C). These data demonstrate that more exogenous stem/progenitor cells were recruited into chronically injured livers compared with the controls, but that YGJ treatment decreased the number of exogenous stem/progenitor cells in the liver.

YGJ decoction decreases proliferation of liver epithelial progenitor cells and mature hepatocytes

It is important to determine whether the proliferative activity of hepatocytes increased during hepatic injury since the hepatocytes are the most numerous cells in

liver and have potent proliferative activity. Ki-67 nuclear accumulation increased constantly in hepatocytes after liver injury, reaching 23-fold as compared with the basal level of controls at week 13, the end point of the study (Figure 8A and B). This finding means that the number of mature hepatocytes increased steadily and significantly after CCl₄ injury, and indicates that mature hepatocytes also play a part in responding to CCl₄-induced liver injury by increasing their proliferative activity. However, even after 1 wk of YGJ treatment, the number of Ki-67 positive hepatocytes was dramatically reduced ($P < 0.01$), and by the 13th wk, the number accounted for only 12.6% of those in the CCl₄ injection group at the same time point, which is a reduction of approximately 87% compared with the CCl₄-treated group (Figure 8A and B). These findings suggest that YGJ treatment significantly reduced the number of hepatocytes accumulated after CCl₄ injury.

Serum Alb concentration (34.5 ± 6.9 g/L, control group) decreased by week 7 after hepatic injury (29.4 ± 3.5 g/L), and continued to decrease steadily at week 8 (26.4 ± 4.5 g/L, $P < 0.05$), week 9 (25.3 ± 3.2 g/L, $P < 0.01$), week 10 (28.9 ± 2.2 g/L) and week 13 (25.5 ± 2.40 g/L, $P < 0.01$). However, in YGJ-treated mice, the serum Alb concentration increased markedly compared with that at week 9 ($P < 0.05$) and week 13 ($P < 0.01$) (Figure 8). Similarly, Western blotting results of Alb also showed that Alb protein expression in injured livers continually declined and reached its lowest level by the end of the experiment, at only 64% of the basal level. In contrast, treatment with YGJ steadily enhanced its protein level so that it reached 90% of the basal level by the end of the experiment.

During the process of liver regeneration after injury, liver epithelial progenitor cells were induced to proliferate to compensate for the missing number of parenchymal hepatocytes. Hence, hepatic expression of progenitor markers, such as AFP and PKM2^[17,18], were used in our experiment to assess the proliferation of hepatic progenitor cells after liver injury. PKM2 expression increased steadily after CCl₄ injection as shown by immunostaining and Western blotting analysis, spanning 6 time points over 13 wk. Our data revealed that after 7 wk of CCl₄ injection, PKM2 expression increased significantly ($P < 0.01$) and thereafter remained at a high level throughout the entire experimental period (Figure 9A, B and D). These enhancements were accompanied by an increase in AFP expression (Figure 9C and E). Treatment with YGJ significantly attenuated the increase in the progenitor markers PKM2 and AFP (Figure 9). These findings support the concept that after CCl₄ injection, hepatic injury promotes accumulation of liver progenitor cells.

DISCUSSION

YGJ can ameliorate chronic liver injury in some animal models of CCl₄-induced liver damage in mice. In the course of the experiment, serum ALT activity, deposi-

tion of collagen fibers and Hyp content were continuously increased, and had formed pseudo-nodules by the end of the study. In contrast, all these markers decreased significantly after YGJ treatment. These results strongly suggest that YGJ can block CCl₄-induced chronic liver injury and exhibit a favorable therapeutic effect in mice.

Any etiologically chronic liver injury could result in activation of myofibroblasts, which are the main source of extracellular matrix (ECM) and finally lead to fibrosis or cirrhosis. We consider myofibroblasts to be a target of therapeutic liver fibrosis because they play such a key role in liver fibrogenesis. In our mouse model, α -SMA expression in mice increased in a dynamic manner after CCl₄ injection, and reached a peak at week 13. In contrast, α -SMA expression was remarkably and constantly suppressed after YGJ treatment.

Because myofibroblasts are thought to be heterogeneous in origin, both intrahepatic and BM-derived sources are important in the development of fibrosis^[19]. To assess the function of the BM in supplying myofibroblasts in cases of chronic liver injury, we examined the formation of BM-derived myofibroblasts and found that BM-derived EGFP-positive cells time-dependently increased over the course of the study, reaching a peak at week 13, and they were mainly localized along the fibrous septa, in accordance with the course of fibrogenesis. Furthermore, the number of EGFP and α -SMA double positive cells increased time-dependently and they were scattered at the fibrous septa. Therefore, we concluded that the EGFP and α -SMA positive cells are of BM origin and that BM cells can migrate and differentiate into myofibroblasts in the damaged liver. On the other hand, the number of both EGFP-positive cells and the EGFP- α -SMA positive cells decreased steadily after YGJ treatment, which suggested that YGJ inhibited the migration and differentiation of BM cells into myofibroblasts.

Kupffer cells facilitate liver fibrogenic processes either by secreting fibrotic factors or by increasing the production of tissue inhibitor of metalloproteinases to reduce ECM degradation^[20]. The origin of Kupffer cells was thought to be recruitment from the bone marrow to the liver^[21] or from intrahepatic precursor cells that exist in the liver^[21]. However, only the former population can be recruited into inflammatory foci in response to inflammation^[22]. Our results indicate that the number of EGFP and F4/80 double positive cells begins to increase after CCl₄ injection, reaching a peak at week 13, and that the cells are distributed along the fibrotic septa. The number of EGFP and F4/80 double positive cells decreased significantly after YGJ treatment. This revealed that YGJ inhibited liver fibrogenesis by mediating BM differentiation into Kupffer cells in the liver.

It has also been reported that various components of the bone marrow can differentiate into hepatocyte-like cells, causing a decrease in liver fibrosis^[23,24]. The results from previously published reports concerning whether hepatocytes were BM-derived or not were conflicting.

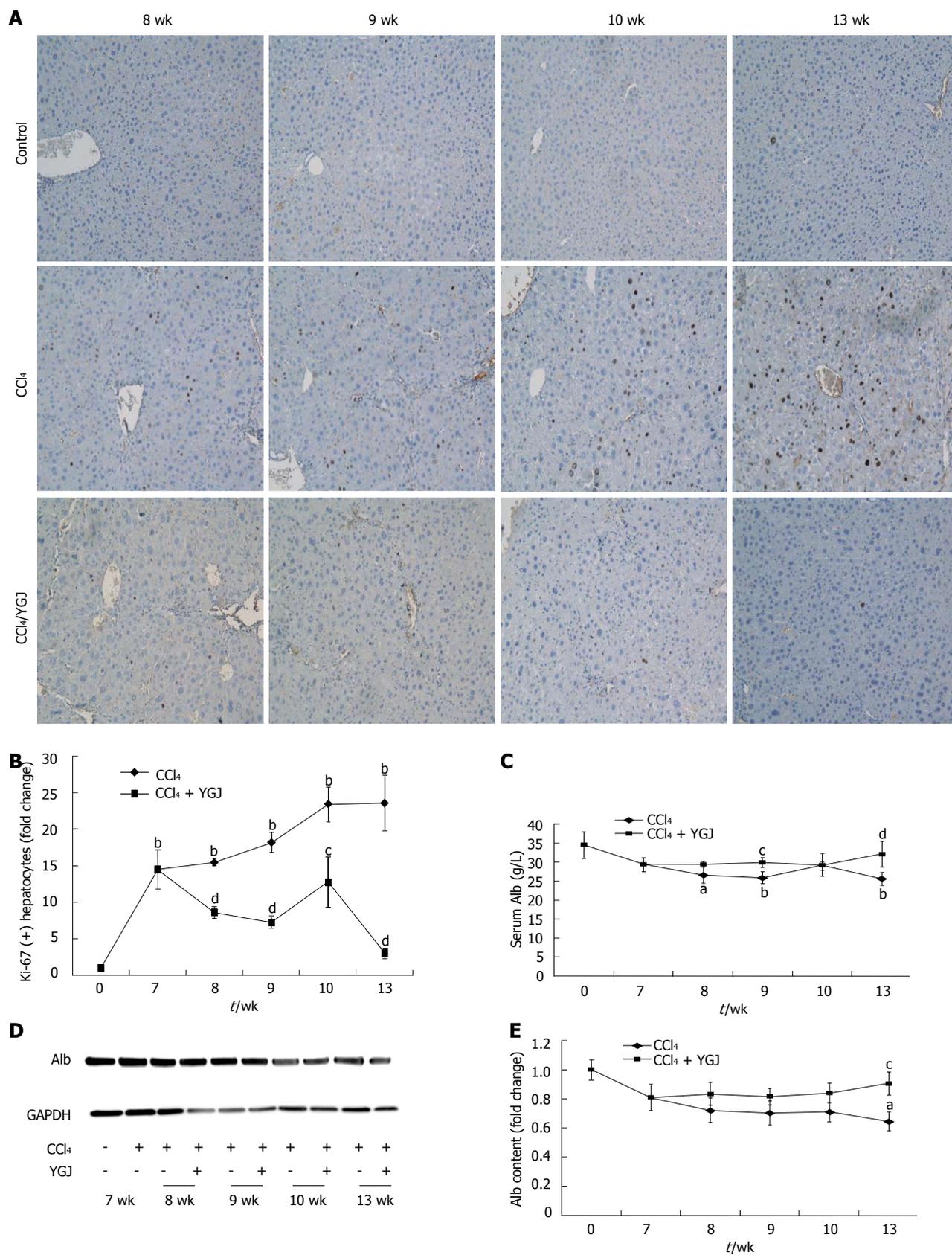


Figure 8 Hepatocyte function. A: Ki-67 immunostaining in liver tissues, $\times 200$; B: Semi-quantification of Ki-67 staining, with the week 0 control group level as the basal level; C: Serum Alb content; D: Liver Alb Western blotting bands; E: Semi-quantification of Alb based on Western blotting results, with the week 0 control group level as the basal level. ^a $P < 0.05$, ^b $P < 0.01$ vs week 0 control group; ^c $P < 0.05$, ^d $P < 0.01$ vs the same time-point CCl₄ group. Results are presented as mean \pm SD. GAPDH: Glyceraldehydes-3-phosphate dehydrogenase; YGJ: Yiguanjian; Alb: Albumin.

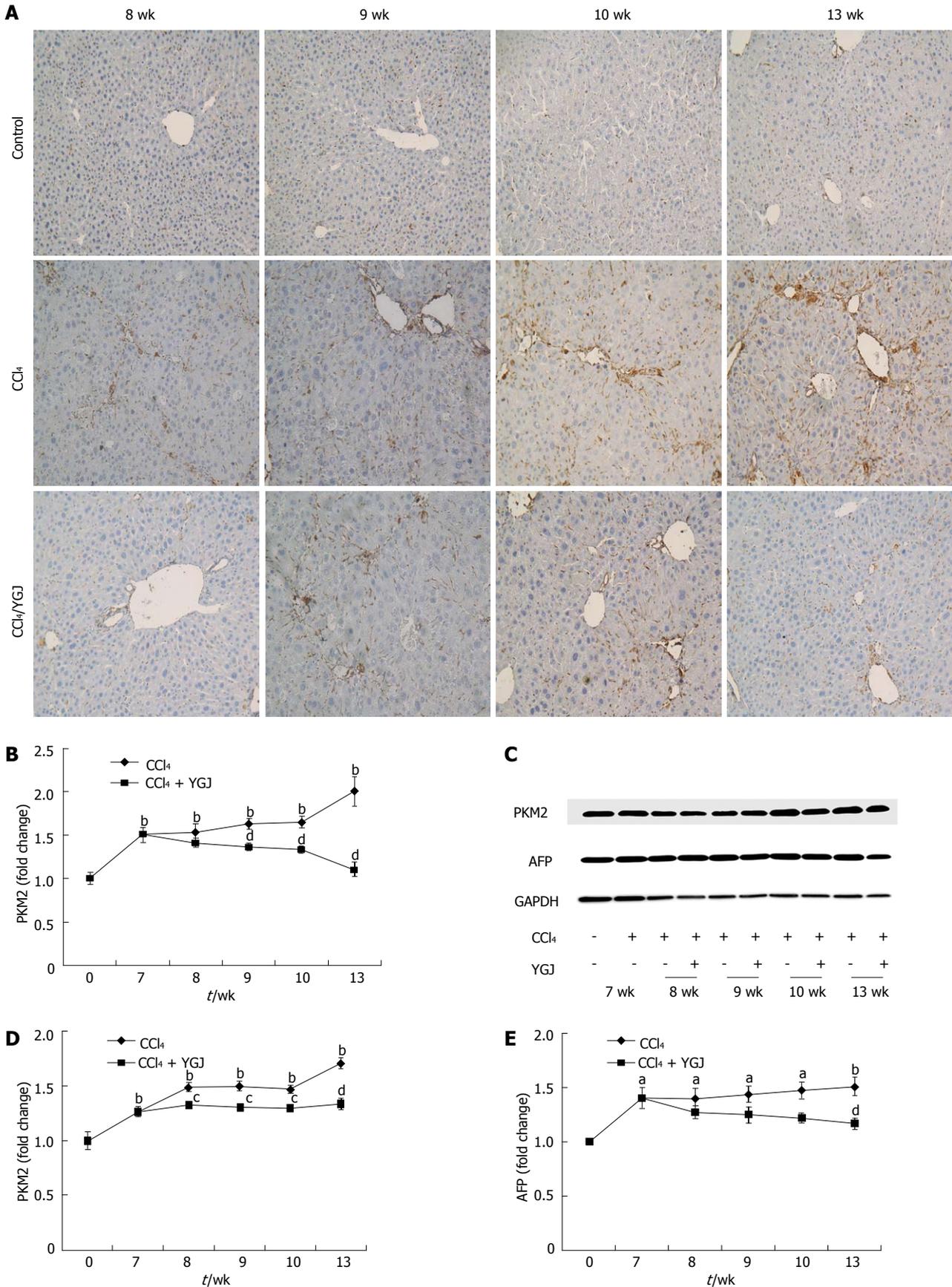


Figure 9 Expression of progenitor markers in liver tissues. A: PKM2 immunostaining, $\times 200$; B: Semi-quantification of PKM2 staining, with the week 0 control group level as the basal level; C: PKM2 and AFP Western blotting bands; D: Semi-quantification of PKM2 in Western blotting results, with the week 0 control group level as the basal level; E: Semi-quantification of Western blotting results of PKM2, with the week 0 control group level as the basal level. ^a $P < 0.05$, ^b $P < 0.01$ vs week 0 control group; ^c $P < 0.05$, ^d $P < 0.01$ vs the same time-point CCl₄ group. Results are presented as mean \pm SD. GAPDH: Glyceraldehydes-3-phosphate dehydrogenase; YGJ: Yiguanjian; PKM2: Mitogen-activated protein kinase-2; AFP: α fetoprotein.

Our results revealed virtually no EGFP-Alb double positive cells in any of the mice, whether they were in the control, CCl₄-damaged or YGJ treatment group.

Chemokines and their receptors play a central role in the regulation of cell migration. MCP-1 and its receptor CCR2 not only contribute to the recruitment of BM cells to liver but also have been identified as profibrotic mediators^[25,26]. Increased MCP-1 is associated with HSCs and macrophage recruitment^[27,28]. In the present study, MCP-1 increased constantly over the course of fibrogenesis. Bone marrow F4/80⁺ cells are the major producers of MCP-1^[29], and we observed that the number of bone marrow F4/80⁺ cells increased following liver injury. These results revealed that production of MCP-1 by bone marrow F4/80⁺ cells recruited into liver was one of the mechanisms underlying CCl₄-induced liver fibrosis. YGJ treatment significantly decreased the number of bone marrow F4/80⁺ cells and the level of MCP-1 expression in accordance with the improvement of liver fibrosis.

CCR2 promotes fibrosis^[30,31] and is expressed on resident liver cells including hepatic stellate cells and Kupffer cells, but not hepatocytes^[16]. CCR2 expression increased over the time-course of CCl₄ injection, similarly to MCP-1 expression. YGJ treatment markedly inhibited CCR2 expression.

In order to further investigate the mechanisms of YGJ antifibrotic function in CCl₄ injury, we took into account the role of mature hepatocytes. Because various etiologies of liver injury involve hepatocytes as the target, mature hepatocytes have stem cell functions, giving them the ability to re-enter the cell cycle rapidly after liver injury has occurred^[32]. The number of proliferative hepatocytes increased constantly after CCl₄ injection, reaching a peak at week 13. Unlike the proliferative behavior of mature hepatocytes during injury, both the serum content and expression of Alb protein, which is uniquely synthesized by hepatocytes, in the liver declined continuously over the time course of liver injury. It would be reasonable to assume that the proliferation of hepatocytes in injured liver is a kind of compensatory response to their reduction in number. In contrast, hepatocyte proliferation was suppressed steadily after YGJ administration, but the Alb content in both serum and liver increased continuously. It seems unnecessary to initiate mature hepatocyte proliferation after YGJ treatment, since YGJ works as an inhibitor of hepatocyte apoptosis in CCl₄-induced liver fibrosis^[4]. Together with the ALT results, these findings infer that the improvement in hepatocyte function in the YGJ group may result from attenuation of hepatocyte necrosis or apoptosis.

Hepatic progenitor cells (or oval cells) are activated in response to persistent liver injury when the hepatocytes are unable to mount a proliferative response to injury^[32]. In the present study, AFP and PKM2 protein expression increased continuously with time after CCl₄ injection, reaching a peak at the 13th week of CCl₄ injury. The results demonstrated that hepatic progenitor cells were

activated in the CCl₄-injured liver, but reduced steadily after oral YGJ administration. These results suggest that hepatic progenitor cells are activated by persistent CCl₄-induced liver injury, but the hepatic progenitor cells are not activated after YGJ treatment.

In summary, our results revealed that YGJ improves CCl₄-induced liver injury by inhibiting BM migration into injured liver and suppressing the proliferation of hepatic progenitor cells and hepatocytes.

COMMENTS

Background

Liver fibrosis is usually progressive and reversible, but up to now, the molecular and cellular mechanisms responsible for the reversibility of liver fibrosis have been poorly understood. Yiguanjian (YGJ) decoction has been employed clinically in China for centuries to treat various chronic hepatic injuries, but its mechanisms of action remain unclear.

Research frontiers

YGJ decoction is a traditional Chinese medicine complex containing six herbs which has a long history of clinical use. In the search for therapies for chronic liver diseases, the research hotspot focuses on its mechanisms of eliminating liver fibrosis.

Innovations and breakthroughs

In previous published papers, research focuses on how YGJ improves liver fibrosis, and especially how it induces apoptosis of hepatic stellate cells while exerting anti-apoptotic effects in hepatocytes. In the present study, the authors not only investigated the homing of bone marrow cells to the liver and their differentiation, but also investigated the behavior of intrahepatic progenitors and mature hepatocytes during liver injury. The number of bone marrow cells which differentiate into fibrogenic cells increased in the liver following injury, but was decreased by YGJ treatment. Furthermore, the proliferation of intrahepatic progenitors and mature hepatocytes was stimulated during CCl₄-induced liver injury, and YGJ also suppressed their proliferation.

Applications

The study results suggest that oral administration of YGJ decoction is a potential therapy for the prevention of chronic liver injury of varying etiologies.

Terminology

Liver fibrosis is a pathological progress characterized by an excessive deposition of extracellular matrix especially collagen; YGJ is a traditional Chinese medicine containing six herbs, which has a long history of clinical use in China.

Peer review

This is a good descriptive study in which authors analyze the effects of Yiguanjian decoction in liver fibrosis, induced by CCl₄. The results are interesting and suggest that YGJ decoction improved liver fibrosis by inhibiting the migration of bone marrow cells into the liver as well as inhibiting their differentiation and suppressing the proliferation of both progenitors and hepatocytes in the injured liver. The paper is interesting and deserves to be published.

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Carbon dioxide insufflation during colonoscopy in deeply sedated patients

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Abstract

AIM: To compare the impact of carbon dioxide (CO₂) and air insufflation on patient tolerance/safety in deeply sedated patients undergoing colonoscopy.

METHODS: Patients referred for colonoscopy were randomized to receive either CO₂ or air insufflation during the procedure. Both the colonoscopist and patient were blinded to the type of gas used. During the procedure, insertion and withdrawal times, caecal intubation

rates, total sedation given and capnography readings were recorded. The level of sedation and magnitude of patient discomfort during the procedure was assessed by a nurse using a visual analogue scale (VAS) (0-3). Patients then graded their level of discomfort and abdominal bloating using a similar VAS. Complications during and after the procedure were recorded.

RESULTS: A total of 142 patients were randomized with 72 in the air arm and 70 in the CO₂ arm. Mean age between the two study groups were similar. Insertion time to the caecum was quicker in the CO₂ group at 7.3 min vs 9.9 min with air ($P = 0.0083$). The average withdrawal times were not significantly different between the two groups. Caecal intubation rates were 94.4% and 100% in the air and CO₂ groups respectively ($P = 0.012$). The level of discomfort assessed by the nurse was 0.69 (air) and 0.39 (CO₂) ($P = 0.0155$) and by the patient 0.82 (air) and 0.46 (CO₂) ($P = 0.0228$). The level of abdominal bloating was 0.97 (air) and 0.36 (CO₂) ($P = 0.001$). Capnography readings trended to be higher in the CO₂ group at the commencement, caecal intubation, and conclusion of the procedure, even though this was not significantly different when compared to readings obtained during air insufflation. There were no complications in both arms.

CONCLUSION: CO₂ insufflation during colonoscopy is more efficacious than air, allowing quicker and better caecal intubation rates. Abdominal discomfort and bloating were significantly less with CO₂ insufflation.

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Key words: Colonoscopy; Carbon dioxide; Air; Insufflations; Patient tolerance; Safety; Efficacy

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INTRODUCTION

Colonoscopy is now widely used as a screening tool for colorectal neoplasia screening. It is generally necessary to distend the colon adequately during colonoscopy to allow safe navigation and permit thorough inspection of the mucosa. Abdominal discomfort attributed to gas insufflation during the procedure can occasionally lead to significant patient discomfort. The perception of the procedure being relatively painless is hence of paramount importance. In order to make the procedure more comfortable, deep sedation with Propofol have been used^[1]. Patients given Propofol had a more rapid onset of sedation and recovery. The use of carbon dioxide (CO₂) instead of air has also been shown to reduce abdominal discomfort and pain in several randomized controlled trials^[2-8]. These studies however utilized no or only minimal sedation when assessing the efficacy and safety of CO₂ in colonoscopy. The additional benefit of CO₂, if any, in patients who are deeply sedated has not been gauged. We therefore embarked on this study to assess the true impact of CO₂ in deeply sedated patients undergoing colonoscopy.

MATERIALS AND METHODS

This was a randomized, double blind study conducted over a period of 10 mo from May 2008 to March 2009. All patients undergoing screening and surveillance colonoscopy were invited to participate. Patients with previous colonic resections were excluded. Randomization was performed by whole sessions rather than individually by a clinical research coordinator. Sealed envelopes marked with either CO₂ or air insufflation was used. If the first patient on a given list was randomized to receive CO₂, the air insufflation button was switched off and the CO₂ delivery system was activated by depressing a lever which was set up just above the CO₂ tank. The whole gas delivery system was intentionally located out of the view of the colonoscopist, the nurse assigned to grade the level of sedation and patient discomfort during the procedure as well as the patient. Air insufflation was delivered through standard means whereas CO₂ was delivered at a rate of 4 L/min with a pressure of 50 kilopascals using a standard CO₂ delivery system.

All colonoscopies were performed by experienced gastroenterologists or colorectal surgeons whom had each individually performed more than 5000 procedures. Patients received sedation in combinations of Propo-

fol, Midazolam and Fentanyl. This was administered by a trained nurse sedationist under the direction of the proceduralist. The total amount of sedation given was recorded at the end of each procedure. During the procedure, insertion and withdrawal times were recorded. Insertion time was defined as the time taken from the commencement of colonoscopy until the caecum was reached whilst the withdrawal time, time from the caecum up to the anus. The stopwatch was stopped during biopsies and polypectomies. Cecal intubation rates were also recorded.

End tidal CO₂ measurements were used to measure the CO₂ levels. Continuous measurement was possible using a nasal cannula which was connected to a capnograph. Capnography readings were charted at the 3 different time frames: at the commencement of colonoscopy, upon intubation of the caecum and at the conclusion of the procedure. The level of sedation during the procedure was assessed by the nurse sedationist using a visual analogue scale (VAS) (0-wide awake, 1-mild sedation/easy to rouse, 2-moderate sedation/unable to stay awake, 3-difficult to rouse) while the magnitude of patient discomfort was also assessed using a similar VAS (0-nil, 1-mild, 2-moderate, 3-severe).

At the conclusion of each procedure, the colonoscopists were asked to determine whether they thought the gas used was CO₂ or air. The patients were then observed in the recovery bay using a standardized post procedure recovery protocol. Prior to discharge from the unit, patients graded their level of discomfort and abdominal bloating using a similar VAS as described above.

Statistical analysis was performed using the Stata V10 Statistical software (StataCorp. 2007 College Station, TX, United States). *P* value < 0.05 was used to indicate statistical significance.

RESULTS

A total of 150 patients participated in the study. Eight patients were excluded due to failure to give consent (3), history of previous colonic resection (3) and poor bowel preparation (2) leaving a total of 142 patients of which 72 were randomized in the air arm and 70 in the CO₂ arm. The two patient groups were of similar age with a mean of 59.97 (range: 22-88 years) in the air group and 58.26 (range: 22-84 years) in the CO₂ group. Of the 72 patients in the air group, 33 were male and 39 female whilst in the CO₂ group, 45 were male and 25 female (*P* < 0.05). The average insertion time was 9.88 min in the air group and 7.29 min in the CO₂ group (*P* = 0.0083). The average withdrawal times were 6.69 min and 7.29 min in the air and CO₂ groups respectively (*P* > 0.05). The caecal intubation rates were 94.4% in the air group and 100% in the CO₂ group (Table 1).

The amount of sedation given to patients during the procedure was similar in both groups. The level of sedation as assessed by a nurse during the procedure was 1.0 in the air group and 1.09 in the CO₂ group (*P* > 0.05).

Table 1 Patient characteristics, procedure duration, sedation parameters, and capnographic readings with air and CO₂ insufflation during colonoscopy

	Air	CO ₂	P value
Total procedures	72	70	NS
Age (mean) (yr)	59.97	58.26	NS
Male:female ratio	33:39	45:25	NS
Insertion time (min)	9.88	7.29	0.0083
Withdrawal time (min)	6.69	7.29	NS
Cecal intubation rate (%)	94.4	100	0.012
Sedation used			
Propofol (mg)	185.97	184.14	NS
Midazolam (mg)	0.78	0.66	NS
Fentanyl (mg)	32.64	34.93	NS
Sedation (nurse assessment)	1.00	1.09	NS
Discomfort (nurse assessment)	0.69	0.39	0.0155
Pain (patient assessment)	0.82	0.46	0.0228
Bloating (patient assessment)	0.97	0.36	0.001
Capnography at commencement	18.53	20.21	NS
Capnography at caecum	15.1	19.28	NS

NS: Not significant.

Conversely, the discomfort level as assessed by the nurse (during the procedure) was 0.69 (air) and 0.39 (CO₂) ($P = 0.0155$) and post procedure by the patient 0.82 (air) and 0.46 (CO₂) ($P = 0.0228$). The level of abdominal bloating as assessed by the patient prior to discharge from the endoscopy unit was 0.97 (air) and 0.36 (CO₂) ($P = 0.001$) (Table 1).

Capnography readings were higher in the CO₂ group at commencement of colonoscopy, upon reaching the caecum and at the conclusion of the procedure (Table 1). This, however, was not statistically significant. There were 2 patients with Chronic Obstructive Airway Disease (COPD) in the air arm and 3 in the CO₂ arm ($P > 0.05$). There were no complications observed with both the use of CO₂ or air. The accuracy of the colonoscopists in predicting the type of gas insufflation used was 73.6% in the air group and 54.7% in the CO₂ group.

DISCUSSION

This prospective, randomized study has shown that insufflation of CO₂ during colonoscopy resulted in significantly reduced abdominal discomfort in deeply sedated patients. The rapid absorption of CO₂ from the colon resulted in less distension of the colon and therefore shorter procedural times. This was demonstrated with the quicker caecal insertion times in the CO₂ group compared to patients who were randomized to receive air insufflation. The caecal intubation rate in the CO₂ group was also better.

There have been numerous studies looking at the efficacy and safety of CO₂ in the gastrointestinal tract leading to a recently published systematic review on this subject by Dellon *et al*⁹¹. The authors concluded that CO₂ insufflation was associated with decreased post procedural pain and distension. One of the major drawbacks of this review which the authors concurred with is that 6 of the

9 randomized controlled trials were from the same group. None of the studies however looked at patients who were deeply sedated. With an increasing number of patients opting for a painless procedure especially with deep sedation, we attempted to address this question here.

The different colonoscopists performing the procedure may have arguably influenced the results as the more experienced practitioners would be expected to be more skilled. However, all colonoscopists involved in the study were experienced consultants with recognition in the practice of colonoscopy. No trainees/registrar were involved in performing the procedure. The insufflation of gas during colonoscopy was determined by sealed envelopes and several methods were employed in this study to ensure blinding of all parties involved. The gas coupling was hidden from view during the colonoscopic session thereby eliminating the possibility of unblinding if the changing of gas coupling was done after each patient. The usage of CO₂ or air was also silent further reducing the possibility of any audible sound from either the air or CO₂ delivery systems being heard by the colonoscopist or the nurse sedationist during the procedure. In addition, the possibility of the colonoscopist being able to determine the gas used by observing the distension of the colon during insufflation was not found to be a problem as seen in the accuracy of gauging the type of gas used at the procedure being 73.6% in the air group and 54.7% in the CO₂ group.

There have been concerns expressed with regards to use of CO₂ causing interference with metabolic homeostasis and respiratory complications in certain patient groups. Several studies have confirmed the safety of CO₂ insufflation although patients with COPD were excluded. We specifically did not exclude patients with COPD in this study (air: 2 *vs* CO₂: 3). Although this study showed an increased level of capnography readings in the CO₂ group compared to air, this was not statistically significant. Moreover the baseline CO₂ was slightly higher than the air group (18.53 mmHg *vs* 20.21 mmHg). There were no respiratory complications seen. One of the major limitations though is that the measurement of CO₂ levels by capnography is not performed in a closed system and may have therefore not been very accurate. The level of CO₂ should ideally be measured by performing arterial blood gases. However, this was not thought to be appropriate given that the patients would have to undergo multiple arterial punctures during the procedure. We were also vigilant with the continuous monitoring of the CO₂ levels during the procedure although actual documentation was only done at 3 set time frames. Another drawback of the study is that there were a higher number of male patients compared to female patients in the CO₂ group which may have affected the result of bloating and pain level. However, the patient gender was found to not have any statistically significant difference.

In conclusion, the use of CO₂ insufflation during colonoscopy in deeply sedated patients was more efficacious than air, resulting in a quicker procedural time and

better patient tolerance with regards to less abdominal bloating and pain. It was also safe with no complications observed. It is recommended that CO₂ be considered for routine use in colonoscopy in deeply sedated patients to further increase patient comfort.

COMMENTS

Background

Colonic distention with gas during colonoscopy allows thorough inspection of the mucosa, at the expense of causing abdominal discomfort. In order to make the procedure more comfortable, apart from anaesthetic agents, the use of carbon dioxide (CO₂) instead of air has also been shown to reduce abdominal discomfort and pain in several randomized controlled trials.

Research frontiers

Previous studies however utilized no or only minimal sedation when assessing the efficacy and safety of CO₂ in colonoscopy. This study aimed to assess the true impact of CO₂ in deeply sedated patients undergoing colonoscopy.

Innovations and breakthroughs

Use of CO₂ insufflation during colonoscopy in deeply sedated patients was not only safe, but more efficacious than air, resulting in a quicker procedural time and better patient tolerance with regards to less abdominal bloating and pain.

Applications

It is recommended that CO₂ be considered for routine use in colonoscopy in deeply sedated patients to further increase patient comfort while allowing safe adequate colonic distention.

Terminology

CO₂ insufflation is the distention of the colon with carbon dioxide gas during the performance of colonoscopy, to allow better visualization of the mucosa surface.

Peer review

It is well known that patients who are not sedated or minimally sedated have significant benefits when CO₂ is used to insufflate the bowel. This study evaluated the effect of CO₂ on deeply sedated patients and further adds to the available data in the literature with regards to the its application in colonoscopy.

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Intestinal alkaline phosphatase in the colonic mucosa of children with inflammatory bowel disease

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Abstract

AIM: To investigate intestinal alkaline phosphatase (iAP) in the intestinal mucosa of children with inflammatory bowel disease (IBD).

METHODS: Colonic biopsy samples were taken from 15 newly diagnosed IBD patients and from 10 healthy controls. In IBD patients, specimens were obtained

both from inflamed and non-inflamed areas. The iAP mRNA and protein expression was determined by reverse transcription-polymerase chain reaction and Western blotting analysis, respectively. Tissue localization of iAP and Toll-like receptor (TLR) 4 was investigated by immunofluorescent staining.

RESULTS: The iAP protein level in the inflamed mucosa of children with Crohn's disease (CD) and ulcerative colitis (UC) was significantly decreased when compared with controls (both $P < 0.05$). Similarly, we found a significantly decreased level of iAP protein in the inflamed mucosa in CD compared with non-inflamed mucosa in CD ($P < 0.05$). In addition, the iAP protein level in inflamed colonic mucosa in patients with UC was decreased compared with non-inflamed mucosa in patients with CD ($P < 0.05$). iAP protein levels in the non-inflamed mucosa of patients with CD were similar to controls. iAP mRNA expression in inflamed colonic mucosa of children with CD and UC was not significantly different from that in non-inflamed colonic mucosa with CD. Expression of iAP mRNA in patients with non-inflamed mucosa and in controls were similar. Co-localization of iAP with TLR4 showed intense staining with a dotted-like pattern. iAP was present in the inflamed and non-inflamed mucosa of patients with CD, UC, and in control biopsy specimens, irrespective of whether it was present in the terminal ileum or in the colon. However, the fluorescent signal of TLR4 was more pronounced in the colon compared with the terminal ileum in all groups studied.

CONCLUSION: Lower than normal iAP protein levels in inflamed mucosa of IBD patients may indicate a role for iAP in inflammatory lesions in IBD. Based on our results, administration of exogenous iAP enzyme to patients with the active form of IBD may be a therapeutic option.

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Key words: Intestinal alkaline phosphatase; Toll-like receptor; Colonic biopsy; Children; Inflammatory bowel disease

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INTRODUCTION

The etiology of inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), remains unclear. It is hypothesized that, in genetically susceptible individuals, inappropriate and ongoing activation of a mucosal immune response against luminal antigens is a major cause of the inflammation^[1,2]. In active IBD, the tolerance towards the resident intestinal flora is decreased. The balance between protective and commensal luminal bacterial species is lost and, due to increased mucosal permeability and insufficient mucosal clearance, the commensal flora and pathogenic bacteria enter into the lamina propria and destructive inflammatory responses are unavoidable^[3-6]. This prompts an exaggerated immune response with the activation of the two arms of mucosal immune system, the innate and adaptive elements^[7,8].

The activation of the innate immune system heavily depends on the recognition of microbes by pattern recognition receptors such as Toll-like receptors (TLRs). The TLR family consists of 13 members, and each has different type of ligands. One of them is TLR4, which is responsible for recognition of lipopolysaccharide (LPS), a principal component of the bacterial outer membrane. Uncontrolled activation of TLR4 may lead to the loss of mucosal barrier integrity, aggravation of the inflammatory response within the gut epithelial mucosa, increased expression of TLR-ligands and tumorigenesis^[9-13]. Previously, we found increased TLR4 protein and mRNA levels in the inflamed mucosa of children with IBD and celiac disease^[14,15].

An increasing body of evidence also supports the regulatory role of intestinal alkaline phosphatase (iAP) in TLR activation. iAP is expressed on the apical surface of enterocytes and exists in membrane-bound and soluble forms^[16]. iAP plays an essential role in the inactivation of LPS through dephosphorylation of its lipid A moiety, thus generating a non-toxic monophosphoryl section. This dephosphorylated monophosphoryl lipid A is not able to form a complex with TLR4^[17,18].

There is only one human study where expression of iAP (mRNA) in adult IBD patients was analyzed, and lower than normal iAP mRNA expression was found

in epithelial specimens^[19]. It should be noted, however, that no data on the level of iAP protein in IBD mucosa is available. The aim of our study was to investigate iAP protein and mRNA levels in affected and non-affected colon mucosa of children with newly diagnosed IBD. In addition, our secondary aim was to determine the localization of iAP enzyme with TLR4.

MATERIALS AND METHODS

Patients and colonic biopsies

Ten children (7 boys, 3 girls; median age: 10.5 years, range: 1.5-15 years) with newly diagnosed CD, 5 children (3 boys, 2 girls; median age: 11 years, range: 6-17 years) with newly diagnosed UC, and 10 control children (5 boys, 5 girls; median age: 9.5 years, range: 1.5-16 years) were enrolled in the study (Table 1). IBD was diagnosed according to the Porto criteria^[20,21]. The presenting symptoms in CD were perianal fistula, hematochezia, abdominal pain, diarrhea-bloody diarrhea, or anemia. All of the patients later diagnosed with UC had hematochezia, and some had abdominal pain and weight loss. Colonic biopsy samples were taken from macroscopically inflamed and non-inflamed sites of the colonic mucosa in children with CD. As each UC children had pancolitis, only inflamed mucosa was obtained from UC patients (Table 1). The activity score was calculated by means of the Pediatric Crohn's Disease Activity Index (PCDAI) and Pediatric Ulcerative Colitis Activity Index (PUCAI)^[22,23]. Measuring disease activity in pediatric CD is based on disease history (abdominal pain, stools per day and general well-being), laboratory findings, weight, abdominal and perianal examination, extra-intestinal manifestations, and growth rate^[24]. PUCAI requires no laboratory measurements^[25]. The mean PCDAI of our patients was 33.75, and the mean PUCAI was 35. This means that both groups had moderate-to-severe disease activity. Control children were referred to the outpatient clinic with rectal bleeding, constipation or weight loss. Colonoscopy was part of their diagnostic procedure and the biopsy specimens showed normal macroscopic appearance and histology. Written informed consent was obtained from parents prior to the procedure, and the study was approved by the Semmelweis University Regional and Institutional Committee and Research Ethics.

RNA isolation and real-time polymerase chain reaction

Total RNA was isolated from the colonic biopsy samples by RNeasy Total RNA Isolation Kit (Qiagen GmbH, Hilden, Germany), according to the instructions of the manufacturer. One μ g of total RNA was reverse-transcribed and iAP mRNA expressions were determined by real-time polymerase chain reaction (PCR) on Light Cycler480 (Roche Diagnostics, Mannheim, Germany). PCRs were performed containing RealTime ready Catalog Assay primer (Roche Diagnostics), Light Cycler 480 Probes Master (Roche Diagnostics, Mannheim, Germany), and cDNA. Conditions for iAP mRNA measurements: one

Table 1 Clinical characteristics of newly diagnosed patients with Crohn's disease and ulcerative colitis

Patient	Form of IBD	Gender	Age (yr)	Main complaints at presentation	Activity score	Duration (symptoms) (mo)
1	CD	F	15	Perianal fistula	25	1
2	CD	M	4	Hematochezia	20	3
3	CD	M	11	Abdominal pain, diarrhea	45	2
4	CD	F	9	Bloody diarrhoea	25	2
5	CD	F	4	Diarrhoea, anaemia	30	3
6	CD	M	14	Hematochezia	50	6
7	CD	F	1.5	Bloody diarrhoea	35	1.5
8	CD	M	11	Bloody diarrhoea	20	2
9	CD	M	12	Diarrhoea, anaemia	35	3
10	CD	M	10	Weight loss, diarrhea	52.5	1.5
11	UC	F	12	Hematochezia, abdominal pain	20	4
12	UC	M	9	Hematochezia, anaemia	35	1
13	UC	M	17	Hematochezia, abdominal pain	25	7
14	UC	F	12	Hematochezia, weight loss	55	4
15	UC	M	6	Hematochezia	40	2.5

The activity score was calculated by means of pediatric Crohn's disease activity index and pediatric ulcerative colitis activity index. IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; M: Male; F: Female.

cycle, 95 °C, 10 min (denaturation), followed by several cycles at 95 °C, 10 s and 30 s, 72 °C 1 s (annealing and extension). The mRNA expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control was determined using Brilliant II Fast SYBR Green quantitative polymerase chain reaction Master Mix (Stratagene, Cedar Creek, TX, United States), PCR primers (Forward: 5'-CAC CAC CAT GGA GAA GGC TG-3'; Reverse: 5'-GTG ATG GCA TGG ACT GTG-3', Invitrogen, CA, United States) and cDNA. Conditions for GAPDH: one cycle, 95 °C, 2 min, 50 cycles at 95 °C 20 s and 60 °C, 40 s. Results were analyzed by Light-Cycler software 480 (Roche Diagnostics).

Protein isolation and Western blotting

Colonic biopsy specimens were homogenized in lysing solution, and protein concentrations were determined by DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, United States); 0.5 µg protein from each sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (120 V, 40 mA, 120 min) (Penguin™ Dual-Gel Water Cooler Systems, Owl, NH, United States) and transferred to nitrocellulose membrane (GE Healthcare, Little Chalfont, United Kingdom) (70 V, 220 mA, 120 min) (MiniTank™ electroblotter, Owl). Membranes were blocked in 1% non-fat dry milk solution (1 h) and

incubated with iAP specific rabbit polyclonal antibody (1:1000, 1 h) (AbCam, Cambridge, United Kingdom). Equal protein loading was confirmed by β-actin specific (C-11) goat polyclonal IgG antibody (1:100) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States). Peroxidase-conjugated secondary anti-rabbit IgG or donkey anti-goat IgG antibodies (1:2000, 30 min) (Santa Cruz Biotechnology Inc.) were used. Immunoreactive bands were visualized using the enhanced chemiluminescence Western blotting detection protocol (GE Healthcare). Bands were analyzed with software Image J. 1.42q (National Institutes of Health, United States). The values were expressed as relative optical density.

Immunofluorescent staining

Biopsy samples were snap-frozen, embedded in Shandon cryomatrix (ThermoElectron Co., Waltham, United States), cut to 3-4 µm slides and double incubated with TLR4 specific goat polyclonal antibody and iAP specific rabbit polyclonal antibody (1:100, 1 h) (Abcam Plc). Secondary antibodies were Alexa Fluor 488 donkey anti-goat and Alexa Fluor 568 goat anti-rabbit antibodies (Invitrogen). Zeiss LSM 510 Meta confocal laser scanning microscope (Carl Zeiss, Jena, Germany) was used with 20 × Plan Apochromat (NA = 0.8) and 63 × Plan Apochromat oil immersion differential interference contrast objectives (NA = 1.4).

Statistical analysis

Data were analyzed using Statistica 7.0 software (StatSoft Inc., Tulsa, OK, United States). After testing the normality with Shapiro-Wilk's test, non-parametric Mann-Whitney *U* test was used. Data were considered statistically significant if $P \leq 0.05$, and expressed as mean ± standard deviation.

RESULTS

iAP protein levels

Western blotting analysis revealed one distinct band at 60 kDa. The iAP protein level in the inflamed mucosa of children with CD and UC was lower by 22% and 20%, respectively, compared with controls ($P < 0.05$). We found a lower iAP protein level in the inflamed mucosa in CD compared with non-inflamed mucosa in CD ($P < 0.05$). The iAP protein level in the inflamed colonic mucosa in UC patients was decreased by 24% compared with non-inflamed mucosa in CD patients ($P < 0.05$) (Figure 1). iAP protein levels in the non-inflamed mucosa of patients with CD were normal.

iAP mRNA expression

iAP mRNA expression in inflamed and non-inflamed colonic mucosa in IBD was comparable with that in controls (Figure 2).

Mucosal localization of iAP and TLR4

The distribution of iAP was restricted to the epithelial

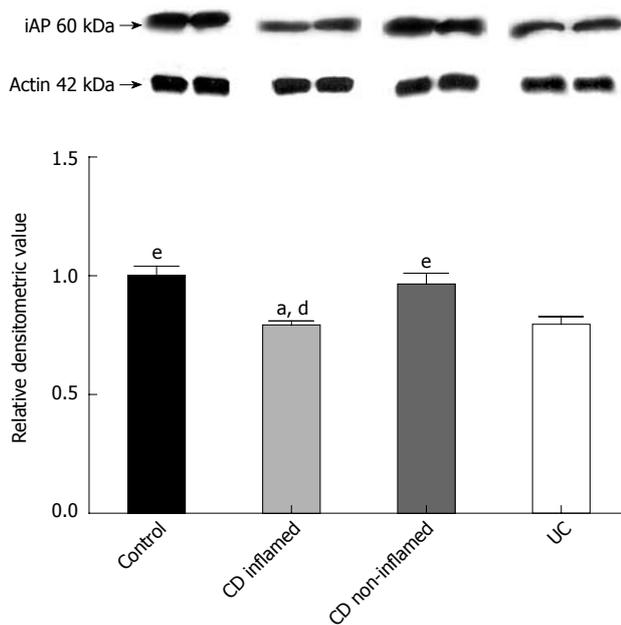


Figure 1 Protein levels of intestinal alkaline phosphatase in the colonic mucosa of children with newly diagnosed Crohn's disease, ulcerative colitis and controls. A: Western blotting analysis of the colonic biopsy specimens using intestinal alkaline phosphatase (iAP)-specific antibody reveals one distinct band at 60 kDa; B: Data for protein levels of iAP were obtained by computerized analysis of the Western blottings and expressed as median interquartile range. Analysis of significance was performed by the Mann-Whitney *U* test. ^a*P* < 0.05 vs control; ^d*P* < 0.01 vs non-inflamed CD; ^e*P* < 0.05 vs UC. CD: Crohn's disease; UC: Ulcerative colitis.

surface of the colonic and terminal ileal mucosa in each group. No fluorescent signal was detected in Lieberkühn crypt cells, in goblet cells, and in lamina propria immune cells. The co-localization of iAP with TLR4 showed intense staining with a dotted-like pattern. iAP was present in inflamed and non-inflamed mucosa of patients with CD, UC, and in control specimens irrespective of whether it was present in the terminal ileum or in the colon. However, the fluorescent TLR4 signal was more pronounced in the colon compared with the terminal ileum in all groups (Figure 3).

DISCUSSION

A dysregulated immune response, involving the innate immunity of the intestinal mucosa plays a role in the pathomechanism of IBD. The maintenance of microbiota and host is supported by the balance of microbiota and immune activation that may be disturbed in IBD^[26]. Previously we and others showed that activation of TLR4 by bacterial lipopolysaccharide contributes to disease progression^[14,27].

Recently, in connection with LPS-activated TLR4, a new enzyme, iAP has received increasing attention as a factor responsible for mucosal defense. iAP dephosphorylates and detoxifies LPS and, hence, generates an inactive, non-toxic form. This may be one of the key factors why dephosphorylated LPS is unable to bind to TLR4 and the innate immune system is not triggered. iAP may control the interaction between TLR4 in the intestinal

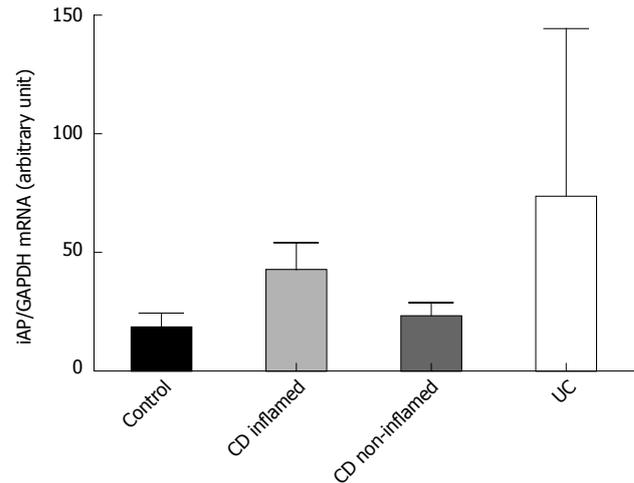


Figure 2 Intestinal alkaline phosphatase mRNA expression in the colonic mucosa of children with newly diagnosed Crohn's disease, ulcerative colitis and controls. iAP mRNA expression data were obtained by computerized analysis of PCR products. Optical density was corrected according to that of GAPDH. Data are expressed as median interquartile range. Analysis of significance was performed by Mann-Whitney *U* test. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; PCR: Polymerase chain reaction; iAP: Intestinal alkaline phosphatase; CD: Crohn's disease; UC: Ulcerative colitis.

mucosa and LPS derived from the bacterial flora^[28,29].

In the present study, we obtained data regarding the protein level, mRNA expression and localization of iAP in the intestinal mucosa of children with IBD. Lower than normal iAP levels were observed in the inflamed mucosa of CD and UC patients.

Previously it was hypothesized that the altered LPS-dephosphorylating activity may be a consequence of decreased iAP activity. We think that, in accordance with Tuin *et al*^[19], our observations also suggest that iAP has a role in the pathogenesis of IBD. Decreased iAP levels in the inflamed mucosa may be associated with decreased LPS detoxification and, consequentially, with increased TLR4 activation. On the other hand, we found no significant difference in iAP mRNA expression that may indicate a possible role of posttranscriptional regulation.

Tuin *et al*^[19] demonstrated decreased iAP mRNA expression in pretreated CD patients compared with controls. However, it should be noted that, in this study, more than half of the patients received immunosuppressive drugs such as infliximab, methotrexate, corticosteroids, and thiopurine at the time of sample collection, which may influence iAP mRNA synthesis^[30]. The unique feature of our study is the investigation of children without prior immunomodulatory therapy, hence, our results can be considered as characteristic for IBD.

Previously, we have demonstrated increased TLR4 mRNA expression and protein levels in the inflamed colonic mucosa of children with IBD^[14]. Therefore, the finding that iAP and TLR4 are co-localized, is particularly important from two aspects. First, it supports a linked role of iAP in the maintenance of mucosal integrity both in healthy and in diseased subjects. Second, the lower than normal iAP in the presence of a higher than

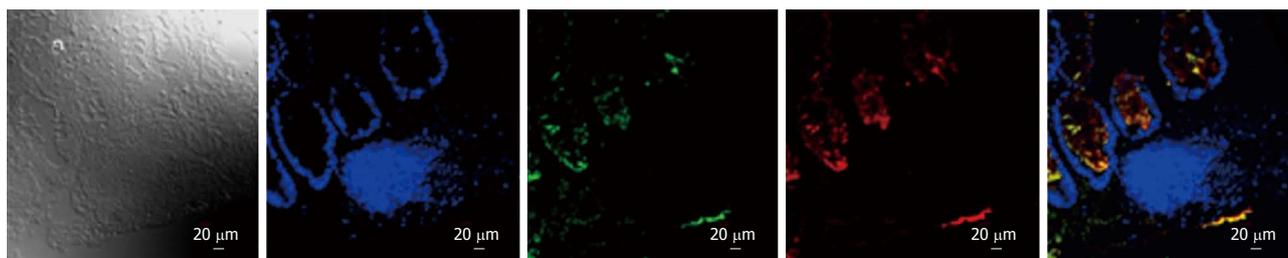


Figure 3 Localization of intestinal alkaline phosphatase and Toll-like receptor-4 in colon of Crohn's disease. Immunofluorescent staining for intestinal alkaline phosphatase (iAP) (red) and Toll-like receptor-4 (TLR4) (green) staining was performed in inflamed colon of patient with Crohn's disease. Yellow color (merge) indicates co-localization of iAP and TLR4. For the observation of non-labeled tissues, differential interference contrast was used. Nuclei are stained with blue.

normal TLR4 expression might indicate an imbalance in iAP/TLR4 that would result in an increased susceptibility of the mucosa to LPS. Indeed, this mechanism is already demonstrated in animal models of induced colitis. Our results are the first to indicate the presence of this phenomenon in man.

The current management of IBD consists of conventional therapy, but severe therapy-resistant cases require more powerful therapies, such as biological treatment^[31]. Therapeutic manipulation to restore the balance of microflora may have a strong impact on mucosal healing of IBD^[32]. In animal models of dextrane sodium sulfate (DSS)-induced colitis, exogenously administered iAP improved the signs of colitis both macroscopically and microscopically^[33]. Microscopic injury scores of DSS-induced colitis in iAP-knockout mice were much higher than in the wild-type group, which may reveal the mucosal defense role of iAP^[34]. In a human study performed in adult subjects a 7-d course of iAP products decreased the activity index of therapy-resistant UC patients^[35]. Oral administration of iAP may have a beneficial effect in the case of severe intestinal epithelial damage^[36]. Our results obtained in a pediatric population might indicate a similar approach may be of benefit in children with IBD.

In summary, to the best of our knowledge, this is the first demonstration of a decrement in the iAP enzyme in the mucosa of patients with IBD. A decreased level of iAP with reduced LPS-detoxifying capacity could be responsible for increased bacterial passage across the intestinal mucosa of patients with IBD, and this may play an important role in pathogenesis. In addition, co-localization of iAP and TLR4 was demonstrated in the epithelial compartment. Based on our results, administration of exogenous iAP enzyme to patients with active form of IBD may be a supplemental therapeutic option.

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COMMENTS

Background

The level of intestinal alkaline phosphatase (iAP) protein in inflammatory bowel disease (IBD) mucosa is very important for the study of IBD. The authors have

demonstrated firstly the presence of iAP enzyme in the colonic mucosa of patients with IBD. The decreased level of iAP enzyme with reduced lipopolysaccharide-detoxifying capacity could be responsible for the increased bacterial passage across the intestinal mucosa in the inflamed mucosa of patients with IBD and this may play a role in the pathogenesis.

Research frontiers

To the best of our knowledge, this is the first demonstration of a decrement in iAP enzyme in the mucosa of patients with IBD.

Innovations and breakthroughs

A decreased level of iAP with reduced lipopolysaccharide-detoxifying capacity could be responsible for increased bacterial passage across the intestinal mucosa of patients with IBD, and this may play an important role in the pathogenesis of IBD. In addition, co-localization of iAP and Toll-like receptor-4 was demonstrated in the epithelial compartment.

Applications

Based on their results, the authors propose administration of exogenous iAP enzyme to patients with the active form of IBD as a supplemental therapeutic option. However, this hypothesis should be tested in future clinical trials.

Terminology

The importance of the mucosal barrier damage is emphasized in IBD due to its potential role in IBD pathogenesis. iAP, a potent factor to maintain or restore mucosal barrier integrity in the gut, could participate in the mucosal healing of IBD.

Peer review

This is a well-written manuscript reporting about significance of intestinal alkaline phosphatase in the colonic mucosa for the pathogenesis of inflammatory bowel disease in children. The manuscript contains clear component of novelty to the best my knowledge, the authors have firstly demonstrated the decrement of iAP enzyme in the mucosa of patients with IBD.

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Incidence and clinical features of endoscopic ulcers developing after gastrectomy

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Abstract

AIM: To determine the precise incidence and clinical features of endoscopic ulcers following gastrectomy.

METHODS: A consecutive series of patients who underwent endoscopic examination following gastrectomy between 2005 and 2010 was retrospectively analyzed. A total of 78 patients with endoscopic ulcers and 759 without ulcers following gastrectomy were enrolled. We analyzed differences in patient age, sex, size of the lesions, method of operation, indications for gastric resection, and infection rates of *Helicobacter pylori* (*H. pylori*) between the nonulcer and ulcer groups.

RESULTS: The incidence of endoscopic ulcers after gastrectomy was 9.3% and that of marginal ulcers was 8.6%. Ulcers were more common in patients with Billroth II anastomosis and pre-existing conditions for

peptic ulcer disease (PUD). Infection rates of *H. pylori* did not differ significantly between the two groups. The patients who underwent operations to treat PUD had lower initial levels of hemoglobin and higher rates of hospital admission.

CONCLUSION: *H. pylori* was not an important factor in ulcerogenesis following gastrectomy. For patients who underwent surgery for PUD, clinical course of marginal ulcers was more severe.

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Key words: Gastrectomy; Marginal ulcer; *Helicobacter pylori*

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Chung WC, Jeon EJ, Lee KM, Paik CN, Jung SH, Oh JH, Kim JH, Jun KH, Chin HM. Incidence and clinical features of endoscopic ulcers developing after gastrectomy. *World J Gastroenterol* 2012; 18(25): 3260-3266 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i25/3260.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i25.3260>

INTRODUCTION

Total or partial gastrectomy is reserved for patients with peptic ulcer disease (PUD) who have failed to respond to therapy or individuals with gastric malignancies. The need to perform gastrectomy on PUD patients has decreased since the discovery of *Helicobacter pylori* (*H. pylori*) and development of proton pump inhibitors^[1,2]. Nevertheless, the total frequency of performing gastrectomy has increased due to the frequent detection of early gastric

cancer and increasing morbid obesity^[3-6]. The complications of gastrectomy are recurrent ulcers, nutritional disturbances, dumping syndrome, *de novo* cancer of the gastric remnant [so-called “gastric stump cancer” (GSC)], and alkaline reflux gastritis^[7].

During follow-up endoscopic examination, the detection of ulcerative lesions including marginal ulcers as well as cancer recurrence and development of GSC are of clinical interest. Therefore, information about the distinct features of these different ulcerative lesions will help discriminate these lesions from one another so that appropriate treatment strategies can be identified. Marginal ulcers are defined as ulceration around anastomosis following gastrectomy. It has been reported that the incidence of marginal ulcers varies from 0.6% to 16%^[8,9]. Although the etiology of marginal ulcers remains obscure, several mechanisms have been postulated. Until now, there are no convincing results, and the exact link between *H. pylori* and the development of marginal ulcers is unclear. GSC was originally defined as a gastric cancer that arises in the remnant stomach > 5 years after primary surgery for benign diseases such as PUD. A minimal latency of 5 years is included in the definition to avoid misdiagnosis^[10,11]. Until now, many features of these lesions were controversial.

The goal of this study was to determine the precise incidence of endoscopic ulcers in patients with a history of gastrectomy. Clinical features of these lesions and patients were evaluated including location, size, types of reconstruction following gastrectomy, causative disease for operation, and history of *H. pylori* infection. In addition, features of GSC were evaluated including frequency, site, time of appearance following gastrectomy, and pathological characteristics of cancer.

MATERIALS AND METHODS

Patients

This study protocol was approved by the Ethics Committee of the Catholic University of Korea. The study was conducted at St. Vincent Hospital, a teaching hospital of the Catholic University of Korea. The medical records, charts, and digitized archived images of consecutive patients who had a history of gastrectomy and underwent diagnostic esophago-gastroduodenoscopy between January 2005 and December 2010 were reviewed.

Each case was classified as *H. pylori*-positive or -negative according to the histological results [rapid urease test (CLO test[®], Kimberly-Clark, Utah, United States) or silver staining] for two biopsy specimens taken from the remnant stomach. Exclusion criteria were endoscopic evaluation within 1 year following gastrectomy and comorbidity of serious systemic disease. Individuals with conditions that might have substantial effects on our study results (e.g., serum creatinine > 2.5 mg/dL, total bilirubin > 3.0 mg/dL), pregnant women, patients with psychiatric diseases, and patients who did not sign a consent form were excluded. Patients were also excluded if they had cancer recurrence.

Study design

The endoscopic, histological, and clinical features of the ulcerative lesions were analyzed. Subsequently, the ulcerative lesions were classified as neoplastic or non-neoplastic lesions. Cases of recurrent cancer were excluded; the remaining lesions included marginal ulcer and GSC. We analyzed differences in patient age, sex, size of lesions, method of operation, indications for gastric resection, and infection rates of *H. pylori* between the non-ulcer and ulcer groups. For patients with marginal ulcers, we analyzed baseline characteristics, clinical manifestation, and rates of *H. pylori* infection according to the location of ulcers. In addition, we evaluated the clinical features of GSC.

Statistical analysis

Continuous variables were expressed as the mean \pm SD and compared using Student's *t*-test. Categorical variables were expressed as percentages and compared using a χ^2 test. Statistical analyses were conducted with SPSS version 12.0 software (SPSS, Chicago, IL, United States). *P* < 0.05 was considered to be statistically significant.

RESULTS

Demographic features

Data for a consecutive series of patients who underwent gastrectomy between 2005 and 2010 were retrospectively analyzed. A total of 2862 endoscopic examinations were performed and 918 patients were enrolled in our study. Among these, endoscopic examinations were performed for 512 patients within 1 year of gastrectomy and were excluded from our study. Each patient underwent two or three endoscopic procedures during the study period. Eighty-one patients were excluded due to underlying chronic illnesses including: liver cirrhosis, heart failure, chronic obstructive pulmonary disease, chronic renal failure (*n* = 37), simple closure (*n* = 12), gastrojejunostomy without gastrectomy (*n* = 7), and recurrence of previous cancer (*n* = 25). The remaining 837 patients were endoscopically examined and included in our study. A total of 78 patients with endoscopic ulcers and 759 without ulcers following gastrectomy were enrolled (Figure 1). Six (0.7%) out of all 837 patients who underwent gastrectomy were diagnosed with GSC.

Basal characteristics of endoscopic ulcers following gastrectomy

The clinical features of patients in both groups are shown in Table 1. There were no significant differences in age or sex ratio in the group of patients with or without ulcers following gastrectomy. Patients with Billroth II (B-II) gastrojejunal anastomosis were more prone to endoscopic ulceration (*P* < 0.01) compared to those with Billroth I (B-I) anastomosis (Figure 2). The formation of endoscopic ulcers was more frequent in patients who had undergone gastrectomy for PUD complications than

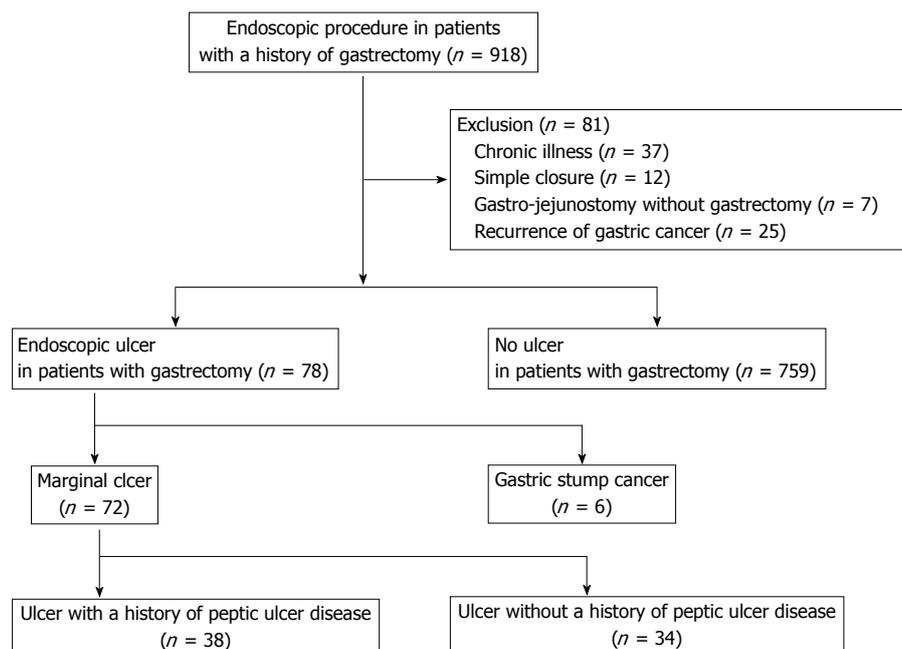


Figure 1 Study design.

	Patients with endoscopic ulcers after gastrectomy (n = 78)	Patients without endoscopic ulcers after gastrectomy (n = 759)	P value
Age (yr)	62.26 ± 12.89	61.00 ± 12.50	0.92
Gender (male/female)	59:19	523:236	0.20
Anastomosis			
Billroth- I	12	237	< 0.01
Billroth- II	63	477	
Other method	3	45	
Causative diseases			
Malignancy	31	645	< 0.01
Complication of PUD	44	102	
Others	3	12	
<i>H. pylori</i> infection (%)	20/58 (34.5)	145/397 (36.5)	0.76

H. pylori: *Helicobacter pylori*; PUD: Peptic ulcer disease.

for other reasons ($P < 0.01$). In patients treated with gastrectomy for PUD complications, the incidence of ulcers was 30.1% (44/146). When patients with GSC were excluded, the incidence of marginal ulcers was 27.1% (38/140). The incidence of ulcers in patients treated with gastrectomy for non-PUD diseases was 4.9% (34/691). The incidence of *H. pylori* infection did not differ significantly between the two groups (34.5% *vs* 36.5%, $P > 0.05$).

Clinical features of marginal ulcers

A total of 72 patients had marginal ulcers that were located on the efferent side or anastomosis. Most of these patients (86.1%, 62/72) complained of specific gastrointestinal symptoms, and 41.7% (30/72) experienced episodes of bleeding or developed anemia. The most frequent symptom was abdominal pain. However, there

were no differences of clinical manifestation according to ulcer location. The rate of patients undergoing therapeutic endoscopy to stop or prevent bleeding was 19.4% (14/72) and the rate of patients receiving admission care was 54.2% (39/72). The recurrence of marginal ulcers was observed in 29.2% (21/72) of the patients. There were no differences in the rate of patients receiving therapeutic endoscopy or recurrence of ulcers according to ulcer location (Table 2).

The patients were divided into two groups according to ulcer location: individuals with ulcers on the efferent side and ulcers at anastomosis sites. The most common location of marginal ulcers was the efferent side (55.6%, 40/72). There were no differences in patient age, sex ratio, tobacco use, alcohol consumption, types of reconstruction, causative disease, initial hemoglobin levels, recurrence rates of marginal ulcers, or *H. pylori* infection rates. Ulcers on the efferent side compared to those at anastomosis sites had a tendency of multiplicity, but a number of marginal ulcers appeared as a single lesion (69.4%, 50/72).

Comparison of marginal ulcers according to causative disease

Thirty-eight patients with marginal ulcers previously underwent an operation to control PUD, whereas 34 patients underwent operations to treat malignancies or other conditions (e.g., hemoperitoneum due to trauma). Clinical features of these two groups are shown in Table 3. There was no significant difference in the clinical characteristics including age, sex, types of reconstruction, or *H. pylori* infection rates. The patients who underwent operations to treat PUD had a higher rate of admission (25/38, 65.8%) than the other group (13/34, 38.2%; $P = 0.01$).

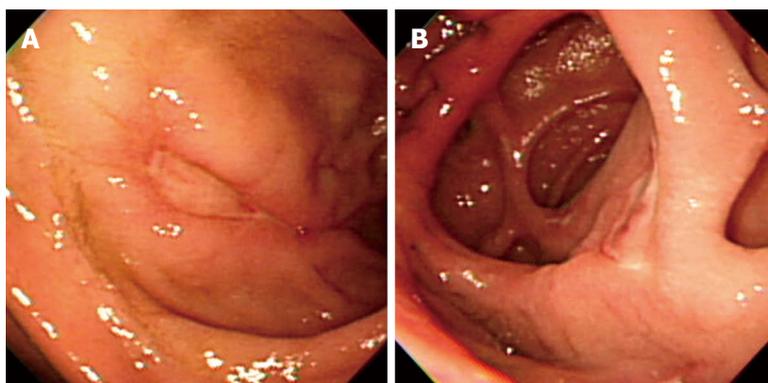


Figure 2 Endoscopic finding of marginal ulcer. In patients with B- I (A) and B- II (B) anastomosis, ovoid shaped ulcerations were observed on the efferent side.

Table 2 Comparison of marginal ulcers according to the location

	Ulcers on the efferent side (n = 36)	Both (n = 4)	Ulcers at anastomosis (n = 32)
Age (yr)	63.45 ± 14.03	62.50 ± 10.25	61.15 ± 12.49
Sex (male/female)	26:10	2:2	25:7
Tobacco use	15	2	10
Alcohol consumption	14	2	12
Clinical symptoms			
Bleeding episode	6	1	10
Anemia evaluation	9	2	2
Abdominal pain	11	1	10
Dyspepsia	5	0	5
Routine check	5	0	5
Anastomosis			
Billroth- I	7	0	6
Billroth- II	27	4	25
Total gastrectomy with RY	1	0	0
Whipple	1	0	1
Causative disease			
Malignancy	14	1	16
Complication of PUD	20	3	15
Others	2	0	1
Ulcer multiplicity	15	4	6
Number of therapeutic intervention	5	1	8
Initial hemoglobin (g/dL)	9.70 ± 3.38	6.70 ± 2.53	9.87 ± 3.79
Admission	21	4	14
Ulcer recurrence	12	2	7
<i>H. pylori</i> infection (%)	7/25 (28)	1/2 (50)	8/25 (32)

RY: Roux-en-Y anastomosis; *H. pylori*: *Helicobacter pylori*; PUD: Peptic ulcer disease.

Initial hemoglobin levels were significantly lower in the PUD group. However, the incidence of rebleeding after primary hemostasis and rate of ulcer recurrence were not different (Table 3).

Clinical features of GSC

GSC was found in six (0.7%) out of 837 patients who underwent gastrectomy. The clinicopathological features of GSC are shown in Table 4. All of these patients were male, and the median interval between the initial gastrectomy and treatment of GSC was 298.8 mo (range, 141-480). Half of these patients underwent gastric reconstruction by B-

Table 3 Comparison of marginal ulcers according to causative disease

	Marginal ulcers in patients with a history of PUD (n = 38)	Marginal ulcers in patients without a history of PUD (n = 34)	P value
Age (yr)	62.87 ± 13.67	63.47 ± 13.03	0.85
Sex (male/female)	31:7	22:12	0.10
Anastomosis			
B- I /B- II /others	3/35/0	6/25/3	0.06
Clinical symptoms			
Bleeding episode	10	7	0.04
Severe anemia	10	3	
Location			
Efferent side/ anastomosis/both	20/15/3	16/17/1	0.50
Initial hemoglobin (g/dL)	8.83 ± 2.83	11.04 ± 3.73	0.03
Admission (case)	25	13	0.01
Rebleeding during admission	4	3	0.87
Ulcer recurrence	9	11	0.41
<i>H. pylori</i> infection (%)	9/25 (36)	7/24 (29.2)	0.61

H. pylori: *Helicobacter pylori*; PUD: Peptic ulcer disease; B: Billroth.

I anastomosis and the remaining patients underwent reconstruction by B- II . In two-thirds of all patients, the location of the ulcerative lesion was the gastric stump (Figure 3). Most of these individuals underwent total gastrectomy and only one patient survived the operation.

DISCUSSION

The etiology of ulcer formation following gastrectomy is often multifactorial. Possible contributing factors include local ischemia, anastomotic tension, increased gastric acidity, *H. pylori* infection, tobacco use, and nonsteroidal anti-inflammatory drug (NSAID) use^[12-14]. Chronic inflammation caused by the suture materials at the anastomosis may lead to ulcer formation. Earlier studies have demonstrated that most marginal ulcers develop in the first 3 mo postoperatively due to prolonged irritation by foreign material, however, no ulcers have been found after the first postoperative year^[9,15].

In this study, we included patients who developed

Table 4 Case profile of gastric stump cancer

Case number	1	2	3	4	5	6
Sex	M	M	M	M	M	M
Age (yr)	73	73	58	61	61	44
Tobacco use	Yes	Yes	No	No	Yes	No
Alcohol consumption	No	Yes	No	No	No	No
Anastomosis	B-II	B-I	B-I	B-I	B-II	B-II
Latency period (mo)	360	480	360	152	432	228
Reason for operation	Pyloric obstruction	GU perforation	GU bleeding	Pyloric obstruction	GU perforation	DU perforation
Chief complaint	Melena	Soreness	Pain	Routine	Pain	Pain
Tumor location	Anastomosis	Anastomosis	Stump	Stump	Stump	Stump
Tumor type	Adenoca	Adenoca	Adenoca	Adenoca	Adenoca	Adenoca
Differentiation	Moderate	Poor	Poor	Poor	Moderate	Poor
<i>H. pylori</i> infection	Negative	Negative	Positive	Positive	Positive	Negative
TNM stage						
T	T4a	T3	T3	T2b	pT3	T3
N	N2	N0	N0	N1	N1	N0
M	M1 (Liver)	M0	M0	M0	M0	M0
Treatment	None	Total gastrectomy	Total gastrectomy	Total gastrectomy	Total gastrectomy	Total gastrectomy
Survival (mo)	No (3)	No (10)	No (10) ¹	Yes (36)	No (8)	No (18)
DFS (mo)	No (0)	Yes (9)	Yes (10)	Yes (36)	No (0)	Yes (15)

¹Expired due to leukopenia and pneumonia. GU: Gastric ulcer; DU: Duodenal ulcer; Adenoca: Adenocarcinoma; DFS: Disease-free survival; *H. pylori*: *Helicobacter pylori*; B: Billroth.

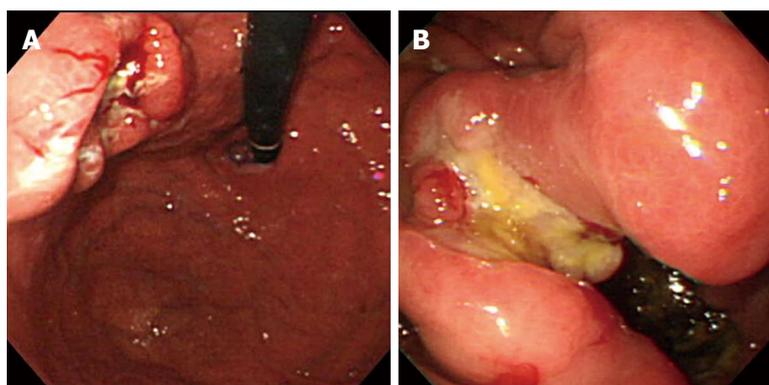


Figure 3 Endoscopic findings of gastric stump cancer. A: An ulcerating lesion of the gastric stump was observed; B: After gastrectomy with Billroth-II reconstruction, a large ulcerating mass was found at the anastomosis site.

ulcers 1 year or more after surgery, and analyzed true marginal ulcers that appeared after gastrectomy. Our results showed that the incidence of endoscopic ulcers after gastrectomy was 9.3% and marginal ulcers was 8.6%. In particular, the incidence of marginal ulcers in patients who had undergone gastrectomy to treat PUD-associated complications was 27.1%. Furthermore, it is noteworthy that most of the patients with marginal ulcers (86.1%) complained of specific gastrointestinal symptoms. Half of these individuals experienced bleeding episodes or developed severe anemia. There was a limitation in this study and we did not consider whether vagotomy was performed. We focused on ulcer development after gastrectomy. Previously, it has been demonstrated that vagotomy prevents ulcer recurrence^[16,17], and most of the enrolled patients had gastrectomy with vagotomy as peptic ulcer surgery.

Marginal ulcer is a well-known complication of partial gastrectomy. It has been reported that marginal ulcer is

evidence of inadequate acid reduction. However, incomplete suppression of gastric acid production is not the sole cause of marginal ulcers. In our endoscopic survey, marginal ulcer was formed following total gastrectomy. On the basis of this result, impaired blood supply or use of NSAIDs can be another mechanism of ulcer development after gastrectomy. In our study, risk factors for ulcer formation after gastrectomy were B-II anastomosis and previous gastrectomy for treating PUD complications. Generally, B-I anastomosis has several advantages. This technique can be performed more easily and rapidly than a B-II anastomosis because fewer steps are required. Normal continuity of the gastrointestinal tract is maintained, thus promoting the mixture of food with bile and pancreatic secretions. The remaining duodenum offers more resistance to recurrent ulceration than does the jejunum and plays a role in preventing the recurrence of ulcers after B-I anastomosis^[18]. These factors explained why ulcerative lesions developed less frequently following

B- I anastomosis.

Infection with *H. pylori* has been shown to be a predictive marker of PUD in the general population, but the exact link between *H. pylori* and the development of marginal ulcers following gastrectomy is unclear. Previously, uncontrolled data have suggested that the frequency of symptomatic marginal ulcers can be reduced by preoperative screening and treatment of *H. pylori* infection in patients undergoing gastric bypass surgery^[19,20]. Although these studies were performed in bariatric patients who have undergone gastric bypass surgery, the results indicate that infection with *H. pylori* may promote marginal ulcer formation. On the contrary, our investigation showed that the rate of *H. pylori* infection did not differ significantly between the ulcer and non-ulcer groups. This suggests that *H. pylori* infection does not play an important role in the pathogenesis of recurrent ulceration after gastrectomy.

However, our study had several limitations associated with our investigation of *H. pylori* infection. First, we did not determine whether patients enrolled in this study were treated when *H. pylori* was diagnosed before they underwent endoscopic examination. In fact, our patients were not routinely tested nor treated for *H. pylori* before gastrectomy. Second, spontaneous clearance of *H. pylori* might occur in a certain number of patients who undergo distal gastrectomy^[21]. Third, the number of patients with B-II was more than twice that with B- I anastomosis. A previous study has shown that resection with B- I has an incidence of *H. pylori* infection of approximately 70%, followed by a significantly lower rate of infection in B- II anastomosis of 45%^[22]. The lower rate of *H. pylori* infection found in B-II may reflect the role of bile reflux, which is more common in B- II than B- I, and may interfere with colonization by *H. pylori*.

To date, many studies have shown that GSC develops after distal gastrectomy, at frequencies of 0.4-2.5%, with an increased incidence 15 years after surgery^[23-25]. Some authors have postulated that patients who have undergone B- II gastrojejunal anastomosis are more prone to developing carcinomas in the gastric remnant^[26,27]. However, GSC has been reported in patients following B- I anastomosis. Our results revealed that GSC was found in three patients after B- I and three after B- II anastomosis. GSC is often described as a tumor with a poor prognosis, with low resectability rates because of extended lymph node metastases and infiltration of adjacent organs^[24,28]. The reported 5-year disease-free overall survival ranges from 7% to 20%^[29,30]. In our study, all patients had advanced stage disease at diagnosis and surgical treatment (total gastrectomy with radical lymph node dissection) was performed in 80% of cases. However, only one patient had 5-year disease-free survival.

Although specific factors responsible for the pathogenesis of GSC have not been identified, chronic inflammation of remnant gastric mucosa is considered to be a risk factor. Enterogastric reflux of bile acids results in increased proliferation of epithelial cells, and hypochlorhydria with subsequent bacterial overgrowth promotes

this condition^[31,32]. It has been previously reported that the gastric mucosa undergoes marked morphological changes in most patients after gastrectomy^[27,33]. Other mechanisms underlying GSC development are effects of hormonal regulation following vagotomy, and *H. pylori* infection^[34-37]. Although *H. pylori* infection may also be a causative factor for GSC, it has reported that the rate of *H. pylori* infection in patients with GSC is lower than in those with primary gastric cancer. The reason is that the gastric stump may be an unsuitable environment for *H. pylori* colonization due to increased bile reflux^[38,39]. However, the exact effect of *H. pylori* is unclear in clinical practice because of the small number of studies on this subject.

In conclusion, we found that endoscopic ulcers following gastrectomy were more common in patients who have undergone B- II anastomosis and had pre-existing cases of PUD. Infection with *H. pylori* did not appear to play an important role in the formation of ulcers following gastrectomy. Patients who underwent operations to treat PUD had lower initial levels of hemoglobin and a higher frequency of admission. Our study demonstrated that patients who undergo operations to treat PUD and subsequently develop marginal ulcer are likely to have a more severe clinical course. Further prospective and detail studies are needed to confirm our findings.

COMMENTS

Background

The total frequency of performing gastrectomy has increased due to the frequent detection of early gastric cancer and increasing morbid obesity. During follow-up endoscopic examination, the detection of ulcerative lesions including marginal ulcers, as well as cancer recurrence, is of clinical interest. Until now, there are limited data currently available on the incidence of marginal ulcer, and the exact link between *Helicobacter pylori* (*H. pylori*) and the development of marginal ulcers is unclear.

Research frontiers

Earlier studies have demonstrated that most marginal ulcers develop in the first 3 mo postoperatively due to prolonged irritation by foreign material. In this study, we excluded the possibility of chronic inflammation caused by the suture materials, and included patients who developed ulcers 1 year or more after surgery. Therefore, true marginal ulcers were analyzed. It was more common in patients in who had undergone Billroth II (B- II) anastomosis and had pre-existing cases of peptic ulcer disease (PUD). It is noteworthy that the incidence of marginal ulcers in patients who had undergone gastrectomy to treat PUD-associated complications was 27.1%. This provided information about the precise incidence and clinical features of marginal ulcers.

Innovations and breakthroughs

The results showed that the incidence of endoscopic ulcers after gastrectomy was 9.3% and that of marginal ulcers was 8.6%. When the patients were divided into two groups according to the presence of ulcer, infection rates of *H. pylori* did not differ significantly between the two groups (34.5% vs 36.5%, $P > 0.05$). Ulcers were more common in patients with B- II anastomosis and pre-existing conditions for PUD. The patients who underwent operations to treat PUD had lower initial levels of hemoglobin and higher rates of hospital admission.

Applications

The researchers drew a conclusion that *H. pylori* infection did not play an important role in the formation of ulcers following gastrectomy. For the patients who underwent operations for PUD, the clinical course of marginal ulcers was more severe.

Peer review

The paper is well written and the findings are of values for gastrectomy evaluation.

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Two-stage resection for malignant colonic obstructions: The timing of early resection and possible predictive factors

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Abstract

AIM: To study potential predictive factors for early radical resection in two-stage resection for left malignant colonic obstruction.

METHODS: Thirty-eight cases of left-sided obstructive colon cancer undergoing two-stage operations were reviewed between January 1998 and August 2008. Patients were classified into two groups ($n = 19$ each): early radical resection (interval ≤ 10 d) and late radical resection (interval > 10 d). Baseline demographics, post-diversion outcome, perioperative data, tumor characteristics, outcome and complications were analyzed.

RESULTS: The baseline demographics revealed no differences except for less pre-diversion sepsis in the early group ($P < 0.001$) and more obstruction days in the late group ($P = 0.009$). The mean intervals of early and late radical resections were 7.9 ± 1.3 d and 17.8 ± 5.5 d, respectively ($P < 0.001$). After diversion,

the presence of bowel sounds, flatus, removal of the nasogastric tube and the resumption of oral feeding occurred earlier in the early group. The operation time and duration of hospital stay were both significant reduced in the early group. Complication rates did not differ between groups.

CONCLUSION: The earlier recovery of bowel function seems to be predictive of early radical resection. In contrast, pre-diversion sepsis and more obstruction days were predictive of delayed radical resection.

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Key words: Colorectal cancer; Colostomy; Diversion; Obstruction; Two-stage resection

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INTRODUCTION

Bowel obstruction occurs in 7%-47% of patients with colorectal cancer (CRC)^[1-9]. This condition usually requires emergency surgical intervention and is associated with high postoperative morbidity, mortality and a poor 5-year survival rate^[4,5,8]. Surgical management for patients with obstructive CRC varies widely according to the tumor location, severity of the patient's condition and experience level of the surgeon. Resection with anastomosis in one stage is now a generally accepted practice

for primary right-sided obstructive CRC^[1,4,6,9-12]. For left-sided obstructive CRC, two types of surgical intervention have been used: primary resection and staged resection (diversion prior to resection). A randomized trial of emergency colostomy versus resection in patients with left-sided obstructive CRS demonstrated that the only advantage of primary resection was a shorter hospital stay^[13]. A Cochrane systematic review found no evidence to suggest a benefit in terms of mortality when comparing staged procedures with primary resection^[11]. Although the optimal choice of treatment for patients with left-sided obstructive CRS remains controversial^[8,11,14-17], several studies have reported better results for staged procedures^[1,2,4,9,18,19].

Two-staged resection consists of a colostomy or ileostomy to resolve the obstruction as a first step, followed by radical resection and simultaneous closure of the stoma. Corman suggested the use of a 10- to 14-d interval between diversion and radical resection^[20]. The mean interval has ranged widely from 11.5 d to 42 d^[1,9,14,19,21,22]. The appropriate interval for two-stage resection for left-sided obstructive CRC remains controversial and ranges widely. In critically ill patients, the interval should be longer than Corman's suggestion. In contrast, we supposed that in patients with early recovery of bowel function or those lacking any pre-diversion septic condition, the interval may be shorter than 10 d. This study aimed to evaluate the timing of early radical resection and possible predictive factors.

MATERIALS AND METHODS

Patients

From January 1998 to August 2008, there were 132 patients diagnosed with complete obstructive CRC who were treated with emergency surgery at the Tri-Service General Hospital in Taipei, Taiwan. These patients were admitted through the emergency department. Patients with the following criteria were excluded: (1) a tumor proximal to the splenic flexure; (2) colonic perforation with peritonitis; (3) primary resection with or without anastomosis; (4) three-stage resection; (5) palliative ileostomy or colostomy for non-resectable tumors, disseminated disease or critical illness; (6) a colonic stent; or (7) rectal cancer. Consequently, 38 cases with left-sided obstructive CRC for which the patients underwent two-staged resection were investigated.

Methods

Cases with intervals of 10 d or less between diversion and radical resection were classified as group A (early radical resection). Cases with intervals of longer than 10 d were classified as group B (late radical resection). Data collected from extensive chart reviews were recorded and compared. These included (1) baseline demographics including baseline data, comorbidities, days of obstruction (no feces passage) and the mean interval; (2) post-diversion outcomes including stoma type, the tim-

ing of oral feeding, the passage of flatus, the presence of bowel sounds, removal of the nasogastric tube and complications; (3) perioperative data and tumor characteristics including surgery type, operation time, blood loss, blood transfusion, tumor location, pathology diagnosis and tumor stage; and (4) the outcome in terms of hospital stay and complications.

The definition of sepsis was adopted from those published by the International Sepsis Definitions Conference^[23]. Significant comorbidities included hypertensive cardiovascular disease, diabetes mellitus, coronary arterial disease, cerebral vascular accidents, chronic obstructive pulmonary disease, liver cirrhosis and chronic renal insufficiency. Complete bowel obstruction was diagnosed based on clinical history, physical examination, a failure to pass flatus, decreased or absent bowel sounds and the X-ray signs of a distended, obstructed colon. Patients with partial bowel obstruction that responded to nasogastric decompression and intravenous fluid replacement were not included in this category of colonic obstruction. After fluid resuscitation, the correction of imbalanced electrolyte levels and optimization of the patient's general condition, emergency T-loop colostomy was performed within 24 h. Prophylactic antibiotic treatment was administered during the induction of anesthesia and maintained for 24 h unless the septic condition persisted. The type of radical resection depended on the tumor location and each surgeon's judgment; meanwhile, the colostomy was closed. The patient's nutrition status, such as their hemoglobin and serum albumin levels, were corrected to 10 dL/mg and 3.5 dL/mg, respectively, before definitive treatment. Tumor, node, metastasis classification was used for tumor staging^[24].

Group A (early radical resection): Nineteen cases in which the patients underwent two-staged resection with an interval of 10 d or less were classified as group A. A T-loop colostomy was performed as a first step, followed by radical resection and simultaneous closure of the colostomy.

Group B (late radical resection): Nineteen cases in which the patients underwent two-stage resection with an interval of more than 10 d were classified as group B. The surgical approach was the same as for group A.

Statistical analysis

Data were processed and analyzed using the Statistical Package for Social Sciences for Windows, Version 15.0 (SPSS, Inc., Chicago, IL, United States). Continuous variables were analyzed using independent Student's *t*-tests. Nominal data were compared using chi-squared or Fisher's exact tests. *P* < 0.05 was assumed to be statistically significant.

RESULTS

Baseline demographics

The baseline demographics of this patient population are shown in Table 1. There were no significant differences between groups A and B except for a higher inci-

Table 1 Baseline demographics

	Group A (n = 19)	Group B (n = 19)	P value
Gender, male/female	14/5	13/6	0.721
Age (yr)	60.6 ± 13	67.9 ± 14.9	0.117
Pre-diversion sepsis (%)	0	10 (53)	< 0.001
Co-morbidities			0.139
0	10 (53)	4 (21)	
1	7 (37)	9 (47)	
2	2 (11)	4 (21)	
≥ 3	0	2 (10.5)	
Days of obstruction Interval (d)	3.6 ± 1	4.8 ± 1.6	0.009
	7.9 ± 1.3	17.8 ± 5.5	< 0.001

Data are presented as n, n (%), mean ± SD or as noted.

Table 2 Post-diversion outcome

	Group A (n = 19)	Group B (n = 19)	P value
Stoma type			
T-loop colostomy	19	19	
Time to presenting with bowel sounds (d)	1.6 ± 0.5	2.7 ± 1.2	0.001
Time to passage of flatus (d)	1.8 ± 0.4	3 ± 1.4	0.001
Time to removal of the nasogastric tube (d)	1.8 ± 0.8	3.1 ± 1.5	0.002
Time to initiation of oral feeding (d)	2.2 ± 0.6	3.5 ± 1.3	0.001
Complications			
Sepsis	0	1 (5)	0.311
Stomal infection	1 (5)	3 (16)	0.290

Data are presented as n, n (%), mean ± SD or as noted.

dence of pre-diversion sepsis in group B (0% *vs* 53%, *P* < 0.001) and more days of obstruction in group B (3.6 ± 1 d *vs* 4.8 ± 1.6 d, *P* = 0.009). The mean intervals for early and late radical resection were 7.9 ± 1.3 d and 17.8 ± 5.5 d, respectively (*P* < 0.001). The molecular markers, such as white blood cell count and levels of hemoglobin, C-reactive protein and serum albumin, were measured and statistically analyzed. The initial white blood cell count and C-reactive protein level (before diversion) were higher in group B, but there was no significant difference in comparison to group A. The initial hemoglobin and serum albumin levels were similar in both groups.

Post-diversion outcome

Post-diversion outcomes are shown in Table 2. All 38 patients underwent T-loop colostomy for emergency diversion. Bowel sounds, the passage of flatus, removal of the nasogastric tube and oral feeding were observed earlier in group A than group B (1.6 ± 0.5 d *vs* 2.7 ± 1.2 d, 1.8 ± 0.4 d *vs* 3 ± 1.4 d, 1.8 ± 0.8 d *vs* 3.1 ± 1.5 d, 2.2 ± 0.6 d *vs* 3.5 ± 1.3 d; *P* = 0.001, 0.002, 0.001 and 0.001, respectively). There were no differences between groups in terms of the complications of diversion.

Table 3 Perioperative data and tumor characteristics

	Group A (n = 19)	Group B (n = 19)	P value
Type of operation			0.201
Left hemicolectomy	16 (84)	11 (58)	
Extended left hemicolectomy	1 (5)	3 (16)	
Anterior resection	2 (10)	5 (26)	
Operation time (h)	4.9 ± 0.4	5.5 ± 0.7	0.003
Blood loss (mL)	213 ± 149	250 ± 92	0.533
Blood transfusion (mL)	26.3 ± 78.8	39.5 ± 93.7	0.642
Tumor location			0.162
Transverse colon	2 (10)	2 (10)	
Descending colon	11 (58)	5 (26)	
Descending/sigmoid colon	2 (10)	7 (37)	
Sigmoid colon	4 (21)	5 (26)	
Pathological diagnosis			
Adenocarcinoma	19	19	
Tumor differentiation			0.734
Well or moderate	15 (79)	13 (69)	
Poor	3 (16)	4 (21)	
Undifferentiated or not known	1 (5.3)	2 (10)	
Stage			0.260
II	6 (32)	11 (58)	
III	11 (58)	7 (37)	
IV	2 (10)	1 (5)	

Data are presented as n, n (%), mean ± SD or as noted.

Perioperative data and tumor characteristics

The perioperative data and tumor characteristics are shown in Table 3. There was no significant difference between the two groups in terms of the type of operation, blood loss, blood transfusion, tumor location, tumor stage or stage of tumor differentiation. The pathology was adenocarcinoma in all 38 cases. Operation time was longer in group B than in group A (4.9 ± 0.4 h *vs* 5.5 ± 0.7 h, *P* = 0.003).

Outcomes and complications

The outcomes and complications are shown in Table 4. Group A had a shorter mean hospital stay than group B (21.2 ± 3.2 d *vs* 36.2 ± 17.4 d, *P* = 0.001). There were no differences between groups in terms of complications.

DISCUSSION

The study conducted on this series of 38 cases showed that early oral feeding, the passage of flatus, the presence of bowel sounds and the time to removal of the nasogastric tube could be predictive factors for early radical resection in patients undergoing a two-stage resection. Pre-diversion sepsis and longer obstruction days, in contrast, were associated with the delay of radical resection. Malignant colonic obstruction usually required emergency surgical intervention. A single-stage strategy has been suggested for patients with primary right-sided obstructive CRC^[1,4,6,9-12] Although the topic is debated, a staged procedure for left-sided obstructive

Table 4 Outcome and complications

	Group A (n = 19)	Group B (n = 19)	P value
Hospital stay	21.2 ± 3.2	36.2 ± 17.4	0.001
Ileus > 4 d	1 (5)	2 (10)	0.547
Anastomotic leakage	0	0	
Wound infection	4 (21)	5 (26)	0.703
Post-resection sepsis	1 (5)	1 (5)	
Other complications			0.548
Urinary tract infection	0	1 (5)	
Pneumonia	1 (5)	1 (5)	
Upper gastrointestinal bleeding	0	1 (5)	

Data are presented as n, n (%), mean ± SD or as noted.

CRC is still considered to be the best option due to its safety^[8,15,18,19,21,25]. Corman suggested that a 10- to 14-d interval be used for two-stage procedures. The mean interval has ranged from 11.5 d to 42 d. The optimal interval has not been thoroughly investigated^[1,9,14,19,21,22]. The present study grouped patients based on the timing of radical resection. The mean intervals were 7.9 ± 1.3 d and 17.8 ± 5.5 d in the early and late radical resection groups, respectively.

Tumors cause obstruction by two major mechanisms: mechanical or adynamic obstruction^[26]. In our study, the late radical resection group had significantly more pre-diversion sepsis than the early group (0% vs 53%, P < 0.001) and a longer period of obstruction (3.6 ± 1 d vs 4.8 ± 1.6 d, P = 0.009). As is known, sepsis is associated with adynamic obstruction. We consider that pre-diversion sepsis and longer durations of obstruction-especially more than 5 d, which exacerbates mechanical obstruction-may be the predisposing factors necessitating a delay in radical resection. In contrast, the absence of pre-diversion sepsis or a shorter period of obstruction may lead to more rapid recovery of bowel function and early radical resection.

A nasogastric tube is usually placed temporarily to decompress the proximal bowel and to alleviate acute bowel obstruction symptoms^[26]. The passage of flatus and the presence of bowel sounds are generally considered to be signs of the recovery of bowel movement. In this series, the initiation of oral feeding, flatus, the presence of bowel sounds and removal of the nasogastric tube were observed sooner in the early radical resection group. These signs may imply the early elimination of edematous bowel, the early recovery of bowel movement and the early timing of any further surgical intervention required. However, more evidence is necessary to clarify the relationship between the recovery of bowel function or edematous status and the timing of the initiation of oral feeding, flatus, the presence of bowel sounds and removal of the nasogastric tube.

In our data, the operation time was significantly longer in cases that underwent delayed radical resection. This may have been caused by more severe post-inflammatory bowel adhesion in group B. Post-inflammatory bowel adhesion makes radical surgery difficult, even after a longer interval. However, further studies will be necessary to demonstrate the severity of bowel adhesion in patients with obstructive CRC.

The early radical resection group tended to stay in the hospital for less time than the late group (21.2 ± 3.2 d vs 36.2 ± 17.4 d, P = 0.001). No anastomotic leakage was noted in either group. The rates of wound infection, post-resection sepsis and complications, such as urinary tract infection, pneumonia and upper gastrointestinal bleeding, were similar between groups. The five-year disease-free survival rates were also similar between groups.

Although a 10- to 14-d interval between diversion and radical resection is generally accepted for the two-stage resection of acute left malignant colonic obstruction, early radical resection (with an interval shorter than 10 d) may be considered when the patients present with the earlier recovery of bowel function or the lack of any pre-diversion septic condition.

The present study found that earlier oral feeding, the presence of bowel sounds, the passage of flatus and removal of the nasogastric tube, all of which indicate the recovery of bowel function, seem to predict early radical resection. In contrast, pre-diversion sepsis and obstructions longer than five days may indicate the need to delay radical resection. The early radical resection group benefited from a shorter hospital stay with no difference in terms of complications compared to the late group. Further prospective or multicenter studies are recommended to clarify the relationship between such factors and the optimal timing of radical resection.

COMMENTS

Background

Surgical management for patients with obstructive colorectal cancer (CRC) varies widely according to tumor location, severity of the patient's condition and experience of the surgeon. Resection with anastomosis in one stage is now generally accepted for primary right-sided obstructive CRC. For left-sided obstructive CRC, two types of surgical intervention have been used: primary resection and staged resection (diversion prior to resection). Two-staged resection consists of colostomy or ileostomy to resolve the obstruction as a first step, followed by radical resection and closure of the stoma at the same time. Corman suggested a 10- to 14-d interval between diversion and radical resection. In critical illness patients, the interval would be longer than the Corman's suggestion. In contrast, the authors supposed that the patients with early recovery of bowel function or no pre-diversion septic condition, the interval may be earlier than 10 d.

Research frontiers

Signs of earlier oral feeding, presence of bowel sound, passage of flatus and removal of the nasogastric tube, which indicating recovery of bowel function, seem to be predictive factors for early radical resection. In contrast, pre-diversion sepsis and obstructions longer than five days might indicate a later timing for radical resection.

Innovations and breakthroughs

The present study found signs of earlier oral feeding, presence of bowel sound, passage of flatus and removal of the nasogastric tube, which indicating recovery of bowel function, seem to be predictive factors for early radical resection. In contrast, pre-diversion sepsis and obstructions longer than five days might

indicate a later timing for radical resection. The early radical resection group had an advantage of a shorter hospital stay with no difference in terms of complications comparing to the late group.

Applications

Earlier oral feeding, presence of bowel sound, passage of flatus and removal of the nasogastric tube, which indicating recovery of bowel function, seem to be predictive factors for early radical resection in two-staged resection for obstructive CRC.

Peer review

This paper describes two-stage resection for acute left-sided malignant colonic obstructions: timing of early radical resection and possible predictive factors. This work provides useful information on the early group had significantly earlier presence of bowel sounds, flatus, and removal of nasogastric tube and resumption of oral feeding.

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Preoperative predictors of short-term survival after hepatectomy for multinodular hepatocellular carcinoma

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Abstract

AIM: To investigate preoperative factors associated with poor short-term outcome after resection for multinodular hepatocellular carcinoma (HCC) and to assess the contraindication of patients for surgery.

METHODS: We retrospectively analyzed 162 multinodular HCC patients with Child-Pugh A liver function who underwent surgical resection. The prognostic significance of preoperative factors was investigated by univariate analysis using the log-rank test and by multivariate analysis using the Cox proportional hazards model. Each independent risk factor was then assigned points to construct a scoring model to evaluate the indication for surgical intervention. A receiver operating characteristics (ROC) curve was constructed to assess the predictive ability of this system.

RESULTS: The median overall survival was 38.3 mo (range: 3-80 mo), while the median disease-free survival was 18.6 mo (range: 1-79 mo). The 1-year mortality was 14%. Independent prognostic risk factors of 1-year death included prealbumin < 170 mg/L [hazard ratio (HR): 5.531, $P < 0.001$], alkaline phosphatase > 129 U/L (HR: 3.252, $P = 0.005$), α fetoprotein > 20 μ g/L (HR: 7.477, $P = 0.011$), total tumor size > 8 cm (HR: 10.543; $P < 0.001$), platelet count < $100 \times 10^9/L$ (HR: 9.937, $P < 0.001$), and γ -glutamyl transpeptidase > 64 U/L (HR: 3.791, $P < 0.001$). The scoring model had a strong ability to predict 1-year survival (area under ROC: 0.925, $P < 0.001$). Patients with a score ≥ 5 had significantly poorer short-term outcome than those with a score < 5 (1-year mortality: 62% vs 5%, $P < 0.001$; 1-year recurrence rate: 86% vs 33%, $P < 0.001$). Patients with score ≥ 5 had greater possibility of microvascular invasion ($P < 0.001$), poor tumor differentiation ($P = 0.003$), liver cirrhosis with small nodules ($P < 0.001$), and intraoperative blood transfusion ($P = 0.010$).

CONCLUSION: A composite preoperative scoring model can be used as an indication of prognosis of HCC patients after surgical resection. Resection should be considered with caution in patients with a score ≥ 5 , which indicates a contraindication for surgery.

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Key words: Hepatectomy; Hepatocellular carcinoma; Multinodular; Prognosis; Treatment outcome

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths worldwide, with the highest incidence rates reported in East Asia^[1,2]. Multinodular HCC accounts for a large number of HCC cases. For these patients, hepatic resection may lead to an increased risk of postoperative liver failure and the recurrence rates after resection are higher than those for a single HCC. Hence, multinodular HCC have been considered a controversial indication for hepatic resection, especially for cases involving liver cirrhosis^[3].

However, the fact that patients with multinodular HCC have poorer survival than those with a single small tumor should not be considered a sufficient reason for excluding them from undergoing resection. Although liver transplantation is considered another curative treatment, its application is hampered by the lack of liver donors and high drop-out rates^[4]. The efficacy of radiofrequency ablation (RFA) is greatly limited by tumor size and location. Chemoembolization has been regarded as a palliative method with lower rates of tumor necrosis^[5,6]. With developments in surgical techniques and perioperative treatment, the safety of hepatic resection has markedly improved. Surgical mortality rates have been reduced to less than 5.0%^[7]. Many previous studies have demonstrated that resection can provide survival benefits for patients with multinodular HCC^[4,8-12]. Therefore, hepatic resection remains the widely accepted mainstay of curative treatment for multinodular HCC.

Better patient selection plays a crucial role in the improvement of postoperative outcome^[7]. Although it is widely accepted that Child-Pugh A cirrhosis can be treated safely in elective surgery, poor short-term survival still exists in these patients. It is reported that patients with multinodular HCC have a 1-year mortality ranging from 14% to 26% after hepatectomy^[4,13]. Several previous studies reported that HCC patients undergoing non-surgical therapy had a total 1-year survival rate of 40%-62%^[2,14-16], and patients without adverse factors who only received supportive care had a 1-year survival rate of 80%^[16], thus patients who die in the first year after surgery may not have gained a benefit from hepatic resection. Hepatectomy should not be indicated for these patients even though there is acceptable perioperative safety.

Several recent studies showed that traditional scoring systems of liver function such as the Child-Pugh score have limitations^[17,18], and routine parameters may not describe hepatic impairment sufficiently. Most current staging systems for HCC focus on predicting perioperative or

long-term survival but neglect the short-term outcome. The risk factors for poor short-term survival in patients with satisfactory liver function remain unclear. The present study was designed to investigate the independent predictive factors that are associated with poor short-term outcome after resection of multinodular HCC. We only focused on the preoperative clinical data that were most helpful in choosing the optimal initial treatment. The results may supplement the classical estimation system for surgical indication.

MATERIALS AND METHODS

Patients

Between January 2004 and December 2008, we retrospectively accumulated 208 consecutive patients with multinodular HCC underwent hepatic resection at the Department of Surgery, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University (Shanghai, China). We regarded a tumor with a surrounding conodule(s) as a single tumor only when the co-nodule(s) was attached to the main tumor^[4,13]. Multinodular HCC was diagnosed when the tumor number was ≥ 2 . Fifteen patients with extrahepatic metastasis and 31 patients with macroscopic cancerous emboli were excluded. The remaining 162 patients (28 female, 134 male; mean age, 50.16 years; range: 27-76 years) who underwent curative hepatic resection were enrolled in this study. The baseline clinical features are listed in Table 1. All patients had liver function of Child-Pugh class A. Among them, 44 patients were Barcelona Clinic Liver Cancer (BCLC) stage A and 118 BCLC stage B. A total of 156 patients were diagnosed with hepatitis B virus (HBV) infection, which was defined as positivity of hepatitis B surface antigen (HBsAg) in the serum. No patient had a background of other chronic liver disease. The study protocol was approved by the clinical research ethics committee of the hospital. Written informed consent was obtained from all patients according to the policies of the committee.

Diagnosis of hepatocellular carcinoma and preoperative assessment

The preoperative diagnosis of multinodular HCC was based on the findings of focal lesions with signs of early hyperenhancement and delayed hypoenhancement on 2 different imaging modalities such as enhanced spiral computed tomography (CT) and magnetic resonance imaging (MRI), or on the combination of imaging findings and α fetoprotein (AFP) level^[5,13,19]. Tumor size was defined as the maximal diameter. Routine biopsy of the lesions was not performed before resection if the lesion had typical characteristics of HCC. No patient enrolled in this study received a fine-needle biopsy. The diagnoses of all patients were confirmed definitively by pathological examination after resection. Histological tumor differentiation was determined using the Edmonson-Steiner grading.

Each patient underwent a complete blood count.

Table 1 The baselines of clinical features at the time of diagnosis of hepatocellular carcinoma

Variable	mean/n
Age (yr)	50.16 ± 10.65 (range: 27-76)
Gender (male/female)	134:28
HBsAg-positive	156 (96.30%)
HCV-Ab positive	0
HBV-DNA	
> 10 ⁴ /L	88 (140, 62.85%)
> 10 ⁶ /L	32 (140, 22.86%)
Child-Pugh score	
5	148 (97.53%)
6	10 (2.47%)
TBIL (μmol/L)	15.76 ± 5.72 (range: 4.9-33)
DBIL (μmol/L)	5.51 ± 2.26 (range: 1.5-15.5)
IBIL (μmol/L)	10.28 ± 4.09 (range: 1-23)
ALB (g/L)	41.32 ± 3.59 (range: 32-53)
Prealbumin (mg/L)	217.73 ± 65.83 (range: 110-500)
PT > 13 s (n)	41 (25.31%) (range: 10.4-17.6)
ALT > 44U/L (n)	48 (29.63%) (range: 12.9-235)
AST > 38 U/L (n)	88 (54.32%) (range: 12.8-207)
AFP > 20 μg/L	102 (62.96%) (range: 2.5-4537)
AFP > 400 μg/L	59 (36.42%)
Diabetes	14 (8.64%)
Cardiovascular hypertension	16 (9.88%)
History of smoke	50 (30.86%)
History of alcoholism	24 (14.81%)
History of other operations	20 (12.35%)
BCLC Staging	
Stage A	44 (27.16%)
Stage B	118 (72.84%)
With Milan criteria	44 (27.16%)
With UCSF criteria	86 (53.09%)
TNM staging (6th, 2002)	
T2	103 (63.58%)
T3	59 (36.42%)
Total tumor size (cm)	7.58 ± 4.66 (range: 1.4-23)
The largest tumor size (cm)	5.37 ± 4.00 (range: 0.7-21)
Number of tumors	
2	110
3	36
4	10
> 4	6
Serum creatinine (mmol/L)	68.65 ± 14.67 (range: 36-119)
Serum urea nitrogen (μmol/L)	5.86 ± 5.92 (range: 2.6-57)
WBC (× 10 ⁹ /L)	5.23 ± 1.69 (range: 1.66-11.6)
RBC (× 10 ¹² /L)	4.53 ± 0.52 (range: 3.3-6.59)
HGB (g/L)	142.8 ± 21.32 (range: 94-146)
PLT (× 10 ⁹ /L)	148.3 ± 75.93 (range: 31-476)
PLT < 100 × 10 ⁹ /L (n)	44 (27.16%)
Intraoperative transfusion	14 (8.64%)
Postoperative transfusion	6 (3.70%)
Death in 3 mo after operation	4 (2.47%)

HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFP: α fetoprotein; TBIL: Total bilirubin; DBIL: Direct bilirubin; IBIL: Indirect bilirubin; ALB: Albumin; PT: Prothrombin time; ALT: Alanine transaminase; AST: Aspartate aminotransferase; BCLC: Barcelona Clinic Liver Cancer; UCSF: University of California, San Francisco; WBC: White blood cell; RBC: Red blood cell; PLT: Platelet; HGB: Hematoglobulin; TNM: Tumor, nodes, metastasis.

Liver impairment was evaluated in all patients by liver biochemistry, including serum total bilirubin (TBIL), direct bilirubin, indirect bilirubin, albumin (ALB), globulin, prealbumin, alanine transaminase, aspartate aminotransferase (AST), alkaline phosphatase (ALP) and

γ-glutamyl transpeptidase (GGT). Prothrombin time (PT) and activated partial thromboplastin time were also determined to evaluate liver function and surgical safety. Tumor markers, including AFP, carcinoembryonic antigen, carbohydrate antigen 19-9 and α fucosidase (AFU), were used to identify the tumor origin. The parameters of HBV infection were tested. We evaluated kidney function by measuring serum urea nitrogen, creatinine, uric acid and electrolytes. All patients received an upper gastrointestinal endoscopic examination for portal hypertension and hemorrhage. Patients also underwent thoracic X-ray to examine metastasis to the lungs. Abdominal ultrasonography and enhanced CT or MRI were used to evaluate the size and location of the tumors. For patients older than 60 years or who had a relevant disease history, pulmonary function tests and cardiovascular Doppler ultrasound were performed to determine any contraindications to resection.

Hepatic resection

Hepatic resection was considered the first-line therapy for patients with Child-Pugh A liver function. The indications for hepatic resection included the technical feasibility of resection, the absence of macroscopic cancerous thrombi in vessels, absence of distant metastasis and sufficient future remnant liver volume in the preoperative evaluation. Patients with resectable multinodular HCC received resection immediately without any preoperative anti-HCC therapy. We defined curative hepatic resection as complete removal of all the detectable tumors combined with tumor-free margins confirmed by histopathology^[4,8,13]. In China, most HCC patients had underlying liver disease such as hepatitis, fibrosis and cirrhosis, which result in limited capacity for regeneration. Surgery must balance resectability with preservation of hepatic function to reduce the risk of hepatic failure^[20]. For multinodular HCC, concomitant resection may result in more blood loss, a longer Pringle time, and a significant change in blood perfusion and drainage. The limits for safe resection of multinodular HCC should be smaller than that of a single tumor. Therefore, all patients received local nonanatomic resection. The surgical procedure has been reported previously^[21]. The tumors of 44 patients were resected *en bloc* (27.16%), and 118 patients underwent multinodular hepatic resections (72.84%). Intraoperative ultrasonography was always used to detect non-visible, nonpalpable nodules and to check the resection plane. Resection margins were examined by a microscopic histological test.

Follow-up

All patients were regularly followed up for recurrence by determination of AFP, liver enzymes, complete blood count, and CT or MRI scan monthly for the first 3 mo after resection. If there was no recurrence, the frequency of routine examination was changed to once every 3 mo. Tumor recurrence was identified by new lesions on imaging with appearances typical of HCC, a rising AFP

level, or rapid enlargement of lesions without typical HCC characteristics. If tumor recurrence was diagnosed, patients received a second hepatectomy, chemoembolization and locoregional ablation, such as RFA or percutaneous ethanol resection.

Statistical analysis

Patient demographics, tumor parameters, liver function and hepatitis-associated characteristics were evaluated. Continuous data are expressed as mean \pm SD. Categorical data were compared using the χ^2 test and Fisher's exact test as appropriate. Survival analyses were performed using the Kaplan-Meier method and compared using the log-rank test. For continuous data with statistical significance in the univariate analysis, a series of receiver operating characteristics (ROC) curves were used to identify the cutoff values with optimal discriminatory ability for 1-year survival. Multivariate analysis was performed using the Cox proportional hazard ratio model to identify independent prognostic factors. The factors with a *P*-value less than 0.1 in univariate analysis were included in the multivariate Cox regression analysis. In order to estimate the clinical value of the independent factors, a prognostic scoring system was constructed. We assigned points to each independent predictor according to the value of the partial regression coefficient, because each factor had different importance in the final Cox model^[22]. Each patient's score was the total points of 6 factors. A ROC curve was constructed to estimate the prognostic ability of the new scoring model. Overall survival was the time from the day of surgery to the day of death or to the most recent follow-up visit. Disease-free survival was the time from the day of surgery to the most recent follow-up visit at which evidence of a tumor was clear. The deaths caused by non-HCC-associated factors were included in the overall survival analysis, but not in the disease-free survival analysis. A *P* value $<$ 0.05 was considered statistically significant. All statistical processing was performed by SPSS 18.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Survival, outcome and morbidity after liver resection

The mean postoperative hospitalization period was 10.6 d (range: 5-28 d). The overall morbidity was 25.31% ($n = 41$). Pleural effusion ($n = 24$) and ascites ($n = 14$), which required diuretics or paracentesis, were the most common sequelae, with both occurring in 7 patients. Two patients developed bile leakage and one developed transitory arrhythmia. The median overall survival was 38.3 mo (range: 3-80 mo); overall survival rates at 1 year, 3 years and 5 years were 86%, 51% and 35%, respectively; the median disease-free survival was 18.6 mo (range: 1-79 mo); 1-year, 3-year and 5-year disease-free survival rates were 56%, 40% and 31%, respectively. The survival outcome was similar to that in 2 previous retrospective studies^[4,8]. During the entire follow-up, a total of 100 (61.73%) patients were diagnosed with tumor recurrence during the follow-

up period. Among them, twelve patients underwent a second hepatic resection, 65 received transarterial chemoembolization (TACE) alone, 18 received TACE combined with locoregional ablation and 5 received locoregional ablation alone. A total of 32 patients died within the first year after resection. Four patients died at the 3rd month, one died of acute severe hepatitis and 3 of unrecovered liver impairment after chemoembolization for recurrence. Of the remaining 28 patients, one died of incidental hemorrhage of the upper digestive tract at the 7th month after resection, 27 died of liver failure, including 4 patients within 4-6 mo, 2 within 7-9 mo, and 9 within 10-12 mo. All 27 patients had diffuse tumor recurrence leading to liver failure.

Predictive factors for 1-year survival

In the evaluation of preoperative factors that may have potential prognostic ability for short-term outcome, we limited the endpoint of follow-up to 1 year. The univariate Kaplan-Meier analysis showed that TBIL, prealbumin, AST, AFP, HBeAg, HBV-DNA, GGT, AFU, total tumor size, largest tumor size, ALP, and platelet count were significantly associated with 1-year mortality after hepatic resection (Table 2).

For the above factors, we analyzed continuous data by ROC curves to determine cutoff values that had the optimal sensitivity and specificity (i.e., the largest sum of sensitivity and specificity). The cutoff values of platelet count, AST, TBIL, AFU, AFP, GGT and ALP ($92.5 \times 10^9/L$, 41.8 U/L, 17.25 $\mu g/L$, 36 U/L, 32.6 $\mu g/L$, 75.5 U/L, 119.5 U/L, respectively) were similar to the limits of normal values, thus we defined the normal upper or low limitations as the best cutoff points (Table 3).

Multivariate analysis showed that 6 variables were significant predictive factors for 1-year survival status after resection: prealbumin $<$ 170 mg/L [hazard ratio (HR): 5.531, $P <$ 0.001]; ALP $>$ 129 U/L (HR: 3.252, $P =$ 0.005); AFP $>$ 20 $\mu g/L$ (HR: 7.477, $P =$ 0.011); total tumor size $>$ 8 cm (HR: 10.543, $P <$ 0.001); platelet count $<$ $100 \times 10^9/L$ (HR: 9.937, $P <$ 0.001), and GGT $>$ 64 U/L (HR: 3.791, $P <$ 0.001) (Table 4). Although factors of tumor invasiveness cannot be detected before surgery, the data showed that microvascular invasion and poor tumor differentiation were associated with tumor size (Figure 1).

Construction of a scoring system to determine indication for surgery

We assigned points for each prognostic factor (Table 4). The theoretical range of the prognostic score was 0 to 9. In the total population in our study, 10 patients scored 0, 16 patients scored 1, 44 patients scored 2, 32 patients scored 3, 18 patients scored 4, 22 patients scored 5, 10 patients scored 6, 6 patients scored 7, and 4 patients scored 8. A higher score implied a lower chance of 1-year survival. We assessed the prognostic value of the score on 1-year survival using a ROC curve (Figure 2). The area under the curve was 0.925 [95% confidence interval (CI): 0.864-0.985, $P <$ 0.001]. The data indicated that,

Table 2 Univariate Cox analysis of potential prognostic factors

Variables	n	The 1-year survival rates %	P value
Age			< 0.001
> 40 yr	128	85.9	
≤ 40 yr	34	64.7	
Parameters of liver function			
TBIL			< 0.001
> 17.1 μmol/L	49	58.33	
≤ 17.1 μmol/L	113	91.22	
ALB			0.530
< 35 g/L	8	75.00	
≥ 35 g/L	154	81.82	
GLB			0.565
> 30 g/L	79	79.48	
≤ 30 g/L	83	83.33	
Prealbumin			< 0.001
< 170 mg/L	33	58.3	
≥ 170 mg/L	129	90.6	
ALT			0.281
> 38 U/L	82	78.04	
≤ 38 U/L	80	85.00	
ALT			0.993
> 76 U/L	10	80.00	
≤ 76 U/L	152	81.58	
AST			0.001
> 38 U/L	88	75.00	
≤ 38 U/L	74	89.21	
AFU			0.004
> 40 U/L	31	64.31	
≤ 40 U/L	131	85.89	
ALP			0.001
> 129 U/L	27	58.33	
≤ 129 U/L	135	85.50	
GGT			< 0.001
> 64 U/L	82	65.00	
≤ 64 U/L	80	97.56	
PT			0.428
> 13 s	41	75.00	
≤ 13 s	121	82.00	
APTT			0.566
> 37 s	72	77.80	
≤ 37 s	90	82.20	
Parameters of blood test			
WBC			0.389
< 4 × 10 ⁹ /L	35	76.5	
≥ 4 × 10 ⁹ /L	127	82.5	
PLT			< 0.001
< 100 × 10 ⁹ /L	44	59.1	
≥ 100 × 10 ⁹ /L	118	89.7	
Parameters of tumors			
Total tumor size			0.010
> 5 cm	108	75.9	
≤ 5 cm	54	92.6	
Total tumor size			< 0.001
> 8 cm	54	66.7	
≤ 8 cm	108	88.9	
Largest tumor size			0.050
> 5 cm	58	72.41	
≤ 5 cm	104	84.61	
Largest tumor size			0.001
> 8 cm	22	54.54	
≤ 8 cm	140	84.28	
AFP			< 0.001
> 20 μg/L	102	72.00	
≤ 20 μg/L	60	96.78	
AFP			0.014
> 400 μg/L	59	72.41	

≤ 400 μg/L	103	86.54	
CA19-9			0.287
> 39 U/L	35	72.22	
≤ 39 U/L	127	83.87	
Location in same segment			
Yes	20	80.00	0.924
No	142	81.96	
Location in same lobe			
Yes	34	88.2	0.238
No	128	79.7	
Location in same hemiliver			
Yes	74	83.8	0.530
No	88	79.5	
Number of tumors			0.529
≤ 3	146	80.82	
> 3	16	87.50	
Parameters of hepatitis B			
HBsAg			0.517
Positive	156	80.81	
Negative	6	100.00	
HBsAb			0.803
Positive	8	100.0	
Negative	154	80.52	
HBeAg			
Positive	64	71.88	0.005
Negative	98	87.76	
HBeAb			
Positive	101	84.31	0.041
Negative	61	74.19	
HBV-DNA ¹			0.228
> 10 ⁴ /L	88	79.54	
≤ 10 ⁴ /L	50	88.00	
HBV-DNA ¹			0.019
> 10 ⁶ /L	32	68.75	
≤ 10 ⁶ /L	106	86.79	

¹There were only 138 patients received HBV-DNA examinations. GLB: Globulin; ALP: Alkaline phosphatase; AFU: α fucosidase; GGT: γ-glutamyl transpeptidase; PLT: Platelet; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; HBsAb: Hepatitis B surface antibody; HBeAb: Hepatitis B e antibody; CA19-9: Carbohydrate antigen 19-9; PT: Prothrombin time; APTT: Activated partial thromboplastin time; TBIL: Total bilirubin; ALB: Albumin; ALT: Alanine transaminase; AST: Aspartate aminotransferase; WBC: White blood cell.

for patients with multinodular HCC, the scoring system had a satisfactory ability to predict 1-year mortality after hepatic resection. A score of 5 was the cut-off value with optimal specificity and sensitivity. For patients with a score of 5 or more, the 1-year mortality was 62% which was similar to that of patients who received nonsurgical treatment^[2,15-17], while patients with a score of 0-4 had 1-year mortality of only 5%.

We also compared the long-term survival of patients with a score 0-4 (*n* = 120, group 1) and those with a score of 5-8 (*n* = 42, group 2). In group 1, the median survival was 53.55 mo (range: 6-80), the median disease-free survival was 37.14 mo (range: 1-79). In group 2, the median survival was 12.86 mo (range: 3-51), the median disease-free survival was 7.00 mo (range: 1-45). The 1-year and 3-year survival rates of group 1 were 95% and 60%, respectively, while the 1-year and 3-year survival rates of group 2 were 38% and 8%, respectively. The 1-year and 3-year disease-free survival rates of group 1 were 67% and 35%, respectively, while the 1-year and 3-year

Table 3 Area under the receiver operating characteristics curves and cut-off points

Test variable(s)	Area	SE	Asym-ptotic Sig	Lower bound	Upper bound	Cut-off point 1 ¹	Cut-off point 2 ²
TBIL	0.694	0.054	0.001	0.588	0.801	17.25	17.1
Prealbumin	0.817	0.043	< 0.001	0.099	0.266	170	170
AST	0.707	0.048	0.001	0.613	0.802	41.8	38
ALP	0.695	0.056	0.001	0.586	0.804	119.5	129
GGT	0.792	0.040	< 0.001	0.714	0.870	75.5	64
AFU	0.615	0.064	0.058	0.489	0.740	36	40
Total tumor size	0.693	0.057	0.001	0.581	0.805	8.1	8
AFP	0.626	0.050	0.037	0.528	0.725	32.6	20
PLT	0.721	0.058	< 0.001	0.166	0.393	92.5	100
Largest tumor size	0.597	0.064	0.089	0.472	0.722	5.9	6

¹The actual results defined by the ROC curves; ²The values adopted for multivariate analysis. AFP: α fetoprotein; TBIL: Total bilirubin; AST: Aspartate aminotransferase; PLT: Platelet; ALP: Alkaline phosphatase; AFU: α fucosidase; GGT: γ -glutamyl transpeptidase; Sig: Significance.

Table 4 Results of multivariate Cox analysis

Variables	β	SE	Sig	HR	95% CI for HR	Assigned points
Prealbumin < 170 mg/L	1.711	0.420	< 0.001	5.531	3.122-13.196	1
ALP > 129 U/L	1.132	0.420	0.005	3.252	1.413-7.480	1
AFP > 20 μ g/L	2.014	0.792	0.011	7.477	1.419-31.234	2
Total tumor size > 8 cm	2.334	0.542	< 0.001	10.543	3.611-30.157	2
PLT < 100 $\times 10^9$ /L	2.310	0.484	< 0.001	9.937	3.770-26.121	2
GGT > 64 U/L	1.291	0.460	< 0.001	3.791	1.476-9.960	1

β : Partial regression coefficient; SE: Standard error; Sig: Significance; HR: Hazard ratio; CI: Confidence interval; AFP: α fetoprotein; PLT: Platelet; ALP: Alkaline phosphatase; GGT: γ -glutamyl transpeptidase.

disease-free survival rates of group 2 were 14% and 12%, respectively. Patients in group 2 had much poorer long-term overall and disease-free survival ($P < 0.001$ for both, Figure 3). Table 5 shows the comparison of pathological status between group 1 and group 2. Patients in group 2 had a greater possibility of microvascular invasion ($P < 0.001$) and poor tumor differentiation ($P = 0.003$). Liver cirrhosis with small nodules was more likely to be detected in group 2 ($P < 0.001$). In addition, group 2 patients were more likely to receive intraoperative blood transfusion ($P = 0.010$).

DISCUSSION

This study evaluated the short-term prognosis of patients with multinodular HCC after curative resection and developed a scoring system to determine contraindication to surgery. In patients with well-preserved liver function, hepatic resection remains the optimal and effective treatment. Although liver transplantation provides an alternative curative treatment option for small HCC, organ shortages and long waiting times have prohibited it as initial treatment^[4,5]. Several studies have proved that the benefit of resection was similar to that of transplantation. However, patients who received transplantation had undergone a natural selection process in which patients with more aggressive tumor phenotypes, such as the presence of microvascular invasion and microscopic metastasis, had been ruled out because of tumor progression^[23]. The efficacy of RFA is limited by several factors, such as sub-

capsular location, tumor size and tumor differentiation, which have been proved to be associated with increased risk of bleeding, peritoneal seeding and residual vital tumor tissue^[5,6,24]. Several studies have demonstrated that patients who underwent resection had better survival than those who received RFA^[25,26]. Chemoembolization, as a palliative treatment, results in a high incidence of residual viable tumor tissue, even after repeated treatment^[27,28]. Its poor performance on blood-deficient and larger tumors restricts its application in HCC treatment. Previous studies have demonstrated that resection brought more benefit to patients of intermediate stage than TACE^[4,10]. As surgical techniques have advanced, many studies have explored the possibility of broadening the surgical criteria for HCC. Ng *et al*^[4] demonstrated the safety and effectiveness of hepatic resection for multinodular HCC, although the survival rate was lower than for a single tumor. Ishizawa *et al*^[8] concluded that resection can provide a survival benefit for patients with multinodular HCC on the background of Child-Pugh A cirrhosis, as well as for those with portal hypertension. Torzilli *et al*^[9] confirmed that patients with BCLC stage B and stage C HCC can tolerate hepatic resection with low mortality, acceptable morbidity, and a survival benefit, if resection is performed under strict intraoperative ultrasonographic guidance.

However, the benefit of hepatic resection in the treatment of multinodular HCC remains controversial. In spite of the exciting conclusions above, we can also observe in clinical practice that there are patients who

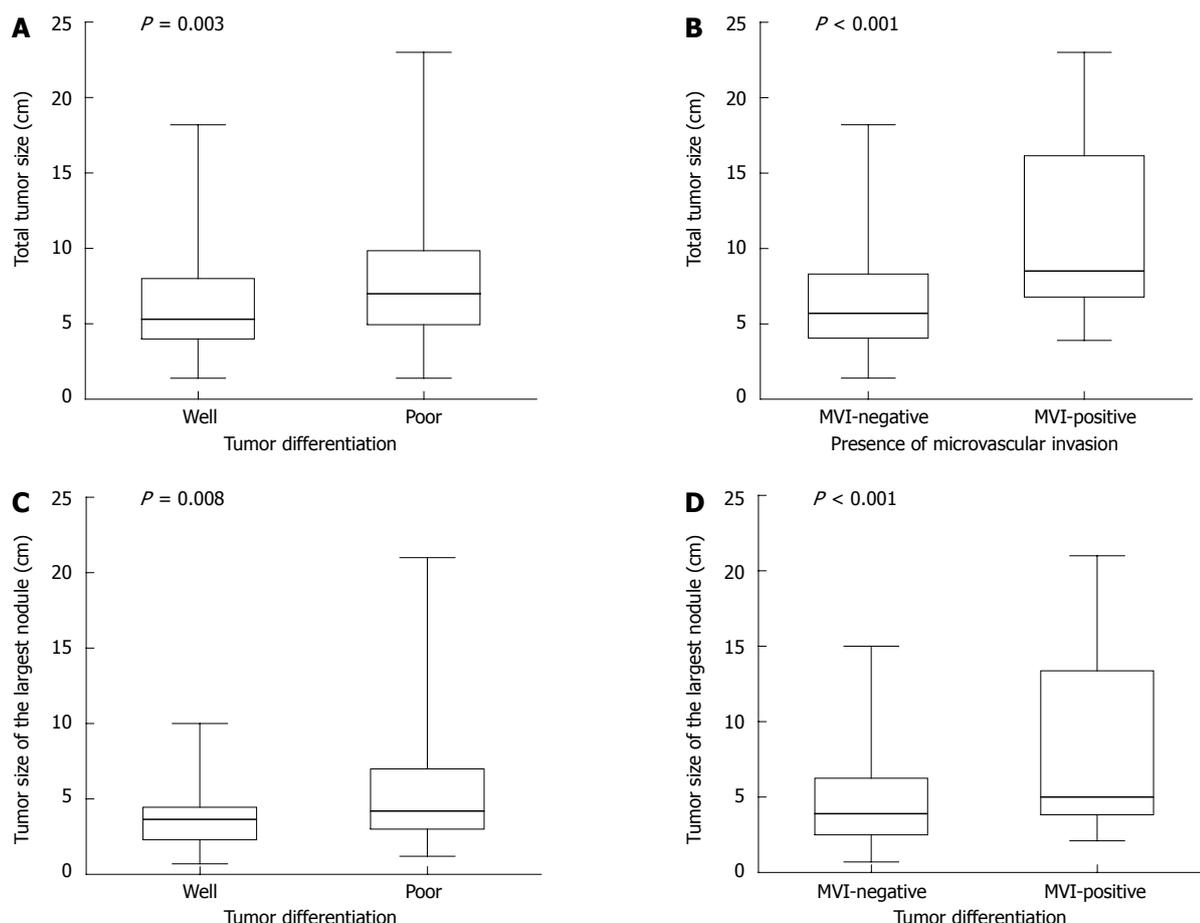


Figure 1 Distribution of tumor size according to tumor differentiation and presence of microvascular invasion, showing median, 25th-75th percentile box and complete range of measurements. A: Distribution of total tumor size according to tumor differentiation ($P = 0.003$); B: Distribution of total tumor size according to presence of microvascular invasion ($P < 0.001$); C: Distribution of the largest tumor size according to tumor differentiation ($P = 0.008$); D: Distribution of total tumor size according to tumor differentiation and presence of microvascular invasion ($P < 0.001$). The tumor size was associated with presence of microvascular invasion and poor tumor differentiation. MVI: Microvascular invasion.

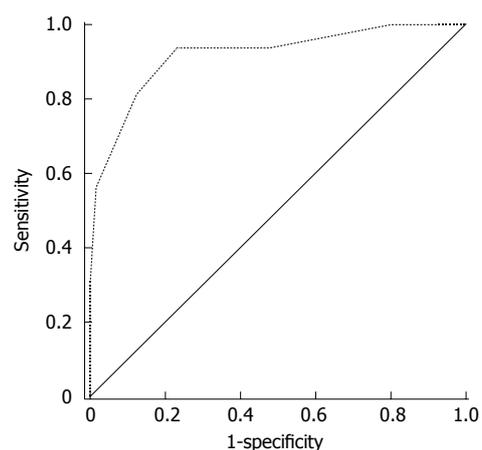


Figure 2 Receiver operating characteristics curve of the predictive scoring system. The area under the curve was 0.925, 95% confidence interval was 0.864-0.985 ($P < 0.001$). The data indicated that the scoring system had a strong ability to predict the 1-year outcome of patients with multiple hepatocellular carcinoma after hepatic resection.

have satisfactory liver function and seemingly optimistic prognosis empirically, but have poor short-term outcome

after resection. This indicates that the classical evaluation systems have their limitations, and that they cannot detect mild liver impairment, which would markedly influence outcome. In our study, no patient died during the hospital stay, and only 4 (2.47%) died in the 3 mo after resection, indicating that the preoperative safety assessment according to the established guidelines was acceptable. However, 32 of the patients died in the first year. For these patients, surgical resection brought no benefit and may even have adversely affected survival. Therefore, identification of these patients may determine that surgical treatment should be contraindicated.

Multivariate analysis showed that 6 variables were independent predictive factors for poor short-term survival. Among them, prealbumin < 170 mg/L, ALP > 129 U/L, GGT > 64 U/L and platelet count $< 100 \times 10^9/L$ were factors associated with liver impairment. Their roles as indicators of liver damage have been reported previously^[29-32]. Platelet count may also be used as a potential marker of portal hypertension^[33]. Conventional blood parameters of liver function, such as TBIL and PT, as well as scoring systems using these values (such as the Child-Pugh score and Model for End-stage Liver Disease score)

Table 5 The comparison of surgical data and tumor biological characteristics between two groups *n* (%)

Variables	Group 1 (<i>n</i> = 120)	Group 2 (<i>n</i> = 42)	<i>P</i> value
Blood transfusion	6 (5.00)	8 (19.05)	0.010
Pringle time > 20 min	52 (43.33)	24 (57.14)	0.151
Liver cirrhosis	68 (56.67)	24 (57.14)	0.854
Type of liver cirrhosis			< 0.001
Small nodules	0 (0)	6 (25.00)	
Large nodules	52 (76.47)	8 (33.33)	
Mixed nodules	16 (23.53)	10 (41.65)	
Differentiation of tumor			0.003
High differentiation (I-II)	30 (25.00)	2 (4.76)	
Low differentiation (III-IV)	90 (75.00)	40 (95.23)	
Microvascular invasion	16 (13.33)	18 (42.89)	< 0.001
Completed capsule of largest tumor			0.468
Yes	72 (60.00)	22 (52.38)	
No	48 (40.00)	20 (47.62)	

have been widely accepted in clinical circles. Many staging systems also adopt them in preoperative estimation of surgical safety. However, decreased ALB and prolonged PT indicate marked liver damage^[29], while ascites and encephalopathy indicate liver failure and serious portal hypertension. Previous studies reported that most patients who underwent elective hepatic resection were classified as Child-Pugh class A, but postoperative liver failure and mortality existed even in this group of patients^[17,18]. This phenomenon indicated that most hepatic impairment in surgical candidates is slight and might not be fully evaluated by classical estimation systems. Our results disclosed that platelet count, ALP, prealbumin and GGT may be considered as supplemental factors for routine liver function scoring systems. Patients who have satisfactory liver function reserve according to the traditional estimation system, but abnormalities in these additional parameters above should be considered with caution for surgery.

Factors associated with tumor burden also have a crucial influence on the short-term survival of patients. The majority of our patients who died in the first year had tumor recurrence and metastasis. Early tumor recurrence and fast hyperplasia adversely affected liver function recovery. Our results included AFP > 20 µg/L and total tumor size > 8 cm as independent risk factors of first year death. AFP > 200 µg/L is the diagnostic level indicating HCC, but only one-third of patients with HCC have AFP levels higher than 100 µg/L, and even mild elevation predicts a worse prognosis^[14]. Hence, the 20 µg/L as cutoff point in the ROC curve was reasonable. The AFP level and total tumor size are widely accepted risk factors affecting surgical outcome^[34]. Several studies have reported that tumor size is related to the presence of microvascular invasion and poor tumor differentiation^[35,36], which are strongly associated with intrahepatic metastasis and greatly increase the risk of tumor recurrence^[5,35,36]. Our analysis observed a similar phenomenon. These results implied that clinicians could estimate tumor invasiveness by preoperative examination. Other tumor-associated fac-

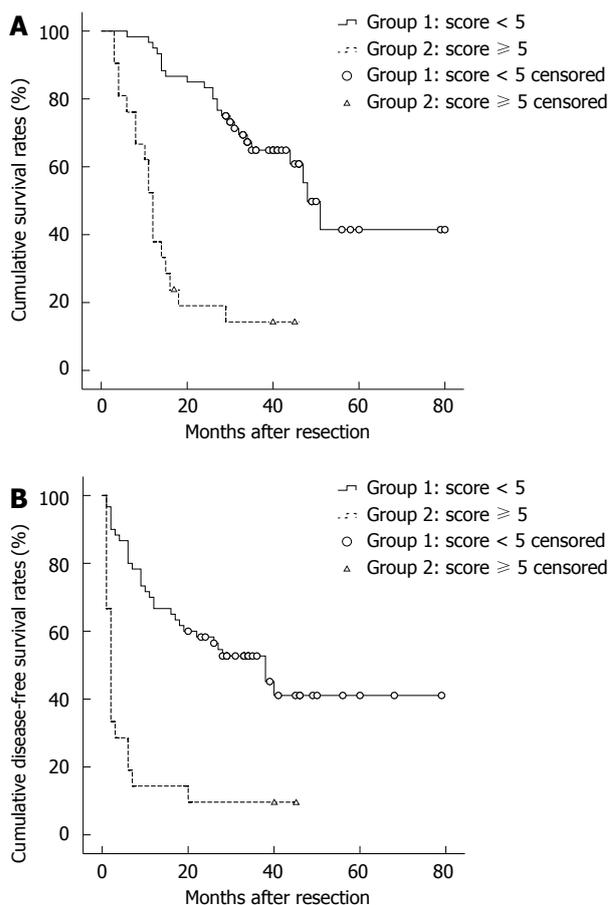


Figure 3 Results of long-term survival of group 1 (score < 5) and group 2 (score ≥ 5). The overall survival and disease-free survival in group 2 were significantly poorer than those in group 1 (both *P* < 0.001).

tors were not included in our results as our study focused on short-term (1-year) survival rather than the long-term outcome.

To apply these risk factors in clinical practice, we constructed a scoring model. A total of 42 patients with high score (≥ 5, group 2) had 1-year outcome similar to that of patients who received non-surgical treatment, and also had significantly poorer long-term overall and disease-free survival. Our analysis also showed that these patients had a greater possibility of microvascular invasion and poor tumor differentiation. They also had a higher percentage of liver cirrhosis with small nodularity, which has been proved to be an independent predictor of clinically significant portal hypertension^[37]. Intraoperative blood transfusion, which significantly influenced short-term survival^[22], was more common in patients with a score ≥ 5, indicating greater surgical difficulty. Although we resected all visible and palpable nodules and a tumor-free margin under guidance of intraoperative ultrasonography, the 3-mo recurrence rate in group 2 patients was 71.4%, indicating the greater possibility of preoperative existence of non-detectable micrometastasis. For these patients, seemingly curative resection does not achieve radical efficacy. This series of data confirmed that this group of patients may derive little benefit from resection and surgery should be

considered a contraindication in spite of satisfactory liver function estimated by classical scoring systems.

Major limitation of our study is that this study was a retrospective analysis with a small sample size in a single center. The potential selective bias that accompanied with this setup was hard to avoid. Patients with hepatitis C virus infection or other chronic liver disease were not included in the analysis. Other limitations of this study were that different types of recurrence were not taken into consideration because of the small sample size. Therefore, further study is needed before a final conclusion is made.

In summary, hepatic resection has been proved to be a safe and effective treatment for some patients with multinodular HCC. However, some patients with good liver function as estimated by traditional scoring systems have poor short-term outcome. Our study focused on these patients and indicated that other factors, namely prealbumin < 170 mg/L, ALP > 129 U/L, GGT > 64 U/L, platelet count < 100×10^9 /L, AFP > 20 μ g/L and total tumor size > 8 cm are independent risk factors for short-term mortality. For patients with these characteristics, 1-year mortality was significantly increased, and resection was associated with an adverse outcome.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality worldwide. Multiple lesions are detected in about 40% patients once HCC is diagnosed. The treatment strategies for multiple HCC remain in controversy.

Research frontiers

Several recent studies reported that surgical resection improves the survival of patients with multiple HCC. However, the indication and contraindication of surgical resection for multiple HCC remain unclear.

Innovations and breakthroughs

Patients with poor short-term (1 year) survival after surgery benefit little from hepatic resection. They should be considered surgical contraindication. The risk factors of poor short-term survival for patients with satisfactory liver function remain unclear. The authors analyzed the preoperative data of 162 multiple HCC patients received surgical resection and found that factors, namely prealbumin < 170 mg/L, alkaline phosphatase (ALP) > 129 U/L, γ -glutamyl transpeptidase (GGT) > 64 U/L, platelet count < 100×10^9 /L, α fetoprotein (AFP) > 20 μ g/L, total tumor size > 8 cm are independent risk factors of short-term mortality. The authors then assigned points to each factor according to its partial regression coefficient and construct a score system. For the patients with score ≥ 5 , 1-year mortality was 62% while only 5% for patients with score < 5.

Applications

The study results suggest that more factors namely ALP, GGT, platelet, prealbumin, AFP and total tumor size should be enrolled in preoperative estimation. Patients with score ≥ 5 should be considered with more cautious attitude towards resection.

Terminology

HCC is the most common primary malignant tumor of the liver which originated from liver parenchymal cells. Multiple HCC refers to the HCC with more than one lesion.

Peer review

The authors analyzed the clinical data of patients with multiple HCCs treated by surgery for detecting prognostic factors of 1 year survival. They determined six prognostic factors from preoperative clinical data. Based on the multivariate analysis, they proposed a prognostic scoring system. This scoring system well predicted poor short outcome after hepatic resection. The study was carefully designed and the evaluation of the results was also appropriate. The manuscript was well organized and well written.

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Electrical bioimpedance gastric motility measurement based on an electrical-mechanical composite mechanism

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Abstract

AIM: To introduce a bioimpedance gastric motility measurement method based on an electrical-mechanical composite concept and a preliminary clinical application.

METHODS: A noninvasive gastric motility measurement method combining electrogastrogram (EGG) and impedance gastric motility (IGM) test was used. Preliminary clinical application studies of patients with functional dyspepsia (FD) and gastritis, as well as healthy controls, were carried out. Twenty-eight FD patients (mean age 40.9 ± 9.7 years) and 40 healthy volunteers (mean age 30.9 ± 7.9 years) were involved. IGM spectrum was measured for both the healthy subjects and FD patients, and outcomes were compared in the FD patients before treatment and 1 wk and 3 wk after treatment. IGM parameters were obtained from 30 erosive gastritis patients (mean age 50.5 ± 13.0 years) and 40 healthy adults, and IGM and EGG results were compared in the gastritis patients before treatment and 1 wk after treatment.

RESULTS: There were significant differences in the IGM parameters between the FD patients and healthy subjects, and FD patients had a poorer gastric motility [percentage of normal frequency (PNF) 70.8 ± 25.5 in healthy subjects and 28.3 ± 16.9 in FD patients, $P < 0.01$]. After 1 wk administration of domperidone 10 mg, *tid*, the gastric motility of FD patients was not improved, although the EGG of the patients had returned to normal. After 3 wk of treatment, the IGM rhythm of the FD patients became normal. There was a significant difference in IGM parameters between the two groups (PNF 70.4 ± 25.5 for healthy subjects and 36.1 ± 21.8 for gastritis patients, $P < 0.05$). The EGG rhythm of the gastritis patients returned to normal (frequency instability coefficient 2.22 ± 0.43 before treatment and 1.77 ± 0.19 one wk after treatment, $P < 0.05$) after 1 wk of treatment with sodium rabeprazole tablets, 10 mg, *qd*, *po*, *qm*, while some IGM parameters showed a tendency toward improvement but had not reached statistical significance.

CONCLUSION: The electrical-mechanical composite measurement method showed an attractive clinical application prospect in gastric motility research and evaluation.

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Key words: Gastric motility; Electrical bioimpedance; Electrical-mechanical composite; Electrogastragram

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INTRODUCTION

An electrical bioimpedance technique is a method used to extract physiological and pathological information from the human body according to the electrical property of the tissue and organs. This method has the advantage of being noninvasive and convenient and can provide functional information. It is a powerful tool in clinical diagnosis and medical research^[1]. There are many applications that use bioimpedance signals for different pathological conditions, but their use in gastric motility assessment needs further exploration^[2].

Gastrointestinal motility as an interdisciplinary subject has developed rapidly in recent years. Gastric motility is one of the most critical physiological functions of the human body. Coordinated gastric motility is necessary for digestion and the absorption of dietary nutrients. Impairment of gastric motility results in delayed gastric emptying and symptoms such as nausea, vomiting, abdominal pain and discomfort^[3]. The research on gastric motility function has fallen behind as compared with the studies on gastric endocrine and exocrine secretion functions and gastric morphology. One of the important reasons for this is the absence of convenient and effective measurement methods for gastric motility function^[4].

Stomach volume distends gradually after the ingestion of food. In the period of digestion, such as contraction and peristalsis after a meal, the stomach content changes greatly and so does the impedance of the stomach. When measuring the impedance of the stomach during digestion, information reflecting the stomach volume (gastric emptying) and gastric motility can be extracted. Sutton *et al.*^[5] in 1985 reported results on extracting the signal of gastric movement by an electrical impedance method. The curve reflecting gastric emptying was obtained and, from the curve, the gastric peristaltic information was extracted showing a rhythm of 2-4 cycles per minute (cpm), which is in accordance with gastric contraction. In 1987, Familoni *et al.*^[6] presented a technique to monitor gastric electrical activity and mechanical activity as an aid in assessing gastric motor function. In 1992, Kothapalli *et al.*^[7] established a three-dimensional abdomen model to study the origin of changes in the epigastric signal, and analyzed the relationship between the gastric impedance signal and food capacity, resistivity of a test meal, and gastric contraction when the exciting current electrodes and the measuring voltage electrodes were located at different positions.

Early research using an impedance method to measure the digestion course mainly focused on gastric emptying measurement^[8-10]. There have been few researches on the extraction of gastric motility information^[11-13]. One of the primary reasons is that the rhythm of gastric motility is much lower; i.e., about 3 cpm. It is difficult to extract the gastric motility signal and eliminate respiration interference. In 1991, Chen *et al.*^[14] reported their work on obtaining an electric impedance signal, which reflected gastric contraction, and measurement devices

were subsequently developed^[15]. However, the impedance signals obtained by the devices were all sine waveforms that were similar for both healthy and diseased subjects because of an incorrect filter processing. It was difficult to differentiate normal or abnormal conditions from the signals. In 2007, we proposed a noninvasive electrical impedance method for gastric motility measurement and evaluation^[16]. Multi-resolution analysis of the wavelet was adopted to separate the gastric motility signal from the mixed impedance signal obtained on the body surface^[17].

In this paper, a gastric motility measurement method based on an electrical-mechanical composite concept was introduced and results from some preliminary clinical application studies of gastric motility measurement for patients with functional dyspepsia (FD) and gastritis, as well as normal controls, were presented.

MATERIALS AND METHODS

Electrical-mechanical composite method for gastric motility assessment

Gastric contraction is a mechanical behavior that follows electrical activity occurring on the cell membrane surface of smooth muscles. It begins with electrical activity of the smooth muscle, followed by evoked contraction of the gastric corpus and antrum, and is then transmitted to the distal pylorus. It is a composite course from electrical activity to mechanical contraction, which then leads to gastric peristalsis and transmission. Gastric contraction complies with the rhythm of electrical activity and is affected by the amplitude, time limitation, transmission direction and distance of the transmission contraction. Gastric motility is a complex electrical-mechanical composite course. It is very important to measure and evaluate gastric motility according to an electrical-mechanical composite mechanism^[18].

There are two kinds of gastric myoelectric activities: slow wave and spike potential. The gastric antrum contracts only when the slow wave occurs accompanied by the spike potential. The spike potential appears during the slow wave phase, and the rhythm of gastric contraction may be determined by the slow wave. Electrogastrogram (EGG) signals recorded from the body surface reflect the myoelectric activity of different regions of the stomach. It corresponds to the gastric slow wave accurately and, therefore, can be used to investigate the rhythm of gastric contraction. EGGs reflect myoelectric activity of the stomach and the rhythm of gastric contraction. The information from the impedance measurement of the stomach directly corresponds to gastric motility. A combined measurement of EGG and impedance gastric motility (IGM) is a new approach for evaluating gastric motility based on an electrical-mechanical composite mechanism.

IGM measurement

Based on an electrical-mechanical composite mechanism

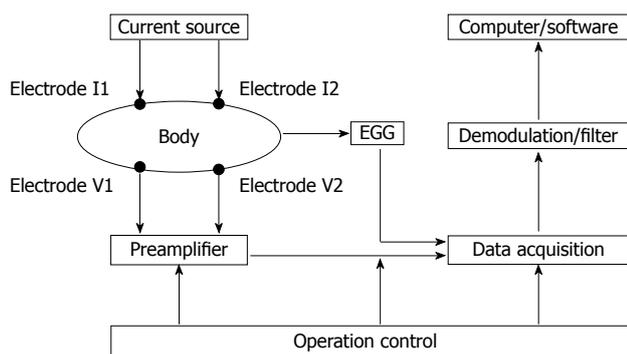


Figure 1 Diagram of gastric motility measurement. EGG: Electrogastragram.

and combined IGM and synchronous EGG measurements, a gastric motility measurement method has been developed, which was reported in our previous paper^[19]. The block diagram of the measurement system is shown in Figure 1.

The measurement system consists of a current source, electrodes, preamplifier, demodulation/filter circuit, data acquisition, operation control and an upper computer. Four electrodes (I₁, I₂, V₁ and V₂) placed on the epigastric surface of the body are used to inject measurement current and measure impedance signals. An electric current of 50 kHz and 1 mA provided by the current source injects into the epigastric area of the subject *via* excitation electrodes I₁ and I₂. The IGM signal picked up from the measurement electrodes V₁ and V₂ is put into the preamplifier and the demodulation/filter, and then goes into the data acquisition system for analog/digital (A/D) conversion and numeralization processing of the signal. The digitized data are transmitted to the computer where proprietary software performs the IGM and EGG information extraction, analysis and calculation of the gastric motility parameters.

IGM and EGG signals are classified according to their rhythm. The rhythm of 2-4 cpm is considered a normal rhythm, while that below 2 cpm is bradygastria, and above 4 cpm is tachygastria. Based on this classification, frequency spectra, energy spectra, dynamic spectra, running spectra, percentage of normal frequency (PNF), percentage of normal power (PNP), the frequency instability coefficient (FIC) and the power instability coefficient (PIC), for both IGM and EGG, were analyzed as temporal features for the subjects. FIC and PIC represent the stability of the frequency (e.g., the rhythm of the gastric motility signals) and power of the rhythm for both IGM and EGG, respectively. The higher FIC or PIC means that the stability of the rhythm and the power of gastric motility signals are worse.

Subjects

The subjects included FD patients, erosive gastritis patients and healthy volunteers. All patients with FD and erosive gastritis came from the First Affiliated Hospital of Chongqing University of Medical Sciences. Twenty-eight FD patients (mean age 40.9 ± 9.7 years) who self-

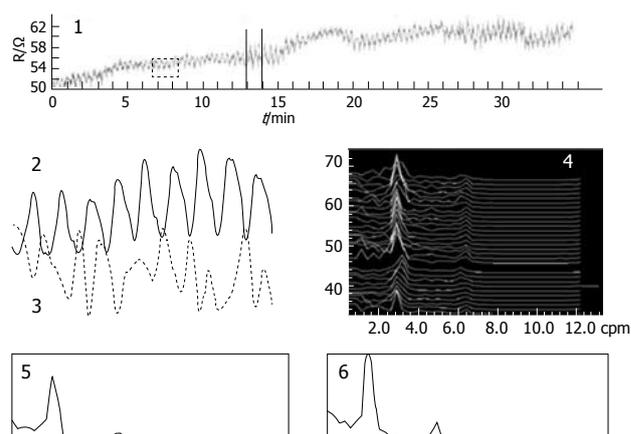


Figure 2 Results of impedance gastric motility measurement and spectra analysis for a healthy volunteer.

reported the symptoms of abdominal bloating, belching and gastric acid reflux and were diagnosed according to the Rome III criteria of FD. All selected FD patients were taking domperidone (10 mg, *tid*) for treatment. The 30 erosive gastritis patients (mean age 50.5 ± 13.0 years) were diagnosed through gastroscopy and were taking sodium rabeprazole tablets (10 mg, *qd, po, qm*) for treatment. The 40 healthy volunteers were university students and teachers (mean age 30.9 ± 7.9 years) who served as a control group.

Preliminary application of IGM measurement

In order to validate the feasibility and effects of the proposed method in this study, a preliminary clinical application study of gastric motility measurement for healthy volunteers and patients with FD and gastritis was conducted. The IGM measurement is a noninvasive method. The study was approved by the ethical committee of the institute and all subjects in the study signed a consent form. The statistical software SPSS 13.0 was used to analyze the data. The data were expressed as mean ± SD. Variance analysis between the study and control groups was undertaken and a significant difference was accepted at $P < 0.05$.

RESULTS

IGM measurement in healthy volunteers

IGM measurement results for healthy volunteers are illustrated in Figure 2. Part 1 represents the original mixed impedance signal picked up from the abdominal surface, part 2 is the IGM signal extracted from the mixed signal, part 3 is the synchronous EGG, part 4 is the dynamic spectrum of IGM, and parts 5 and 6 are the power spectrums before and after a test meal. The dominant frequency of the IGM signal was 2.8 cpm in the dynamic spectrum of part 4.

IGM measurement in FD patients

The IGM parameters for 40 healthy volunteers and 28

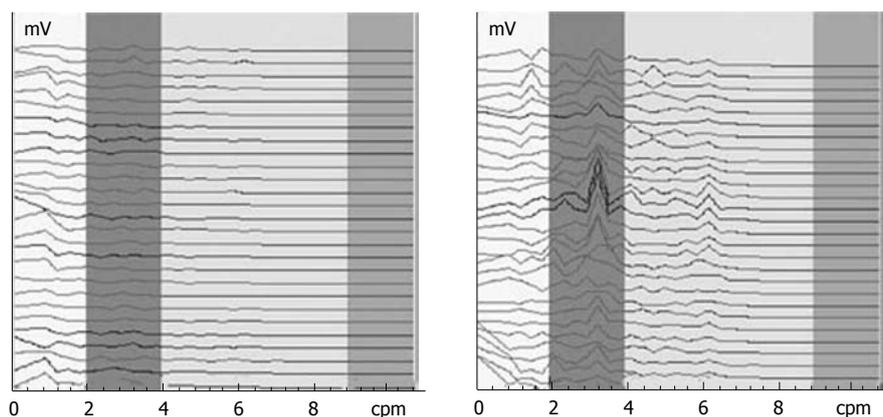


Figure 3 Electrogastrogram dynamic spectra of functional dyspepsia patients before treatment (left) and one wk after treatment (right).

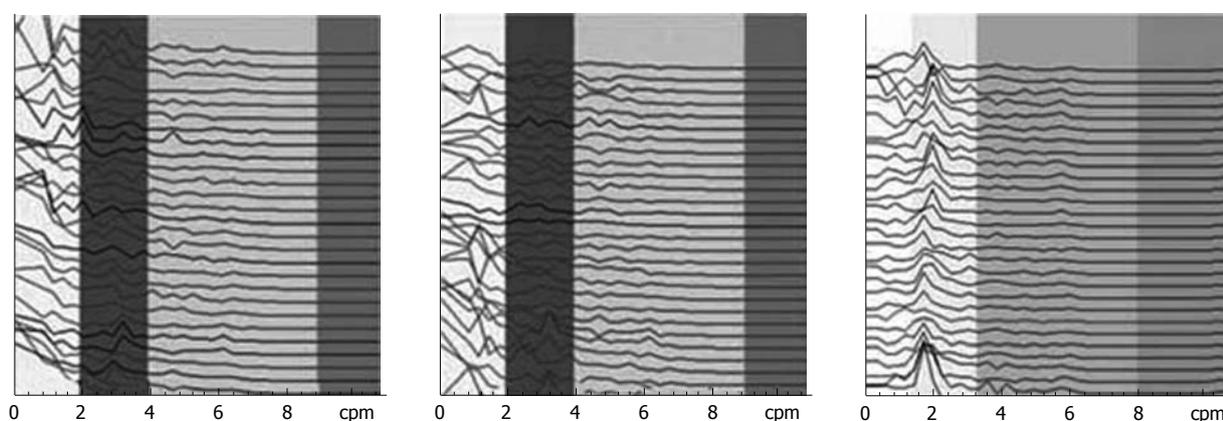


Figure 4 Impedance gastric motility dynamic spectra of functional dyspepsia patients before treatment (left) and 1 wk (middle) and 3 wk (right) after treatment.

Table 1 Impedance gastric motility measurement results in healthy controls and patients with functional dyspepsia and gastritis (mean ± SD)					
Group	Case	PNF	PNP	FIC	PIC
Healthy	40	70.8 ± 25.5	59.3 ± 4.4	1.58 ± 0.48	0.18 ± 0.06
FD	28	28.3 ± 16.9 ^b	50.8 ± 9.9 ^b	2.08 ± 0.55 ^b	0.23 ± 0.05 ^b
Gastritis	30	36.1 ± 21.8 ^a	44.6 ± 4.8 ^a	2.23 ± 0.55 ^a	0.24 ± 0.05 ^a

^a*P* < 0.05, ^b*P* < 0.01 vs healthy group. FD: Functional dyspepsia patients; PNF: Percentage of normal frequency; PNP: Percentage of normal power; FIC: Frequency instability coefficient; PIC: Power instability coefficient.

FD patients are shown in Table 1. It can be seen that PNF in the FD group was obviously lower than that in the control group (*P* < 0.01), and FIC was higher than that in the control group (*P* < 0.01). The PNP and PIC showed similar differences between the two groups (*P* < 0.01). These results suggest that FD patients have a poorer rhythm in gastric motility and energy deficiency. This result is consistent with digestion pathology and physiology in FD.

Figure 3 shows the EGG dynamic spectrum of FD patients before and 1 wk after administration of domperidone tablets, 10 mg, *tid, po*, taken half an hour before meals. The interval for each spectrum line was 1 min in

duration. In Figure 3, the EGGs of FD patients were weak and the rhythms were disordered before treatment (left). After 1 wk of treatment, the EGG improved and the rhythm returned to 2-4 cpm (right). This suggested that the GEA of FD patients tended towards normal after 1 wk of treatment.

The IGM signal spectrums of one FD patient before treatment and 1 wk and 3 wk after treatment are shown in Figure 4. Compared with the EGG dynamic spectrum in Figure 3, the IGM spectrum after 1 wk of treatment in Figure 4 showed little change and the rhythm was also disordered. After 3 wk of treatment, the IGM rhythm returned to 2-4 cpm.

IGM measurement in gastritis patients

The parameters for IGM and EGG in the gastritis patients before and 1 wk after administration of sodium rabeprazole tablets, 10 mg, *qd, po, qm* are shown in Tables 1 and 2.

It can be seen from Table 1 that there was a significant difference in IGM parameters between the gastritis patients and healthy controls. The PNF and PNP of the gastritis patients were significantly lower than that of the healthy controls (*P* < 0.05). The FIC and PIC of the gastritis patients were evidently higher than the healthy

Table 2 Electrogastrogram and impedance gastric motility parameters in 30 gastritis patients before treatment and 1 wk after treatment (mean \pm SD)

Treatment	0-2 cpm PNP	2-4 cpm PNP	> 4 cpm PNP	FIC	PIC
EGG parameters					
Before	24.0 \pm 5.6	51.5 \pm 11.1	24.4 \pm 5.5	2.22 \pm 0.43	0.34 \pm 0.03
After 1 wk	22.7 \pm 3.4	54.3 \pm 6.7	23.1 \pm 3.3	1.77 \pm 0.19 ^a	0.23 \pm 0.02 ^a
IGM parameters					
Before	27.5 \pm 2.4	44.6 \pm 4.8	27.9 \pm 2.4	2.23 \pm 0.55	0.24 \pm 0.05
After 1 wk	27.4 \pm 2.2	44.9 \pm 4.4	27.8 \pm 2.2	1.91 \pm 0.65	0.21 \pm 0.06

^a $P < 0.05$ vs EGG before treatment. PNP: Percentage of normal power; FIC: Frequency instability coefficient; PIC: Power instability coefficient; IGM: Impedance gastric motility; EGG: Electrogastrogram.

controls ($P < 0.05$). This suggests a weakening gastric motility function and disorder of stomach peristalsis in gastritis patients. Table 2 indicates that the EGG power of the normal rhythm (2-4 cpm) for the patients was raised and the power of the abnormal rhythm (0-2 cpm and > 4 cpm) declined after 1 wk of treatment, although this was not statistically significant ($P > 0.05$). It should be noted that the FIC and PIC of the patients decreased significantly ($P < 0.05$). It suggests that the EGG of gastritis patients tended to be normal and stable after 1 wk of treatment, and the rhythm of the EGG improved.

IGM parameters in Table 2 show that the power ratios of IGM signals for the patients in all frequency bands did not change much before and after treatment ($P > 0.05$), although the FIC and PIC showed a decreasing trend ($P > 0.05$).

DISCUSSION

The motility function of the stomach is regulated by nerve and body fluid and is accomplished by the coordinated movement of smooth muscle^[4,20]. It can be seen from Figures 3 and 4 that for FD patients the IGM was not improved with 1 wk of treatment, while the EGG returned to normal. After 3 wk of treatment, the regular IGM rhythm of the FD subjects became normal and the contraction function of the stomach was recovered. It is understandable that although the EGG returned to normal by nerve regulation with 1 wk of treatment, improvement of the electric activity had not coupled with or transferred to the mechanical activity of the stomach. After 3 wk of treatment, when the electrical activity had already coupled with or transferred to the mechanical activity of the stomach *via* the regulation mechanism for nerve and body fluid, the normal IGM rhythm could be seen in the spectrum, which suggests the recovery of the contraction function of the stomach.

There were significant differences in IGM parameters between the gastritis patients and healthy controls. After 1 wk of treatment, the EGG rhythm of the gastritis patients returned to normal while the IGM parameters only showed a tendency to improve, which had no statistical significance. This suggests that the influence of the GEA may not have coupled with the mechanical contraction of the stomach after only 1 wk of treatment. On the other

hand, although the patients felt some alleviation after 1 wk of treatment, the cardinal symptoms of gastritis were not completely relieved. This fact coincided with the results of Table 2, and therefore, the patients should be advised to continue the treatment.

The mixed signal acquired from the abdominal surface contains not only IGM information but also the components of impedance blood flow, breath and some other disturbances. The normal rhythm of IGM is about 3 cpm. Within the mixed signal, the rhythm of the breath impedance signal is about 12 cpm, which is close to the rhythm of the IGM. Signals of the IGM and breath both are ultra-low frequency signals and the amplitude of the breath signal is usually much higher than that of the IGM signal. It is a challenge to extract the IGM signal from the mixed signals effectively. A low-pass filter may be good enough to reduce the effect of high-frequency noise and heart activity, however, it is difficult to eliminate respiration influence and separate the IGM signal from the mixed signal. Therefore, a narrow bandpass filter and a high-order active low-pass filter are required. In the measurement system for gastric motility described in this paper, a wavelet transform was introduced and the IGM signal was separated successfully from impedance signals, including breath and blood flow.

In this study, we focused on the stomach contraction rhythm. Some events without a rhythm, such as gastric acid secretion, did not affect the measurement results.

The EGG reflects GEA of the stomach and is sensitive to regulation mechanisms from nerve and electrical activity. The improvement of the EGG after treatment is only the beginning of improvement in gastric motility function and does not indicate a cure of gastric disorder or the recovery of gastric motility. The IGM is a veritable measure of gastric contraction and peristalsis, and reflects the gastric motility function. Gastric motility measurement is based on an electrical-mechanical composite mechanism. The combined measurement of IGM and EGG is a noninvasive, convenient and effective method that extracts information that directly reflects the gastric motility state. It can be used to measure gastric contraction and peristalsis during digestion and evaluate gastric motility function in different physiological and pathological conditions.

COMMENTS

Background

Gastric motility is one of the most critical physiological functions of the human body. The research on gastric motility function has fallen behind that on gastric endocrine and exocrine secretion functions and gastric morphology. One of the important reasons for this is the absence of a convenient and effective measurement method for gastric motility function. There are many applications using bioimpedance signals for different pathological conditions, but their use in gastric motility assessment needs to be explored in detail and there are few researches on the extraction of gastric motility information.

Research frontiers

In the period of digestion, the stomach content changes greatly and so does the impedance of the stomach. When measuring the impedance of the stomach during digestion, the information reflecting the stomach volume (gastric emptying) and gastric motility can be extracted. The method combining electrical bioimpedance measurement with an electrogastragram (EGG) is one of the research frontiers for gastric motility measurement.

Innovations and breakthroughs

Gastric motility is a complex composite course from electrical gastric activity to mechanical gastric activity. The EGG reflects the myoelectric activity of the stomach and the rhythm of gastric contraction. Information from impedance measurement on the stomach directly corresponds to gastric motility. A combination of EGG and impedance gastric motility (IGM) measurements is a new approach for measuring and evaluating gastric motility based on an electrical-mechanical composite mechanism.

Applications

A combination of IGM and EGG measurement is a noninvasive, convenient and effective method that can be used to measure gastric contraction and peristalsis during digestion and evaluate gastric motility function in different physiological and pathological conditions. This method has shown an attractive application prospect in gastric motility research and evaluation.

Terminology

An electrical bioimpedance technique is a method to extract physiological and pathological information from the human body according to the electrical property of the tissue and organs by means of injecting a small current and measuring the electrical potentials from electrodes on the body surface. The method has the advantages of being noninvasive, convenient, and providing considerable functional information.

Peer review

This is a good article illustrating how technology can be used for clinical measurement. The authors have put a lot of efforts to develop and present their work.

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Omega-3 polyunsaturated fatty acids promote liver regeneration after 90% hepatectomy in rats

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Abstract

AIM: To evaluate the effectiveness of omega-3 polyunsaturated fatty acid (ω -3 PUFA) administration on liver regeneration after 90% partial hepatectomy (PH) in rats.

METHODS: ω -3 PUFAs were intravenously injected in the ω -3 PUFA group before PH surgery. PH, sparing only the caudate lobe, was performed in both the control and the ω -3 PUFA group. Survival rates, liver weight/body weight ratios, liver weights, HE staining, transmission electron microscope imaging, nuclear-associated antigen Ki-67, enzyme-linked immunosorbent assay and signal transduction were evaluated to analyze liver regeneration.

RESULTS: All rats in the control group died within 30 h after hepatectomy. Survival rates in the ω -3 PUFA group were 20/20 at 30 h and 4/20 1 wk after PH.

Liver weight/body weight ratios and liver weights increased significantly in the ω -3 PUFA group. The structure of sinusoidal endothelial cells and space of Disse was greatly restored in the ω -3 PUFA group compared to the control group after PH. In the ω -3 PUFA group, interleukin (IL)-4 and IL-10 levels were significantly increased whereas IL-6 and tumor necrosis factor- α levels were dramatically decreased. In addition, activation of protein kinase B (Akt) and of signal transducer and activator of transcription 3 signaling pathway were identified at an earlier time after PH in the ω -3 PUFA group.

CONCLUSION: Omega-3 polyunsaturated fatty acids may prevent acute liver failure and promote liver regeneration after 90% hepatectomy in rats.

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Key words: Omega-3 polyunsaturated fatty acids; Survival rate; Inflammatory cytokines; Signaling pathways

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INTRODUCTION

The liver is a unique organ with ability to regenerate and recover its original function after extended resection or injury. This occurs due to the hyperplasia of the residual lobes and mitosis of the hepatocytes which are quiescent under normal conditions^[1,2]. The development of two-

thirds partial hepatectomy (PH) in the rat liver by Higgins and Anderson represented a milestone in the exploration of liver regeneration^[3]. In this study, we found that 90% PH was lethal for rats: the cause of death may be associated with acute liver failure induced by small residual liver. Hepatocyte growth factor, platelets and 5-hydroxytryptamine were reported to promote liver regeneration and ameliorate acute liver failure after 90% hepatectomy in previous research^[4-6].

Fatty acids, as essential nutrients, have a wide range of biological functions^[7] and omega-3 polyunsaturated fatty acid (ω -3 PUFA) supplementation is also reported to be involved in modifying the organic biochemical environment^[8]. Recently, ω -3 PUFAs were found to play significantly protective roles in the liver, cardiovascular system and kidney^[9-11] and they have been widely used in clinical perioperative total parenteral nutrition^[12]. In this study, we demonstrate that ω -3 PUFA plays an important role in stimulating liver regeneration after 90% hepatectomy in rats.

MATERIALS AND METHODS

Animals

Sprague-Dawley male rats with weights ranging from 180 to 220 g were purchased from Nanjing University. Rats were maintained in a temperature-controlled room on a 12 h light-dark cycle, with free access to water and standard chow. Animals were divided into three groups ($n = 6$ in each group): the sham group; the control group (rats without ω -3 PUFA treatment); and the ω -3 PUFA group (rats with ω -3 PUFA treatment). In the ω -3 PUFA group, rats were injected intravenously (*via* rat tail vein) with ω -3 PUFA (Fresenius Kabi Corp., Germany) at a dose of 2 mL/kg body weight once 1 d for 2 d before PH surgery. Animal experiments were approved by the Institutional Animal Experiment Committee of Affiliated Drum Tower Hospital, Medical College of Nanjing University (China), in accordance with the Regulations for Animal Experiments at Nanjing University and Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology.

Surgical procedure and anesthesia

Ninety percent hepatectomy was performed in the control and the ω -3 PUFA groups. The procedure is modified from the Higgins-Anderson operation by removing the left lateral, left median and right median lobes with a single ligature (70% PH), and subsequently resecting the right lateral lobe (20%) and leaving only the caudate lobe. Hepatectomy was carried out under ether anesthesia.

Liver tissue collection

Six rats from each group were sacrificed and liver tissues were collected to investigate the effect of ω -3 PUFA administration on liver regeneration. At 12 h, 18 h and 24 h

after PH, rats were sacrificed, regenerated liver samples were collected and wet remnant liver weights were measured. In addition, liver weight/body weight ratios were measured (%) for each rat. Mean value was calculated for each group at each time point. Tissues were divided into three specimens with one immediately frozen in liquid nitrogen and the second immersed into OCT compound and quickly frozen in the liquid nitrogen. The third specimen was fixed in 10% buffered formalin.

Serum parameters

Blood was collected from the peripheral vessels in the quantity of 1-1.5 mL. Blood was centrifuged for 10 min at 4 °C at 3500 rpm. Supernatants were collected and stored at -80 °C until tested by a serum multiple biochemical analyzer to measure alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase, total protein, serum albumin, total bilirubin and total bile acids.

Histology and immunohistochemistry

Liver tissues, fixed in 10% buffered formalin, were used for histological and immunohistological analyses. Samples were stained in hematoxylin-eosin (HE). Liver sections were also incubated with Ki-67 antibody. OCT compound-immersed tissue samples were used for detection of liver sinusoidal endothelial cells (SEC) in the residual liver 24 h after PH.

Transmission electron microscopy

Livers were removed 5 min after hepatectomy. The liver was cut into small pieces (approximately 1 mm) and the specimens were fixed in 2% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.4, and post-fixed in 1% OsO₄ in 0.1 mol/L phosphate buffer. The specimens were dehydrated through a graded series of ethanol, passed through propylene oxide and embedded in EPON 812. Ultra-thin sections mounted on copper grids were stained with uranyl acetate and lead citrate, and then were observed under a Hitachi H-7000 transmission electron microscope.

Enzyme-linked immunosorbent assay

Serum samples were collected and stored at -80 °C and serum tumor necrosis factor- α (TNF- α), interleukin (IL)-4, IL-6 and IL-10 were quantified using commercially-available enzyme-linked immunosorbent assay kits (Becton, Dickinson and Company, United States). Serum TNF- α , IL-4, IL-6 and IL-10 levels were measured in the control group and the ω -3 PUFA group.

Western blotting

Liver tissue extracts were prepared from the liquid nitrogen frozen specimens as previously described. Western blotting was developed using polyclonal antibodies: phosphoserine Akt, total Akt, phosphotyrosine signal transducer and activator of transcription 3 (STAT3), total STAT3, phosphotyrosine glycogen synthase kinase (GSK) and total GSK.

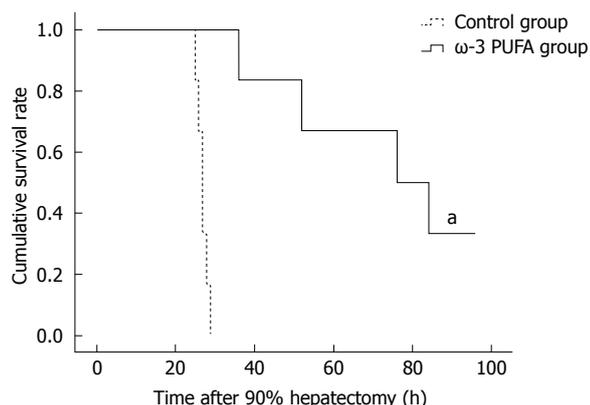


Figure 1 Survival rates in the control and the omega-3 polyunsaturated fatty acid groups. All rats from the control group died within 30 h after hepatectomy. In contrast, in the omega-3 polyunsaturated fatty acid (ω -3 PUFA) group, 20 (100%) and 4 (20%) rats were alive at 30 h and 1 wk after PH, respectively. ^a $P < 0.05$ vs normal group.

Statistical analysis

All data are expressed as the mean \pm SD of samples. Statistical analyses were carried out with the *T*-test for independent samples. In all cases, a *P* value < 0.05 was considered significant.

RESULTS

Survival rates

Twenty rats were subjected to PH surgery to examine the survival rates. All rats from the control group died within 30 h after PH. Four rats from the ω -3 PUFA group survived 1 wk after PH. According to the obtained results, the survival rates in the ω -3 PUFA group after 90% PH were 100% at 30 h after PH and 20% ($P < 0.05$) 1 wk after PH, respectively (Figure 1).

Liver weight/body weight ratios

The ratios of LW/BW and liver weights were significantly increased at 24 h after PH in the ω -3 PUFA group in comparison with the control group ($P < 0.05$) (Figure 2).

Serum parameters

Serum ALT and AST levels were increased in both control and the ω -3 PUFA groups at 24 h after PH. There was a significant difference, with higher levels in the control group ($P < 0.05$) (Figure 3A and B). Serum alkaline phosphatase levels were lower in the ω -3 PUFA group with significant differences at 12 h after PH ($P < 0.05$) in comparison with the control group (Figure 3C). Serum albumin and total protein levels were attenuated in both groups after PH with more rapidly decrease in the control group ($P < 0.05$) (Figure 3D and E). No significant differences were identified between the control and the ω -3 PUFA groups in the levels of total bilirubin, direct bilirubin and total bile acids ($P > 0.05$) (data not shown).

Histological findings

HE staining showed that the structure of liver lobules

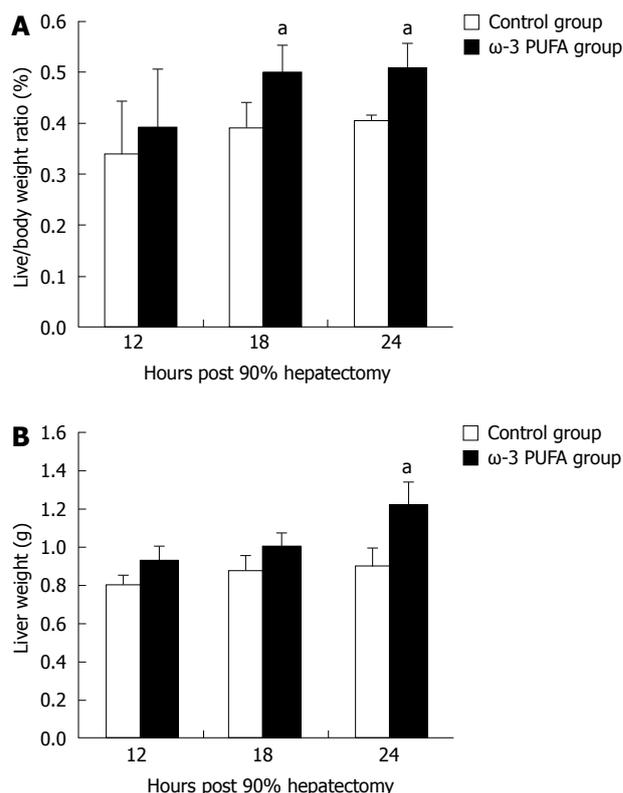


Figure 2 Change in liver weight/body weight ratios and liver weights after partial hepatectomy. Liver weight/body weight ratios (A) and liver weights (B) in the control and the omega-3 polyunsaturated fatty acid (ω -3 PUFA) group at 12 h, 18 h and 24 h post-hepatectomy. Data are expressed as mean \pm SD. $n = 6$ in each group. ^a $P < 0.05$ vs normal group.

was severely damaged; hepatocyte swelling and necrosis of hepatocytes were observed after PH at every time point in the control group. Nevertheless, hepatocytes and the structure of liver lobules were greatly restored at 24 h after PH in the ω -3 PUFA group (Figure 4). The number of Ki-67 positive cells in the liver tissue 24 h after 90% PH was much higher in the ω -3 PUFA group ($P < 0.05$) (Figure 5).

Transmission electron microscopy

In the control group, the space of Disse was enlarged and the structure of sinusoidal endothelial cells (SECs) was severely damaged at 24 h after PH. However, the SEC structure and space of Disse were greatly restored at 24 h after PH in the ω -3 PUFA group (Figure 6).

Serum cytokines

Compared with the control group, the level of pro-inflammatory cytokines IL-6 and TNF- α significantly decreased at 18 h and 24 h after PH ($P < 0.05$). The levels of anti-inflammatory cytokines IL-4 and IL-10 significantly increased in the ω -3 PUFA group at 24 h after PH ($P < 0.05$) (Figure 7).

Phosphorylation of Akt, STAT3, GSK3 β

In the ω -3 PUFA group, Akt was phosphorylated in the samples at 12 h after PH and phosphorylated Akt was

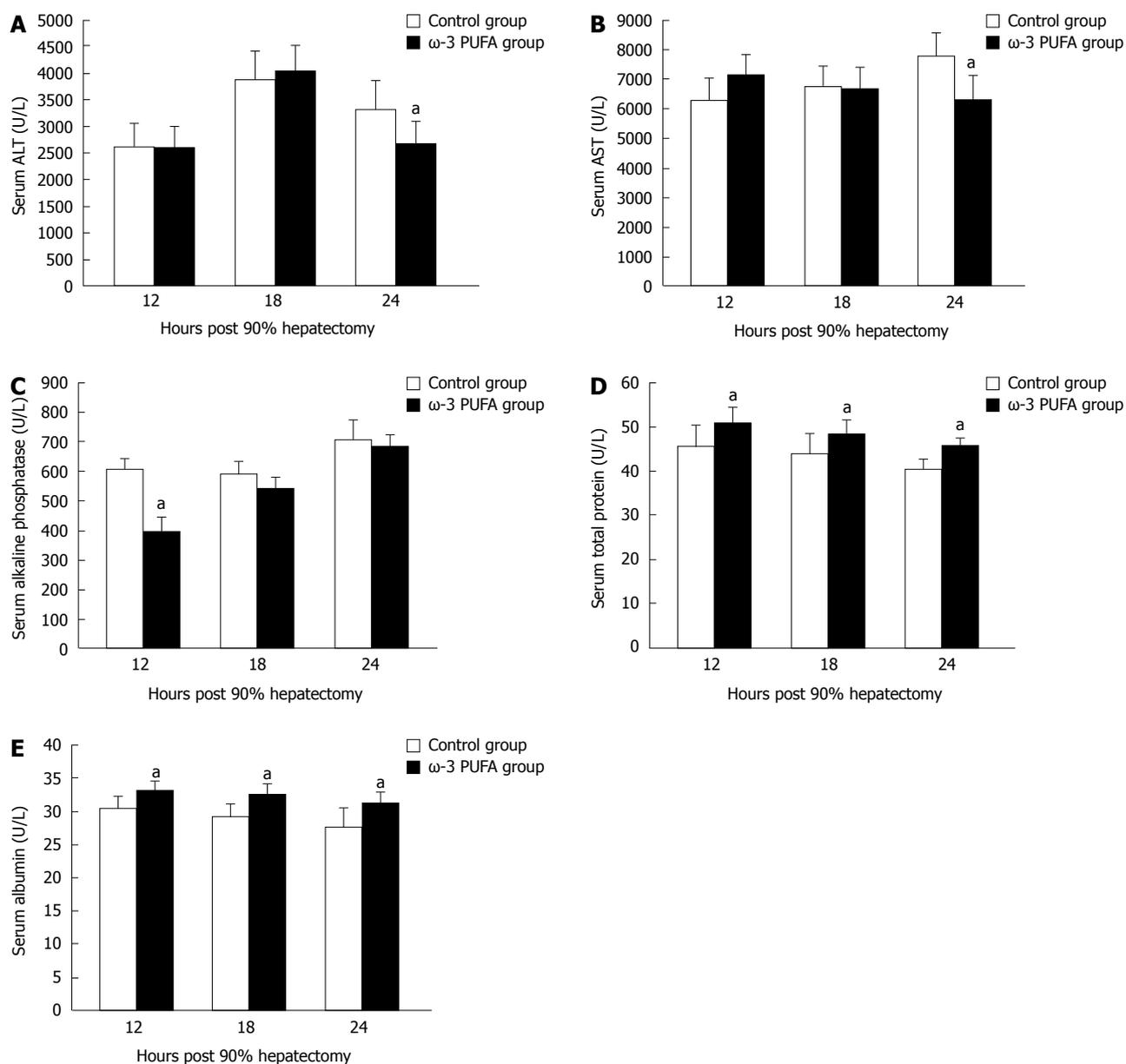


Figure 3 Serum parameters of the control and the omega-3 polyunsaturated fatty acid groups. Serum alanine aminotransferase (ALT) (A), aspartate aminotransferase (AST) (B), alkaline phosphatase (C), total protein (D) and albumin (E) levels at 12 h, 18 h and 24 h post-hepatectomy. $n = 6$ in each group. ^a $P < 0.05$ vs control group. ω-3 PUFA: Omega-3 polyunsaturated fatty acid.

increased in the samples at 18 h after PH. In contrast, phosphorylated Akt expression was much lower at 18 h after PH in the control group. In addition, GSK3 β was expressed at 12 h after PH in the ω-3 PUFA group, which was much earlier than in the normal group. Moreover, the expression of phosphorylated STAT3 was much higher and was sustained longer in the ω-3 PUFA group at 12 h, 18 h and 24 h after PH (Figure 8).

DISCUSSION

PH is the accepted gold standard of treatment for benign and malignant hepatic space-occupying lesions. The precise liver resection, which is based on preoperative evaluation, meticulous surgical procedure and postoperative

management, has been well developed. However, acute liver failure after extended hepatectomy has been shown to be a major contributor to post-operative mortality and morbidity^[13,14]. Thus, how to control post-operative liver failure remains an urgent problem to be solved. It has been shown that ω-3 PUFA plays an important role in gastrointestinal surgery; it can protect hepatocytes through inhibiting liver cell peroxidation *via* anti-oxidation and anti-inflammatory mechanisms of action after PH^[15,16]. Nevertheless, no report has shown the effects of ω-3 PUFA on liver regeneration and acute liver failure after extended hepatectomy. Our study is the first to demonstrate that ω-3 PUFA plays a beneficial role on liver regeneration after 90% hepatectomy, which may offer hope for protecting from post-operative liver failure

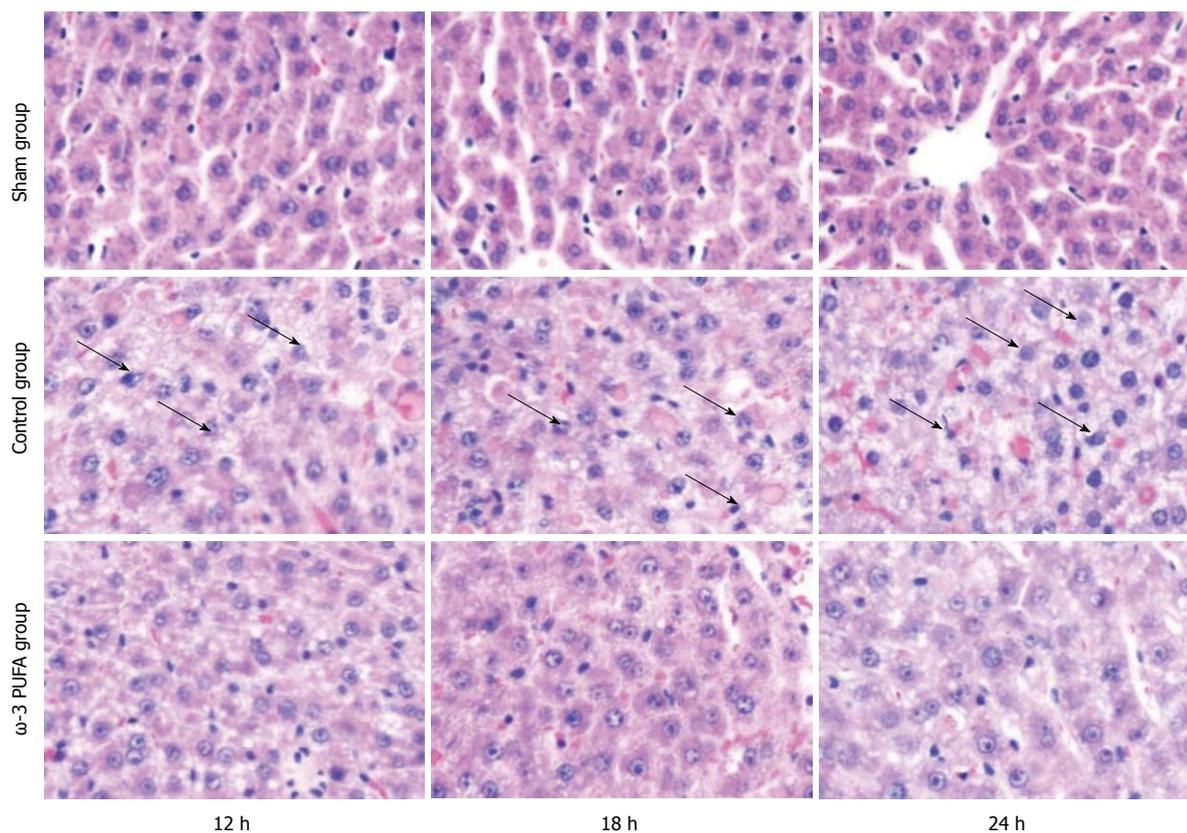


Figure 4 Histopathological examination of rats livers. Swelling and balloon degeneration of hepatocytes were observed at all time points after partial hepatectomy (PH) in the control group (hematoxylin and eosin staining, original magnification $\times 400$). By contrast, no significant hepatocyte swelling or balloon degeneration were observed at 12 h after PH in the omega-3 polyunsaturated fatty acid (ω -3 PUFA) group (arrow). Hepatocytes are normal and structure of liver lobules was obvious at 24 h after PH.

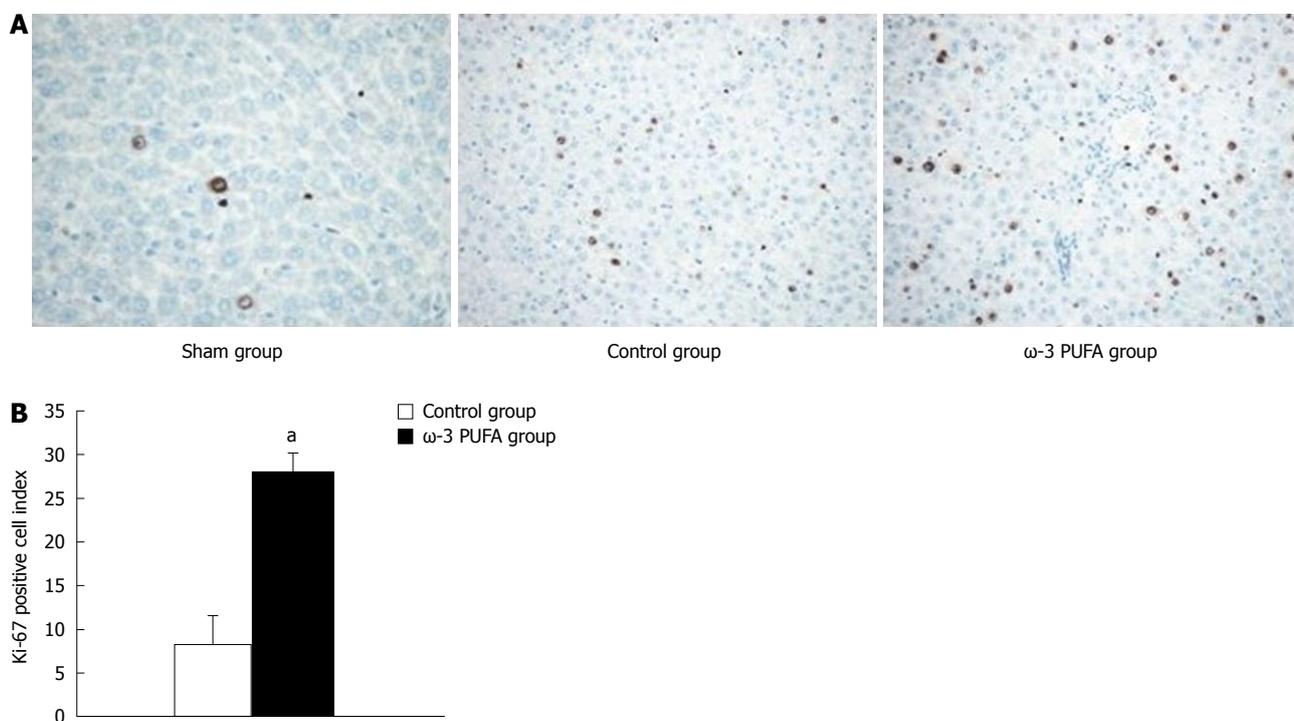


Figure 5 Change in Ki-67-positive cells after partial hepatectomy. A: The number of Ki-67-positive cells in the liver tissue 24 h after 90% partial hepatectomy (PH) was much higher in the omega-3 polyunsaturated fatty acid (ω -3 PUFA) group; B: Compared with control group, Ki-67-positive cells were significantly increased in the ω -3 PUFA group 24 h after PH. $n = 6$ in each group. ^a $P < 0.05$ vs control group.

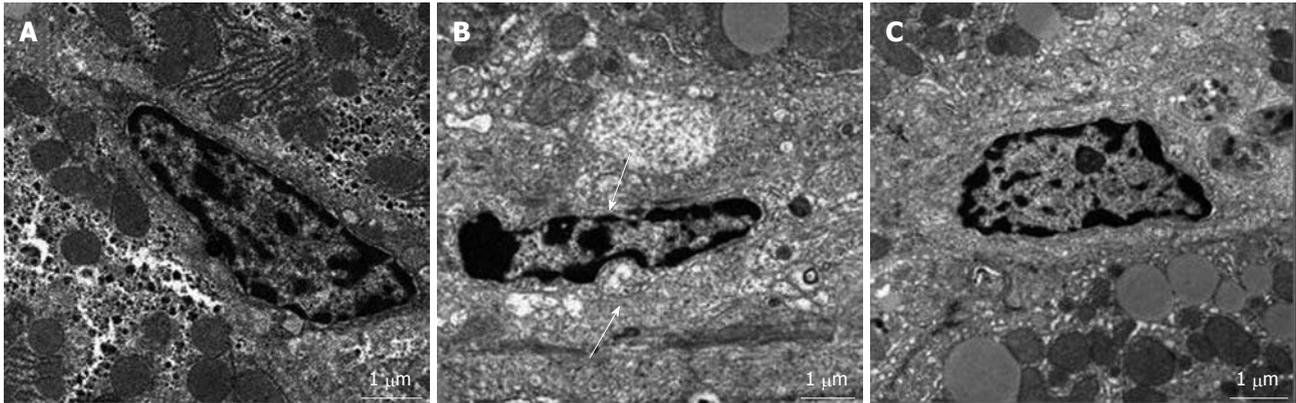


Figure 6 Change in the structure of sinusoidal endothelial cell after partial hepatectomy. A: Normal structure of liver tissue; B: Space of Disse was enlarged and the structure of sinusoidal endothelial cell (SEC) was severely damaged at 24 h after partial hepatectomy (PH) in the control group (arrow); C: Compared with control group, the SEC structure and space of Disse were greatly restored at 24 h after PH in the omega-3 polyunsaturated fatty acid group.

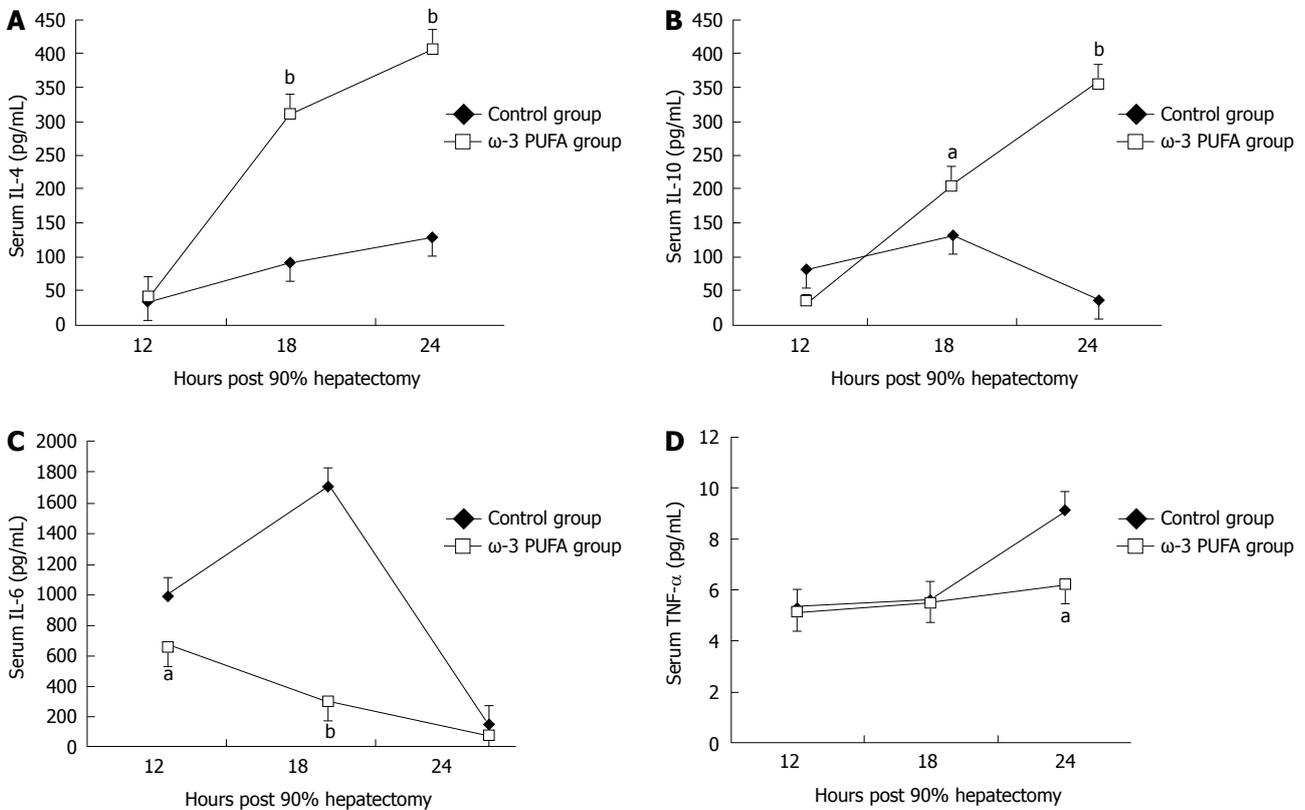


Figure 7 Change in serum cytokines. Cytokines interleukin (IL)-4 (A) and IL-10 (B) significantly increased at 24 h in the omega-3 polyunsaturated fatty acid (ω-3 PUFA) group after partial hepatectomy, while IL-6 (C), and tumor necrosis factor (TNF)-α (D) significantly decreased at 18 h and 24 h after operation in the ω-3 PUFA group. ^a*P* < 0.05, ^b*P* < 0.01 vs control group.

after clinical extended hepatectomy.

In general, serum parameters are a significant index to evaluate liver function. Serum AST and ALT concentrations are inversely related to the severity of liver damage. Albumin is a marker of synthetic function of the liver^[17,18]. In this research, both ALT and AST levels were significantly lower in the ω-3 PUFA group at 24 h after PH. In addition, total protein and albumin levels were higher in the ω-3 PUFA at 12 h, 18 h and 24 h after PH. These results strongly indicate that ω-3 PUFA plays a

protective role in liver function after 90% hepatectomy.

SECs are a type of special endothelium, which not only take part in the formation of blood vessels^[19], but also play a key role in hemodynamic changes of the liver, even physiological and metabolic functions^[20-22]. The structural feature of the SEC is a spindle type of cell with plenty of clusters of cribriform fenestrae^[23], which can accelerate the exchange of substances between hepatocytes and blood plasma^[24]. ω-3 PUFA has been reported to modulate both nitric oxide synthase

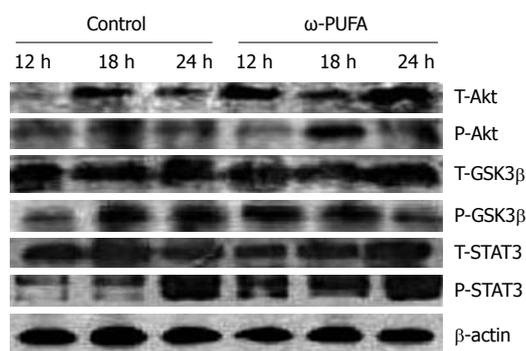


Figure 8 Phosphorylation of activation of protein kinase, signal transducer and activator of transcription 3, glycogen synthase kinase-3 β . Phosphorylation of activation of protein kinase B (Akt), signal transducer and activator of transcription 3 (STAT3), glycogen synthase kinase (GSK)-3 β in the control and the omega-3 polyunsaturated fatty acid (ω -3 PUFA) groups at 12 h, 18 h and 24 h after partial hepatectomy. Liver lysates at 12 h, 18 h and 24 h time points in the quantity of 25 μ g per lane were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene fluoride membrane and then incubated with specific antibodies. There was stronger phosphorylation of Akt at the 18 h time points and earlier phosphorylation of GSK3 β at the 12 h time points compared to the control group. Phosphorylation of STAT3 was stronger and lasted longer at all time points in the ω -3 PUFA group.

(NOS) activity and cyclooxygenase expression as antioxidants^[25,26]. Oxidative stress and the inactivation of NO by superoxide anions play an important role in disease states. NO is synthesized by three distinct NOS isoforms (neuronal, inducible, and endothelial NOS)^[27]. The endothelial NOS III plays physiologically important roles in vascular homeostasis, such as keeping the vasculature dilated, protecting the intima from platelet aggregates and leukocyte adhesion, and combining superoxide^[28]. This procedure can generate non-toxic metabolite NO₃⁻, which could circumvent oxygen-free radical damage to the integrity of the structure and function of SECs^[29]. In our study, the space of Disse was enlarged and the structure of SECs was severely damaged in the control group, in contrast to the favorable integrity of the SEC structure in the ω -3 PUFA group. These results suggest that ω -3 PUFA promotes liver regeneration through protecting the structure of the SEC in the acute phase after hepatectomy.

ω -3 PUFA is known to play a crucial role in the function of the immune system and its receptors such as Toll-like receptors (TLRs). Recently, TLRs were observed to influence the development of adaptive immune responses in T-helper-1 (Th1) and T-helper-2 (Th2) cells which are characterized by their distinct cytokine patterns including IL-4, IL-6, IL-10 and TNF- α . Jerin *et al.*^[30] suggested that the intensity of inflammation could be assessed by the ratio of IL-6/IL-10. In the present study, we showed that pro-inflammatory cytokines IL-6 and TNF- α significantly decreased, and anti-inflammatory cytokines IL-4 and IL-10 significantly increased, in the ω -3 PUFA group in comparison with the control group. TNF- α is produced mainly by resident hepatic macrophages (Kupffer cells)^[31], and the expression of IL-6 has been shown to be stimulated in hepatocytes as well as in Kupffer cells^[32]. Activa-

tion of nuclear factor- κ B induced by TNF- α is a key step in liver regeneration^[33]. IL-6 is also predominantly produced in Kupffer cells. The role of IL-6 in liver regeneration has not been clearly defined^[34]. However, it was found to be one of the strongest inducers of STAT3 in hepatocytes. After IL-6 binds to its receptor on the hepatocyte surface, signal transduction occurs by phosphorylation of cytoplasmic STAT protein 3^[35]. STAT3 is an important signal pathway in regulating cell proliferation, transformation and apoptosis of hepatocytes^[36]. A previous study has shown that a significantly higher mortality rate occurred in the early phase after 70% PH in STAT3-knockout mice^[37]. The Akt pathway is downstream of growth factor receptors^[38], and promotes cell proliferation through activation of GSK3 β to accelerate nuclear accumulation of cyclin D1^[39]. Cyclin D1 can strongly stimulate cell division and thus enhance liver regeneration. In the present study, STAT3 was strongly phosphorylated at 12 h and 18 h after PH in the ω -3 PUFA group. Phosphorylated Akt was also detected at 18 h after PH which was much earlier and stronger than that in the control group. Our results demonstrate that ω -3 PUFA is responsible for earlier and stronger phosphorylation of the STAT3 and Akt in the signaling pathway. However, IL-6 and TNF- α , key cytokines for liver regeneration, were found to decrease in the ω -3 PUFA group. The mechanism of these two opposite effects on liver regeneration is not clear in the present study and needs to be clarified in the future.

Taken together, our results provide strong evidence that intravenous injection of ω -3 PUFA could slow the progress of acute liver failure through an anti-inflammatory action and significantly promote liver regeneration after 90% hepatectomy.

COMMENTS

Background

Liver regeneration after the loss of hepatic tissue is a fundamental parameter of liver response to injury. Omega-3 polyunsaturated fatty acid (ω -3) PUFA could promote liver regeneration as an anti-inflammatory agent.

Research frontiers

Although a previous study has demonstrated that intravenous administration of ω -3 PUFA is beneficial in the treatment of fatty liver in rats, the effect of liver regeneration was not detected.

Innovations and breakthroughs

This study demonstrated that ω -3 PUFA plays a beneficial role in liver regeneration. ω -3 PUFA decreased the expression of pro-inflammatory cytokines and increased the expression of anti-inflammatory cytokines. ω -3 PUFA could slow the progress of acute liver failure through its anti-inflammation effect and promote liver regeneration.

Applications

The results of this study suggest ω -3 PUFA can promote liver regeneration in rats. Further studies are required before applying this treatment to patients with partial hepatectomy.

Terminology

The ω -3 PUFA enter human bodies mainly through consumption of cold-water fish products, and they have been widely clinically used in total parenteral nutrition treatment.

Peer review

It is of interest and importance that ω -3 PUFA may contribute to the recovery of severely damaged livers.

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Expression and significance of homeodomain protein Cdx2 in gastric carcinoma and precancerous lesions

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Abstract

AIM: To investigate the expression and significance of caudal-related homeobox transcription factor (Cdx2) in gastric carcinoma (GC) and precancerous lesions.

METHODS: The expression of Cdx2 in GC, precancerous lesions and normal gastric mucosa were detected using immunohistochemical method. Hematoxylin and eosin staining, alcian blue/periodic acid-schiff and high iron diamine/alcian blue staining were used to classify intestinal metaplasia (IM) and GC.

RESULTS: Cdx2 was not detected in normal gastric mucosa. Cdx2 expression was detected in 87.1% (101/116) of IM, 50% (36/72) of dysplasia and 48.2% (41/85) of GC. The Cdx2-expressing cells in IM were more prevalent than in dysplasia and carcinoma ($P < 0.05$). There was no relationship between Cdx2 expression and the classification of IM or the degree of dysplasia. Expression of Cdx2 was significantly higher in intestinal-type carcinoma than in diffuse and mixed-type carcinoma ($P < 0.05$). Positive expression of Cdx2

was mainly found in moderately to well differentiated GC. There was a negative association between nuclear Cdx2 expression and lymph node metastasis and tumor, nodes, metastasis stage of GC ($P < 0.05$). The patients with Cdx2-positive expression showed a higher survival rate than those with Cdx2-negative expression ($P = 0.038$). Multivariate analysis revealed that the expression of Cdx2 and lymph node metastasis were independent prognostic indicators of GC ($P < 0.05$).

CONCLUSION: Cdx2 may be closely related to IM and the intestinal-type GC and implicate better biological behavior and outcome. Cdx2 is useful for predicting the prognosis of GC.

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Key words: Caudal-related homeobox transcription factor; Stomach neoplasm; Intestinal metaplasia; Dysplasia; Immunohistochemistry

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INTRODUCTION

Gastric carcinoma (GC) is one of the most common malignant diseases and is the second most common cause of cancer-related death in China and in the world^[1]. The pathogenesis of GC is still not very clear. Despite wide acceptance of the gastritis-metaplasia-dysplasia-carcinoma sequence, the precise molecular alterations underlying this progression pathway remain to be delineated^[2].

Caudal-related homeobox transcription factor (Cdx2), which is a member of the caudal-related homeobox gene family, plays an important role in mammalian early intestinal development and the maintenance of intestinal epithelia through its regulation of intestine-specific gene transcription^[3,4]. Normally, Cdx2 is expressed in small intestinal and colonic epithelia, but not in gastric epithelium^[5]. Although Cdx2 expression is restricted to the intestinal epithelium under normal conditions, an ectopic expression has been reported in the gastric mucosa, both in intestine-like metaplasia and in a subset of GCs^[6,7]. Recently, Mutoh *et al*^[8] and Almeida *et al*^[4] reported that the ectopic expression of Cdx2 in the gastric mucosa of transgenic mice is related to the transdifferentiation of gastric mucosal glands into intestinal-like mucosa, and claimed that *Cdx2* gene causes intestinalization in the gastric mucosa. In humans, Cdx2 has been reported to be associated with intestinal metaplasia (IM) in the stomach^[6], in which ectopic expression of Cdx2 is speculated to cause the gastric epithelial cells to transdifferentiate into the intestinal phenotype. Several reports have also suggested a tumor suppressor role for Cdx2 in human colorectal carcinogenesis^[9,11], and this might also be true for gastric cancers. But the question as to whether the ectopic expression of Cdx2 has any influence on cancer initiation and/or progression in the stomach remains unanswered.

GC is a markedly heterogeneous disease in histologic feature and biological characters, especially in the advanced stages. The clinical evidence showed that the biological behavior and prognosis could be significantly different among the patients with the same stage, histological type, or differentiation grade. Therefore, searching for the biomarkers to indicate the biological characters, and predicting the outcome of patients with GC, is the major focus of research on GC. A number of biomarkers have been found to be involved in the development and progression of GC. Although expression of Cdx2 has been detected in some GCs, few studies reported the relationship between Cdx2 expression and prognosis of GC^[12,13].

To better understand the mechanisms underlying malignant transformation and its relationship with developmental processes, we studied and compared the expression of the intestine-specific homeodomain protein Cdx2 in metaplasia, dysplasia and GCs, and the morphologic appearance. Furthermore, in the present study, we analyzed the association between Cdx2 and Lauren's classification, lymph node metastasis, invasion depth, distant metastasis, vascular invasion, tumor size, as well as tumor, nodes, metastasis (TNM) stages, to evaluate the clinical significance of this marker in the histological classification and the prognosis assessment of GC.

MATERIALS AND METHODS

Patients and tissue samples

The present study consisted of 85 cases with surgically resected gastric specimens and 228 cases with endoscopic biopsies were obtained from the Department

of Pathology, the First Affiliated Hospital of Anhui Medical University of China from 2000 to 2005, under a protocol approved by the Institutional Review Board. Slides of GC were reviewed to analyze pathologic parameters, including tumor size, histological grading, depth of invasion, and the presence of nodal metastasis. The 85 patients with GCs (aged 20-87 years, mean 61.75 years; 25 females and 60 males) included 20 early cases and 65 advanced cases. Among them, 10 were classified as well-differentiated adenocarcinoma, 34 as moderately differentiated, and 30 as poorly differentiated adenocarcinoma, and 11 as mucinous cell type. Based on Lauren's classification system, all GCs were categorized into three histological types: intestinal, diffuse, and mixed^[14]. Forty-three cases were classified as intestinal, 35 as diffuse and 7 as mixed. TNM staging was assessed according to the system established by the American Joint Committee on Cancer (AJCC, 19 at pTNM stage I and II, and 66 at pTNM stage III and IV). All patients were followed up until January 2010 for a minimum of 5 years. No patient had received chemotherapy or radiation therapy before surgery. In addition, 228 cases of gastric endoscopic biopsies included 10 cases of normal gastric mucosa, 30 cases of chronic superficial gastritis, 116 cases of gastric IM, and 72 cases of gastric dysplasia (39 cases of mild dysplasia, 20 cases of moderate dysplasia and 13 cases of severe dysplasia). The study was approved by the Research Ethics Committee of Anhui Medical University, China. Informed consent was obtained from all patients. All specimens were handled anonymously according to the ethical and legal requirements.

Histochemistry

The samples were fixed with 10% neutral-buffered formalin and embedded in paraffin. Paraffin-embedded samples were serially sectioned at 4 μ m and mounted on slides. IM was classified into complete type and incomplete type, using Alcian Blue (pH 2.5)/Periodic Acid-Schiff staining and Alcian blue/high-iron diamine (AB/HID) staining (Baso Diagnostics Inc, China). After deparaffinization and rehydration, the sections were incubated with Alcian Blue pH 2.5 for 30 min, followed by 0.5% periodic acid for 10 min and Schiff solution for 10 min. The sections were then counterstained with modified Mayer's hematoxylin for 5 min, dehydrated with graded ethanols and mounted with coverslip. In complete IM, only goblet cells were stained blue, while in incomplete IM, both the goblet cells and the inter-mediate columnar cells appeared blue.

Immunohistochemistry

After routine deparaffinization and rehydration of the slides, antigen retrieval was done by incubation in modified citrate buffer at 121 $^{\circ}$ C for 20 min. The sections were treated with 0.03% hydrogen peroxide for 5min to block the endogenous peroxidase activity, followed by incubation with anti-Cdx2 monoclonal antibody (1:100, Biogenex, San Ramon, CA, United States) at 4 $^{\circ}$ C over-

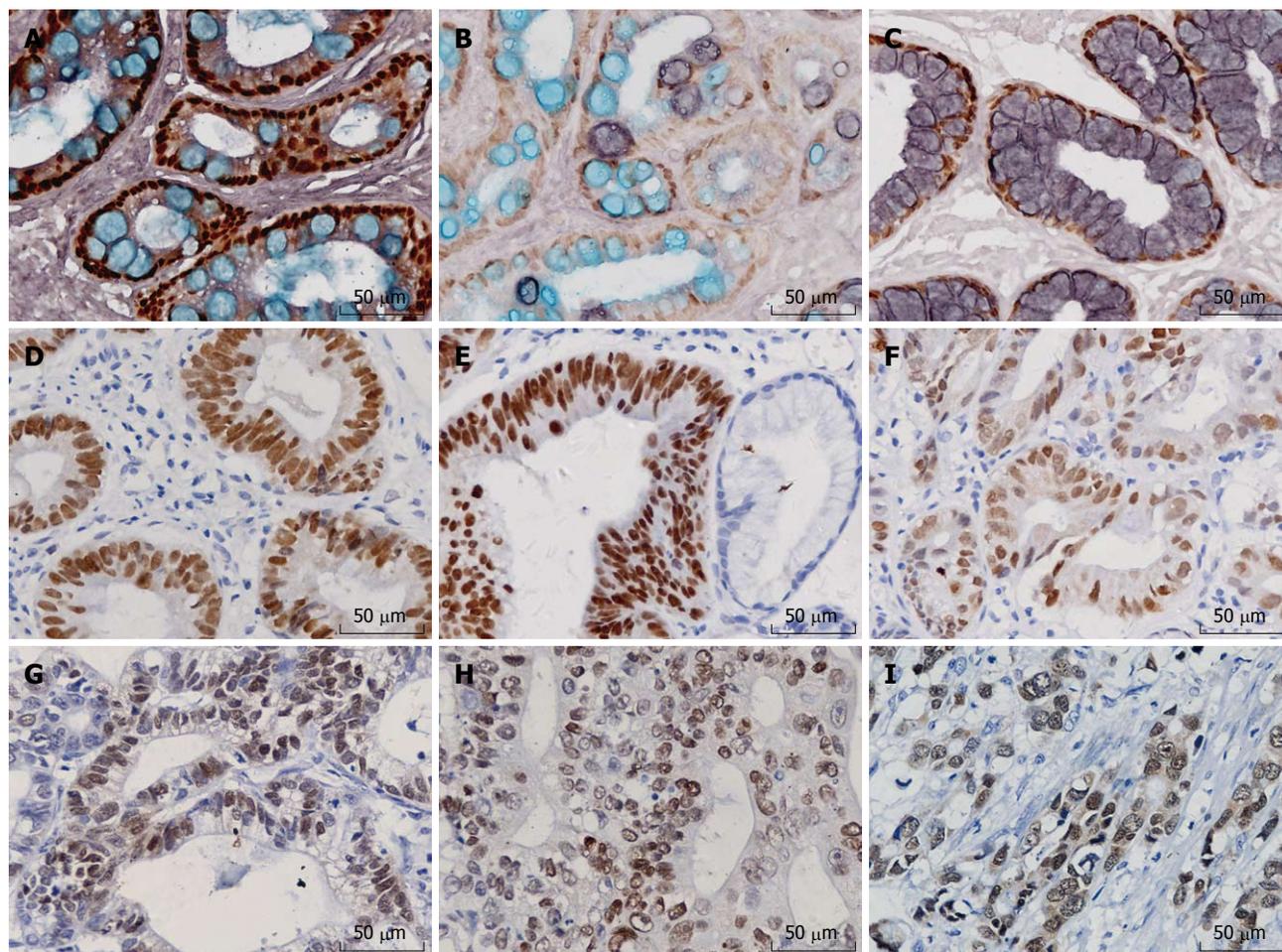


Figure 1 Positive expression of caudal-related homeobox transcription factor in intestinal metaplasia, dysplasia and differentiated gastric cancer. A: Staining of intestinal metaplasia (IM) type I ($\times 400$); B: Staining of IM type II ($\times 400$); C: Staining of IM type III ($\times 400$); D: Staining of mild dysplasia ($\times 400$); E: Staining of moderate dysplasia ($\times 400$); F: Staining of severe dysplasia ($\times 400$); G: Staining of well-differentiated gastric cancer ($\times 400$); H: Staining of moderately-differentiated gastric cancer ($\times 400$); I: Staining of poorly-differentiated gastric cancer ($\times 400$).

night. After washing with $1 \times$ phosphate buffered saline (0.01mol/L, pH 7.4), the sections were subsequently incubated with the biotinylated secondary antibody (Dako, Denmark). The sections were then stained using the Ultra-Sensitive™ Immunohistochemical Staining Kit (Maixin Bio, Fuzhou, China) according to the manufacturer's instructions. After development with 0.05% 3,3'-diaminobenzidine, the sections were counter-stained with hematoxylin, dehydrated with graded ethanols and xylene and then mounted with coverslip. In negative control samples, the primary antibody was omitted and 5% goat serum was used as the primary antibody. Known positive tissue sections served as positive control samples.

Double staining

Double staining of immunohistochemistry and AB/HID staining were applied to detect the Cdx2 expression in different subtypes of IM. Compared to either IHC or AB/HID, combination of both staining was able to detect the protein level and subtypes of IM at the same time.

Assessment of immunostaining in cancer cells

Semiquantitative scores were given as the score of the

percentage of positive cells plus the score of the staining intensity. The scoring criteria of the percentage of positive nuclei (Figure 1A-C) were: score 0: 0%-5% positive cancer cells; score 1: 6%-25% positive cancer cells; score 2: 26%-50% positive cancer cells; score 3: 51%-75% positive cancer cells; and score 4: 76%-100% positive cancer cells. The intensity was scored as follows: score 0, no staining; score 1, mild staining; score 2, moderate staining; and score 3, strong staining. The final scores were from 0 to 7. Specimens with a Cdx2 score of 0-2 were considered negative, whereas specimens with a score of 3-7 were considered positive for Cdx2 expression.

Two experienced investigators independently examined the staining who blinded to the clinicopathological data. Different scores between the two investigators were observed in 15% of the cases, and a consensus was achieved in all the cases after discussion.

Statistical analysis

All data were analyzed using SPSS 13.0 software. Fisher's exact test or χ^2 test was used to calculate the association of Cdx2 expression with various clinicopathological features. Cumulative survival was estimated by the Kaplan-

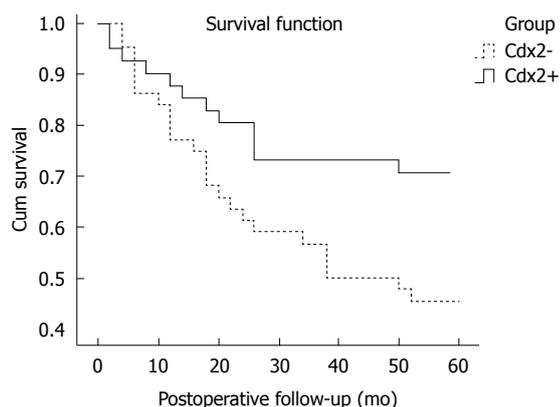


Figure 2 Survival curve of gastric cancer patients with caudal-related homeobox transcription factor positive-expression compared with negative-expression group ($P = 0.038$). Cdx2: Caudal-related homeobox transcription factor.

Meier method and differences between survival curves were analyzed by the log-rank test. The influence of each variable on survival was analyzed by the multivariate Logistic regression analysis. Differences were assumed to be statistically significant when $P < 0.05$.

RESULTS

Cdx2 expression patterns in GC and precancerous lesions

Cdx2 protein expressed in the nuclei of goblet cells of intestinal mucosa, some columnar epithelial cells and some gastric cancer cells. Representative pictures of immunohistochemical staining of Cdx2 are shown in Figure 1.

Expression of Cdx2 protein in GC and precancerous lesions

Gastric IM has been classified as complete (small intestine) or incomplete (colonic) using histochemical staining techniques and into three types based on the staining pattern of its mucins: I (complete), and II and III (incomplete). Type I is the most common type: paneth cells are present and goblet cells secrete sialomucins. Types II and III are characterized by the presence of columnar cells and goblet cells secreting sialomucins and/or sulfomucins; the columnar cells secrete sialomucins in type II and sulfomucins in type III. Cdx2 protein can not be detected in normal gastric mucosa and chronic superficial gastritis. The expression rates of Cdx2 were 87.1% in IM (101/116, Figure 1A-C), 50% in dysplasia tissues (36/72, Figure 1D-F) and 48.2% (41/85, Figure 1G-I) in gastric cancer, respectively. The positive rates of IM, dysplasia and GC were higher than that of normal mucosa ($P < 0.05$). The expression of Cdx2 in IM was much higher than that in dysplasia and carcinoma ($P < 0.05$). There was no significant difference between dysplasia and carcinoma ($P > 0.05$). The expression rates of Cdx2 were 90%, 85% and 75%, respectively in type I, II and III IM, but the difference was not statistically significant ($P > 0.05$). Cases of gastric epithelial dysplasia were graded as mild, moderate and severe dysplasia according to

Table 1 Expression of caudal-related homeobox transcription factor in gastric carcinoma and precancerous tissues

Type	Cases	Cdx2 positive cases	%	χ^2	P value
Different subtypes of intestinal metaplasia	116	101	87.1	33.826	0.000
IM (type I)	80	72	90	31.400	0.000
IM (type II)	20	17	85	7.426	0.003
IM (type III)	16	12	75	5.365	0.012
Dysplasia	72	36	50	0.004	0.873
Mild	39	17	43.5	0.083	0.700
Moderate	20	11	55	0.088	0.637
Severe	13	8	61.5	0.355	0.553
Gastric carcinoma	85	41	48.2		

Cdx2: Caudal-related homeobox transcription factor; IM: Intestinal metaplasia.

the previously established criteria that included the degree of architectural complexity and cytological atypia^[15]. The positive rates of Cdx2 were 43.5%, 55% and 61.5% in mild, moderate and severe dysplasia, showing an increasing trend. But there was no statistically significant correlation between Cdx2 expression and grade of dysplasia ($P > 0.05$, Table 1).

Correlation of Cdx2 expression with clinicopathological parameters in gastric cancer patients

The correlation of Cdx2 expression with clinicopathological parameters in gastric cancer patients is depicted in Table 2. Cdx2 expression was associated with direct Lauren classification, differentiation, lymph node metastasis and clinical stage. The positive rate of Cdx2 in intestinal gastric cancer (67.4%) was significantly higher than that in diffuse and mixed-type gastric cancer (25.7%, 42.9%, $P = 0.001$). The positive rate of Cdx2 in well and moderately differentiated adenocarcinoma (65.9%) was significantly higher than that in the poorly differentiated and mucinous adenocarcinoma (29.3%, $P = 0.001$). In addition, the positive rate of Cdx2 in the group without lymph node metastasis (63.9%) was significantly higher than that with lymph node metastasis (36.7%, $P = 0.017$). The positive rate of Cdx2 of pTNM stage III and IV (40.9%) was also reduced compared with that of pTNM stage I and II (73.7%, $P = 0.018$). Cdx2 expression was not associated with the remaining clinicopathological parameters evaluated, including depth of invasion, sex, and age of patients.

Prognostic implication of Cdx2 nuclear expression in GC

The association between the 5-year survival rate and the expression of Cdx2 was analyzed using the Kaplan Meier method. The results are shown in Figure 2. The patients with nuclear Cdx2 expression showed a significantly higher 5-year survival rate than patients without Cdx2 expression ($P = 0.038$). Based on the Cox regression analysis in the 85 patients, Cdx2 expression and lymph node metastasis seemed to be independent prognostic indicators ($P = 0.001$, $P = 0.019$, respectively, Table 3).

Table 2 Relationship between expression of caudal-related homeobox transcription factor and clinical pathological characteristics of gastric carcinoma

Pathological parameters	n	Cdx2 positive cases	Positive rate (%)	χ^2	P value
Gender				0.071	0.642
Male	60	30	50		
Female	25	11	44		
Age (yr)				0.000	1.000
≥ 55	57	28	49.1		
< 55	28	13	46.4		
Lauren classification				13.544	0.001
Intestinal	43	29	67.4		
Diffuse	35	9	25.7		
Mixed type	7	3	42.9		
Differentiation				9.991	0.001
Well and moderately differentiated	44	29	65.9		
Poorly differentiated and mucinous adenocarcinoma	41	12	29.3		
Depth of invasion				5.630	0.131
T1	9	7	77.8		
T2	11	5	45.5		
T3	25	14	56		
T4	40	15	37.5		
Lymph node metastasis				5.089	0.017
No	36	23	63.9		
Yes	49	18	36.7		
Clinical stages				5.102	0.018
I + II	19	14	73.7		
III + IV	66	27	40.9		

Cdx2: Caudal-related homeobox transcription factor.

Table 3 Association of various factors with overall survival by the logistic regression

Variables	B	SE	Walt	Exp (B)	P value
Cdx2	-1.290	0.397	10.586	0.275	0.001
Lymph node metastasis	0.808	0.344	5.501	2.243	0.019

Cdx2: Caudal-related homeobox transcription factor.

DISCUSSION

It was proposed by Correa^[16] that human gastric carcinogenesis is a multistep process that progresses from chronic gastritis, atrophy, IM, dysplasia and finally leads to gastric cancer. This model, although challenged by a few, is widely accepted, especially for the intestinal type of gastric cancer. IM is most frequently recognized as a pathological condition in the human stomach, where it often develops from progression of chronic gastritis, and has been extensively studied as a putative preneoplastic lesion of intestinal-type GC. Cdx2 was found to be intensively involved in intestinal metaplastic differentiation^[17]. During mouse small intestinal development, Cdx2 is expressed much earlier than Cdx1 in the hindgut^[18], and in human IM, expression of Cdx2 precedes that of Cdx1 during the progression of IM, implying that the expression of Cdx2 may trigger the initiation and devel-

opment of IM^[19,20]. Cdx2 may stimulate intestinal proliferation and differentiation by transcriptional activation of intestine-specific proteins (MUC2, sucrase-isomaltase, carbonic anhydrase I). Aberrant expression of Cdx2 is prominent in intestinal-type gastric adenocarcinoma and Cdx-2 may therefore play an important role in gastric carcinogenesis, especially in the intestinal type^[12]. In this study, we found that Cdx2 was not expressed in normal gastric mucosa and superficial gastritis, but was expressed in 87.1% of IM. The expression of Cdx2 was also higher in intestinal-type than in diffuse-type GC. Cdx2 has been shown to be a key molecule associated with IM and intestinal or differentiated type GC.

IM can be classified into different subtypes by several classification systems. The most widely accepted one is to classify IM into complete type and incomplete type, with the latter carrying a higher risk of gastric cancer. The complete type is characterized by the presence of absorptive cells, paneth cells and goblet cells secreting sialomucins, similar to the small intestinal phenotype. The incomplete type is characterized by the presence of columnar and goblet cells secreting sialomucins and/or sulphomucins, similar to the colonic phenotype. Using alcian-blue/periodic acid Schiff and high-iron diamine-alcian blue technique, Jass *et al*^[21] classified IM into three subtypes. Type I corresponded to the complete type, while type II and type III were classified as the incomplete type according to the mucins secreted by the columnar cells: sialomucins in type II and sulphomucins in type III. Several studies claimed that only type III IM is associated with an increased risk of gastric cancer, but other reports cast doubt on it. In this study, staining of both immunohistochemistry and AB/HID was applied to detect the Cdx2 protein level and subtypes of IM at the same time. The expression rates of Cdx2 were 90%, 85% and 75% respectively in type I, II and III IM. There was no difference of Cdx2 expression among type I, II and III IM. But Liu *et al*^[22] demonstrated that the expression of Cdx2 was significantly decreased in incomplete IM compared with complete IM. This may be explained by the different antibodies and different staining protocols.

So far, there have been a few reports about Cdx2 expression in gastric epithelial dysplasia. Similar to Woodland's finding^[23], we found no relationship between the grade of gastric epithelial dysplasia and Cdx2 expression. But Kim *et al*^[24] demonstrated a positive correlation between Cdx2 expression and the increasing grade of dysplasia.

Our data showed that Cdx2 expression was decreased in gastric cancers, when compared with IM and dysplasia. This collective experience may suggest a potential tumor suppressor role for Cdx2, in view of its sequential decrease in expression along with the stepwise gastric carcinogenesis (IM, epithelial dysplasia and gastric cancer). This opinion is shared by Liu *et al*^[22] and Xin *et al*^[25], who showed that Cdx2 expression is progressively decreased in gastric IM, dysplasia, and cancer. In our study, Cdx2-positive expressing tumors had tendencies towards less invasiveness and fewer lymph node metastases. The Cdx2-

positive patients were found to have a better outcome than Cdx2-negative patients. Multivariate analysis revealed that Cdx2 represents an independent prognostic indicator. The consequences are consistent with the studies by Seno *et al.*^[12] and Mizoshita *et al.*^[13]. We can speculate that, while ectopically expressed in gastric cancers, the Cdx2 gene may play a tumor suppressor role in slowing down cancer progression in the stomach, similar to what happens in the colon. Previous studies suggest that Cdx2 is a tumor-suppressor gene with regard to colorectal carcinogenesis. Several reports^[26,27] have revealed that Cdx2 could promote differentiation, inhibit proliferation, and increase sensitivity to apoptosis of intestinal epithelial cells, and colon cancer derived cells. Moreover, Cdx2 was documented to up-regulate transcription of p21/WAF1/CIP1^[28,29] and PTEN^[30], which plays a critical role in differentiation and tumor suppression, and promotes intestinal differentiation as a cyclin-dependent kinase inhibitor, leading to cell-cycle arrest. The role of Cdx2 in gastric cancer still remains unclear and Cdx2 may have different roles depending on cancer type.

In conclusion, our findings indicated that Cdx2 is a special and sensitive marker for IM. We revealed that Cdx2 might be closely related to the intestinal-type GC and implicate better biological behavior and outcome. Cdx2 might be useful in predicting the prognosis of GC. Further studies are necessary to confirm our findings.

COMMENTS

Background

Intestinal metaplasia (IM) of the stomach is a preneoplastic lesion that confers an increased risk for the development of gastric carcinoma (GC), which remains the second leading cause of cancer death worldwide. Caudal-related homeobox transcription factor (Cdx2) is an intestinal transcription factor responsible for regulating the proliferation and differentiation of intestinal epithelial cells. The role of Cdx-2 in GCs is not clearly understood.

Research frontiers

Human Cdx2 is known as a caudal-related homeodomain transcription factor that is expressed in the intestinal epithelium and is important in differentiation and maintenance of the intestinal epithelial cells. Although Cdx2 expression is restricted to the intestinal epithelium under normal conditions, an ectopic expression has been reported in the gastric mucosa, both in intestine-like metaplasia and in a subset of GCs. Although expression of Cdx2 has been detected in some GCs, few studies reported the relationship between Cdx2 expression and prognosis of GC.

Innovations and breakthroughs

This paper evaluated the immunohistochemical expression of the intestine-specific transcription factor Cdx2 in a large number of GC cases, compared with that in gastric IM, gastric dysplasia, superficial gastritis and normal gastric mucosa. The Cdx2-expressing cells in IM were more prevalent than in dysplasia and carcinoma. In GC, expression of Cdx2 was significantly higher in intestinal-type carcinoma than in diffuse and mixed-type carcinoma. Cdx2 might be closely related to IM and the intestinal-type GC and implicate better biological behavior and outcome. Cdx2 might be useful in predicting the prognosis for GC.

Applications

Cdx2 expression in GC may serve as a useful marker for diagnosis, to judge the degree of malignancy and to predict the prognosis of the patients.

Terminology

The Cdx2, located on chromosome 13 in humans and chromosome 5 in mice, is a member of the ParaHox cluster. Cdx2 is from a class of genes encoding transcription factors that constitute mammalian homologues of the Drosophila

gene Caudal. Caudal homologues have been identified in a number of organisms including humans and mice and play essential roles in intestinal development and maintenance of the epithelia. In mice and humans, there are 3 caudal homologues: Cdx1, Cdx2 and Cdx4, of which only Cdx2 plays a role in development of the gastrointestinal tract.

Peer review

This is an interesting, well written paper evaluating the immunohistochemical expression of the intestine-specific transcription factor Cdx2 in a large number of GC cases, compared with that in gastric IM, gastric dysplasia, superficial gastritis and normal gastric mucosa. It is the first study indicating a prognostic role of Cdx2 expression in GC.

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Increased frequency and clinical significance of myeloid-derived suppressor cells in human colorectal carcinoma

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Abstract

AIM: To investigate the frequency and clinical significance of the myeloid-derived suppressor cells (MDSC) in human colorectal carcinoma (CRC).

METHODS: Samples of peripheral blood and tumor tissue from 49 CRC patients were analyzed. Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation and were subjected to a flow cytometry-based immunophenotypic analysis.

RESULTS: A considerable increase in the percentage of CD33⁺HLA-DR⁺ MDSCs was observed in the peripheral blood (1.89% ± 0.75%) and tumor tissues (2.99% ± 1.29%) of CRC patients as compared with that in the

peripheral blood of healthy controls (0.54% ± 0.35%). This expanded CD33⁺HLA-DR⁺ subset exhibited immature myeloid cell markers, but not lineage markers, and showed up-regulation of CD18/CD11b expression as compared with the MDSCs from healthy donors. Further studies showed that the MDSC proportion in CRC peripheral blood was correlated with nodal metastasis ($P = 0.023$), whereas that in tumor tissues was correlated with nodal/distant metastasis ($P = 0.016/P = 0.047$) and tumor stage ($P = 0.028$), suggesting the involvement of MDSCs in CRC tumor development.

CONCLUSION: Characterization of MDSCs in CRC suggests the clinical significance of circulating and tumor-infiltrating MDSCs and may provide new insights into the CRC immunotherapy targeting MDSCs.

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Key words: Myeloid-derived suppressor cell; Colorectal carcinoma; Tumor metastasis

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INTRODUCTION

Immune escape is not merely a passive process of immune evasion, but is rather an active one in which tumor cells, stroma cells, and immune cells present within the tumor microenvironment actively suppress the antitumor immune response. Thus, although host immune surveil-

lance may prevent tumor outgrowth during the earliest stages of tumor development, locally invasive or metastatic tumors must evade host immunity^[1]. In the tumor microenvironment, the “mission” of immune cells is to execute an antitumor function, but some of these cells are converted to act as confederates of the tumor. The inability of immune cells to mount an effective antitumor response, even following vaccination, is an immunological hallmark of cancer and represents a critical problem for the development of effective immunotherapeutic strategies. Myeloid-derived cells and lymphocytes subsets, such as regulatory T cells (Tregs), collaborate with their malignant counterparts to suppress host immunity^[2,3].

Myeloid-derived suppressor cells (MDSCs) are a large group of myeloid cells comprising immature macrophages, granulocytes, and dendritic cells (DCs) as well as myeloid cells at earlier stages of differentiation^[4-6]. In mice, MDSCs express the myeloid lineage differentiation antigens Gr-1 and CD11b, and undergo dramatic expansion during tumor development. In mouse models, MDSCs are found in tumors and lymph nodes^[2,4,5], and the proportion of MDSCs has been shown to exceed 20% in the spleen. There are some controversies about the phenotype of MDSCs in humans, but these cells are now generally defined as CD11b- and CD33-positive, but lacking mature myeloid and lymphoid cell markers and the major histocompatibility complex (MHC) class II molecule, HLA-DR^[7-9]. In the appropriate cytokine environment, MDSCs can differentiate into mature myeloid cells, but this differentiation is blocked in the presence of tumor-cell-conditioned medium or in tumor-bearing hosts, indicating that MDSC expansion can be induced in a tumor microenvironment. Recent studies have shown that MDSCs inhibit the proliferation and activation of T cells, and suppress maturation of DCs, which together contribute to the negative regulation of the immune responses and promote immune escape of tumors and pathogens^[10,11]. *In vivo* depletion of MDSCs has been shown to improve T cell-mediated immune responses and suppress tumor growth in murine models^[12]. Therefore, depletion of MDSCs in tumor-bearing hosts has been proposed as a new approach for cancer immunotherapy. Colorectal carcinoma (CRC) ranks as the third most common cancer and the fourth leading cause of cancer-related deaths worldwide^[13]. In China, the incidence of CRC is increasing due to changes in diets and lifestyle^[14]. Numerous pathological factors and transformation of multiple genes are involved in tumor genesis and progression. It has been demonstrated that an immune-escape microenvironment shaped by chronic inflammation or autoimmune diseases is clearly associated with increased risk of CRC^[15]. A variety of therapeutic strategies, including conventional surgery, chemotherapy, radiotherapy and immunotherapy, alone or in combination, are currently available for the treatment of CRC patients. However, these therapies lead to different outcomes due to the different physical situation of each patient, which also construct the different tumor microenvironment through immune suppression^[16-18].

Therefore, it is critical that clinicians perform further analyses of immune-suppression status and establish individualized therapeutic strategies for CRC patients. Several studies have described the presence of abnormalities in the immune system of patients with CRC, including defective function of natural killer cells, DCs and Tregs^[19-21], but little is known about MDSCs in CRC.

In the present study, we investigated the frequency and characterized the phenotype of MDSCs in CRC patients and evaluated the clinical significance of MDSCs in CRC clinical status and outcome. The results might suggest a new strategy for efficient, individualized treatment of CRC.

MATERIALS AND METHODS

Patients

Peripheral blood and tumor tissue samples were collected from 49 CRC patients who underwent surgery in the Third People's Hospital of Wuxi, China from January 2010 to January 2011 after the approval by the Ethics Committee of the hospital. All patients were diagnosed with CRC for the first time, and had not been previously treated. Forty age-matched healthy donors were used as controls. Clinical parameters were acquired from the medical records of patients with the permission of the hospital.

Cell isolation from fresh tumor tissues and peripheral blood

Fresh tumor specimens were gently minced over a wire mesh screen to obtain a cell suspension. The cell suspension was layered over Ficoll-Hypaque (Amersham Biosciences, Sweden) and centrifuged at $500 \times g$ for 25 min. After density gradient centrifugation, mononuclear cells were collected and washed with RPMI 1640 media (Gibco, United States) containing 5% fetal bovine serum (FBS; Hyclone, United States) and 1% penicillin/streptomycin (Sigma-Aldrich, United States). Peripheral blood mononuclear cells (PBMCs) were also isolated by Ficoll-Hypaque density gradient centrifugation. PBMCs were collected, washed, and analyzed immediately. Viable cell counts were obtained using trypan blue dye.

Immunophenotypic analysis

Antibodies against the following proteins, purchased from BD Pharmingen or eBioscience, were used for flow cytometry: CD33, HLA-DR, CD3, CD14, CD19, CD56, CD11b, CD18, and CD1a. PBMCs (1×10^5) were suspended in phosphate-buffered saline (PBS) and incubated with antibodies for 30 min at 4 °C, and then washed twice with cold PBS. Fluorochrome-conjugated antibodies were used as isotype controls. Nonspecific staining was prevented by blocking Fc receptors. A Beckman Coulter flow cytometer equipped with Expo 32 software was used to analyze the stained cells. For MDSC marker analysis, the gate was set on the CD33⁺HLA-DR⁻ cell subset.

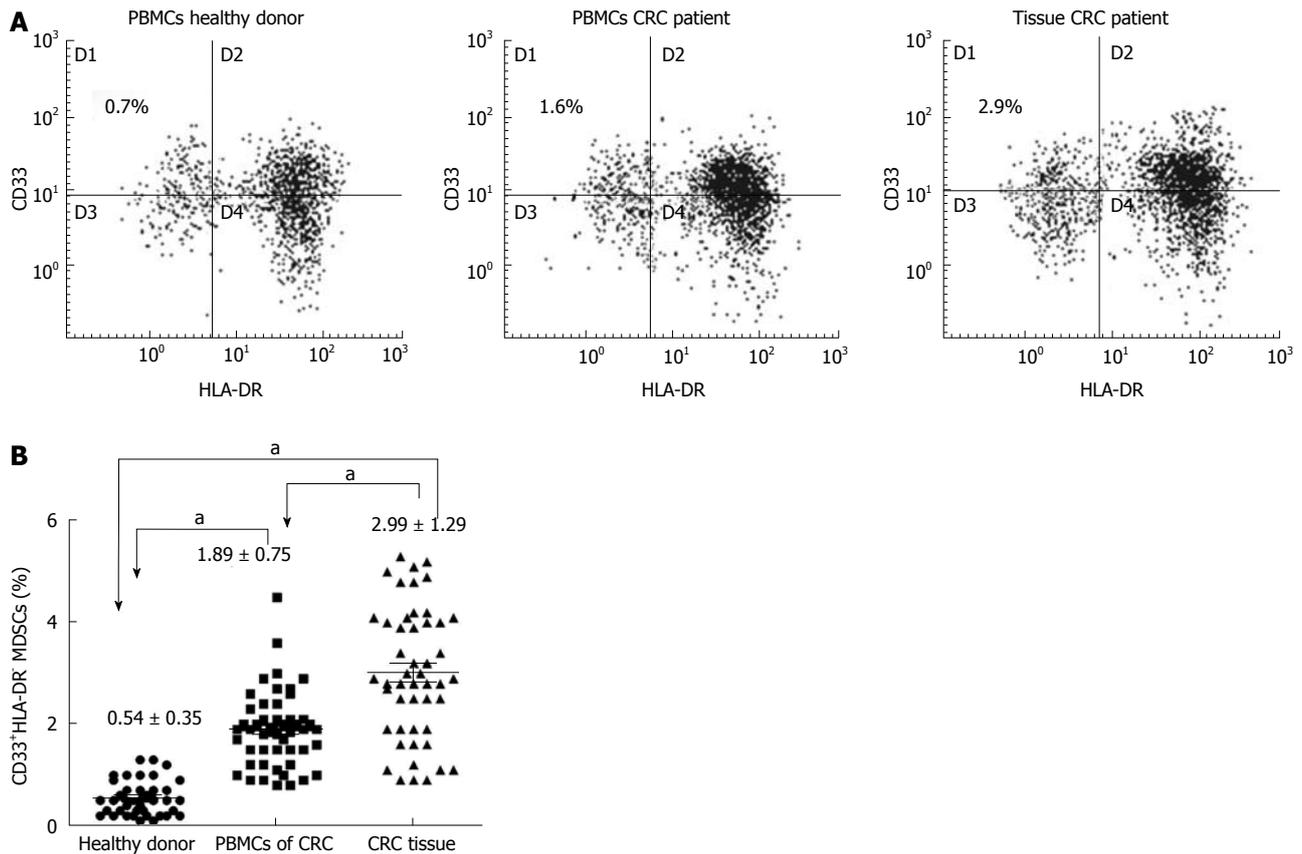


Figure 1 Circulating and tumor-infiltrating CD33⁺HLA-DR⁻ myeloid-derived suppressor cells in colorectal carcinoma patients. A: CD33⁺HLA-DR⁻ myeloid-derived suppressor cells (MDSCs) were present in the peripheral blood of colorectal carcinoma (CRC) patients and healthy donors, as well as in CRC tumor tissues; B: The percentage of CD33⁺HLA-DR⁻ MDSCs was significantly increased in the blood and tumor tissues of CRC patients ($^*P < 0.05$). PBMCs: Peripheral blood mononuclear cells.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, United States). Paired or unpaired Student's *t* tests, Wilcoxon signed-rank tests, and Pearson χ^2 tests were used as appropriate. *P* value < 0.05 was considered statistically significant.

RESULTS

Increased frequency of myeloid-derived suppressor cells in colorectal carcinoma patients

The percentage of MDSCs was analyzed in 49 CRC patients and 40 healthy donors. Circulating and tumor-infiltrating MDSCs were defined as the CD33⁺HLA-DR⁻ double-staining cell subset (Figure 1A). As shown in Figure 1B, MDSCs were present in the peripheral blood of both healthy donors and CRC patients, but the percentages were increased in CRC patients ($1.89\% \pm 0.75\%$) as compared with healthy donors ($0.54\% \pm 0.35\%$, $P < 0.05$). Infiltrating MDSCs were also found in CRC tumor tissues, and the percentage of MDSCs among tumor-infiltrating mononuclear cells ($2.99\% \pm 1.29\%$) was remarkably elevated as compared with that among PBMCs of both healthy donors and CRC patients ($P < 0.05$).

Phenotypic analysis of CD33⁺HLA-DR⁻ MDSC subset

We analyzed the cell surface markers of the CD33⁺HLA-DR⁻ MDSC subset in each CRC patient by immunostaining and flow cytometry. Cells were gated on CD33⁺HLA-DR⁻, and cell surface expression of CD3, CD14, CD19, CD56, CD11b, CD18, and CD1a was analyzed. As shown in Figure 2A, CD33⁺HLA-DR⁻ cells exhibited high expression of CD11b, CD18 and CD1a, but low expression of CD3, CD14, CD19 and CD56. An analysis of phenotypic differences in peripheral blood MDSCs between CRC patients and healthy donors showed that CD11b and CD18 expressions were increased on MDSCs in CRC patients (Figure 2B).

Relationship between percentage of MDSCs in CRC and clinical parameters

As shown in Table 1, patients were divided into high and low MDSC groups according to the median MDSC percentages in peripheral blood and tumor tissues. A statistical analysis showed that the proportion of MDSCs in CRC patient peripheral blood was correlated with distant metastasis ($P = 0.023$): CRC patients with distant metastases showed a higher level of circulating MDSCs than CRC patients without distant metastases. The pro-

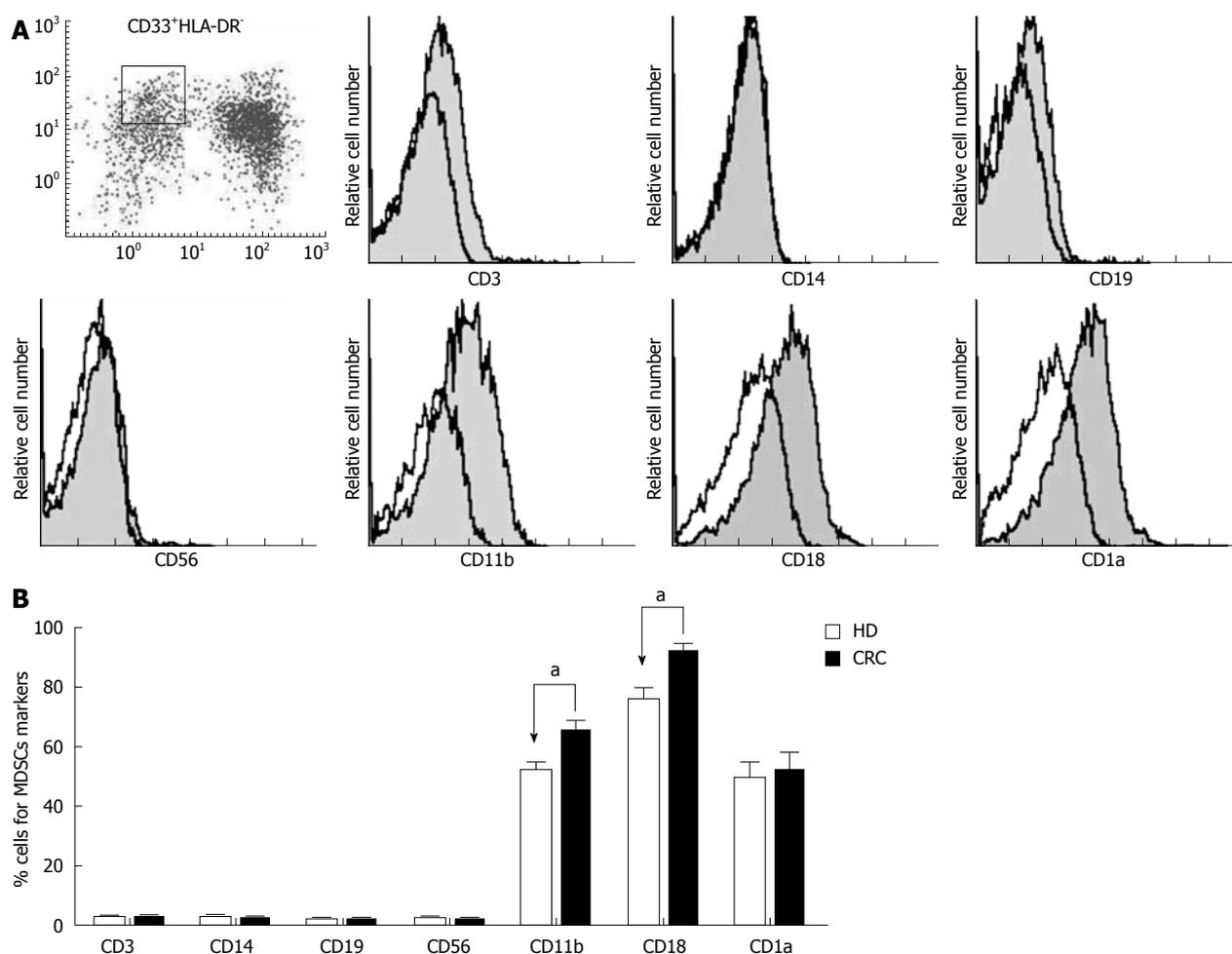


Figure 2 Phenotypic analyses of CD33⁺HLA-DR⁻ myeloid-derived suppressor cells in colorectal carcinoma patients and healthy donors. A: Cell surface markers of peripheral blood myeloid-derived suppressor cells (MDSCs), obtained by gating on the CD33⁺HLA-DR⁻ subset, were detected in colorectal carcinoma (CRC) patients; B: Percentages of cells positive for various markers on the CD33⁺HLA-DR⁻ subset in healthy donors (HD) and CRC patients (^a*P* < 0.05).

portion of MDSCs in tumor tissues was correlated with nodal metastasis, distant metastasis and tumor stages. CRC patients with metastases (nodal/distant) exhibited a higher degree of MDSC infiltration than those without metastases (*P* = 0.016/*P* = 0.047). Among stage IV CRC patients, 60% had a high-level infiltration of MDSCs in tumor tissues (*P* = 0.028), suggesting the involvement of MDSCs in tumor development.

DISCUSSION

Many studies have highlighted the role of MDSCs in cancer immune suppression^[3,4]. Infiltration of these cells in the tumor host is promoted by the tumor microenvironment, and tumor-associated expansion of MDSCs contributes to tumor escape from the immune system. The decline of immune function in the tumor leads to ineffective tumor treatment outcomes, due to the insufficient activity of antigen-specific antitumor responses and the possible extension of immune tolerance in the tumor host. Many studies have addressed MDSCs in solid tumors, but little is known about MDSCs in CRC. In this

study, we investigated circulating and tumor-infiltrating MDSCs in CRC patients, characterizing surface marker expression on MDSCs and demonstrating the clinical significance of MDSCs in CRC.

MDSCs represent 20%-30% of normal bone marrow cells and 1%-4% of all nucleated cells in the spleen^[22,23]. Our study showed that the CD33⁺HLA-DR⁻ subset was present at a very low proportion in the peripheral blood of healthy donors. CD33⁺HLA-DR⁻ MDSCs represent a homogeneous cell population that is significantly elevated in the peripheral blood of CRC patients. Furthermore, CD33⁺HLA-DR⁻ MDSCs were found at a relative high density in CRC tumor tissues compared with peripheral proportions in healthy donors or CRC patients. These data show that MDSCs expansion could be involved in CRC development, and suggest that the tumor tissue microenvironment might serve to promote MDSC expansion. Our data confirm previous studies demonstrating a dramatic expansion of MDSCs during tumor progression, infection, and even following immunization.

MDSCs are identified as a population of myeloid cells at earlier stages of differentiation^[4-6]. In CRC, these

Table 1 Correlations between myeloid-derived suppressor cells percentages and colorectal carcinoma clinical parameters

Clinical parameters	Cases	MDSC proportion in peripheral blood			MDSC proportion in CRC tumor tissues		
		Low	High	P value	Low	High	P value
Gender				0.252			1.000
Male	26	14	12		12	14	
Female	23	8	15		11	12	
Age (yr)				0.229			0.292
< 60	16	5	11		8	18	
≥ 60	33	17	16		15	18	
Tumor size (cm)				1.000			0.571
≤ 5	24	11	13		10	14	
> 5	25	11	14		13	12	
Tumor (T) status ¹				0.179			0.466
pT ₁	4	2	2		1	3	
pT ₂	10	6	4		5	5	
pT ₃	12	9	5		4	8	
pT ₄	23	7	16		13	10	
Nodal (N) status ²				0.248			0.016 ^a
N ₀	31	16	15		19	12	
N ₁	18	6	12		4	14	
Distant metastasis (M) ³				0.023 ^a			0.047 ^a
M ₀	22	14	8		14	8	
M ₁	27	8	19		9	18	
TNM stage				0.207			0.028 ^a
I	6	2	4		2	4	
II	12	7	5		8	4	
III	11	7	4		8	3	
IV	20	6	14		5	15	
Total	49	22	27		23	26	

¹Tumor status is classified as: pT₁, invasion of lamina propria or submucosa; pT₂, invasion of muscularis propria; pT₃, invasion of adventitia; pT₄, invasion of adjacent structures; ²Nodal status is classified as: N₀, no regional lymph-node metastasis; N₁, regional lymph-node metastasis; ³Distant metastasis is classified as follows: M₀, no distant metastasis; M₁, metastasis to cervical nodes, celiac nodes, and other distant metastases. ^aP < 0.05 was considered statistically significant. MDSC: Myeloid-derived suppressor cells; CRC: Colorectal carcinoma; TNM: Tumor, nodes, metastasis.

CD33⁺HLA-DR⁻ MDSCs displayed characteristics of immature myeloid cells, expressing high levels of CD33, CD11b, CD18 and CD1a, but a very low level of HLA-DR. Notably, the lineage markers CD3, CD14, CD19, and CD56 were not expressed in this subset. Thus, the phenotype of MDSCs in CRC is consistent with previous descriptions. Interestingly, however, we found that CD18/CD11b expression was considerably increased in MDSCs from CRC patients compared with those from healthy donors, indicating that the MDSCs in tumors might alter their expression of functional molecules according to the tumor microenvironment. Because CD18/CD11b (also known as Mac-1, CR-3) is critical for cell adhesion and migration^[24,25], MDSCs in CRC might contribute to invasion and metastasis.

We further explored the clinical significance of circulating and tumor-infiltrating MDSCs in CRC. We found that the percentage of MDSCs in the peripheral blood of CRC patients was correlated with distant metastasis, whereas the percentage of MDSCs in tumor tissue was correlated with nodal metastasis, distant metastasis, and tumor stage. These data revealed the clinical significance of MDSCs in CRC. Previous studies have indicated that the main action of MDSCs in tumors is suppression of antigen-specific immune responses. Our demonstration that CD18/CD11b is up-regulated on tumor-associated MDSCs is consistent

with the correlation between MDSC expansion and metastasis, indicating that MDSCs may play an important role in tumor invasion and metastasis. MDSC infiltration was also found to correlate with CRC tumor stage. Taken together, these observations suggest that MDSCs in CRC may substantially contribute to immune invasion, thereby promoting the tumor development.

In summary, we evaluated the frequency, phenotype and clinical significance of MDSCs in CRC. The increased frequency of MDSCs in CRC likely plays an important role in tumor metastasis and progression. Our study is of considerable significance for developing immunotherapeutic strategies *via* targeting and eliminating MDSCs in CRC.

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COMMENTS

Background

In the tumor microenvironment, the principal "mission" of immune cells is to execute an antitumor program, but a portion of such cells become confederates

of the tumor. Myeloid-derived suppressor cells (MDSCs) are a large group of myeloid cells comprising immature macrophages, granulocytes, and dendritic cells (DCs), as well as myeloid cells at earlier stages of differentiation. Recent studies have shown that MDSCs can inhibit proliferation and activation of T-cell and suppress maturation of DCs, which together contribute to the negative regulation of immune responses and the promotion of immune escape of tumors and pathogens. MDSCs have been extensively studied in solid tumors, but little is known about MDSCs in colorectal carcinoma (CRC).

Research frontiers

In humans, the phenotype of MDSCs has been a matter of some controversy, but it is now generally agreed that MDSCs are defined as CD11b- and CD33-positive cells that lack mature myeloid and lymphoid cell markers and do not express the MHC class II molecule, HLA-DR. *In vivo* depletion of MDSCs has been shown to improve T cell-mediated immune responses and suppress tumor growth in murine models. Therefore, depletion of MDSCs in tumor-bearing hosts has been proposed as a new approach for cancer immunotherapy. Several studies have described the presence of abnormalities in the immune system of patients with CRC, including defective function of natural killer cells, DCs, and Tregs, but little is known about MDSCs in CRC patients.

Innovations and breakthroughs

In this study, the authors investigated the circulating and tumor-infiltrating MDSCs in CRC patients and examined the expression of molecules characteristic of MDSCs. A considerable increase was found in the percentage of CD33⁺HLA-DR⁺ MDSCs in the peripheral blood and tumor tissues of CRC patients. The expanded CD33⁺HLA-DR⁺ subset exhibited immature myeloid cell markers, but not lineage markers, and showed up-regulation of CD18/CD11b expression as compared with MDSCs from healthy donors. These data demonstrated the existence of an MDSC population in CRC patients; further studies showed that the proportion of MDSCs in the peripheral blood and tumor tissues of CRC patients was correlated with nodal/distant metastases and tumor stage, strongly suggesting the involvement of MDSCs in CRC tumor development.

Applications

The characterization of the frequency and phenotype of MDSCs in CRC presented in this article suggests the clinical significance of circulating and tumor-infiltrating MDSCs, and it may provide new insights into the CRC immunotherapy targeting MDSCs.

Peer review

In the present paper, the authors evaluated the frequency and clinical significance of MDSCs in human CRC and they found that this population of cells was considerably increased in CRC as compared with the healthy donors. Moreover, they showed that in CRC peripheral blood, the MDSCs frequency was correlated to distant metastasis whereas, in tumor tissues the percentage was correlated to nodal and distant metastasis and tumor stages. Finally, the authors speculate that their study could have clinical implication contributing to the identification of new target for CRC immunotherapy. A major concern is related to the definition of the MDSC population. The authors have defined MDSCs as a large group of myeloid cells consisting of immature macrophages, granulocytes and dendritic cells as well as myeloid cells at earlier stages of differentiation. The description itself is not a definition but a "Pandora box". Each cell type has its own identity and specificity. Two or more cell types interact in unpredictable ways, and in the absence of a clear definition, each observation has little value. Given these premises, the attempt to clarify the confused MDSC issue by the authors is respectable, and their results, albeit flexible, are worth publication.

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Protective effect of nitric oxide on hepatopulmonary syndrome from ischemia-reperfusion injury

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Abstract

AIM: To evaluate immunological protection of nitric oxide (NO) in hepatopulmonary syndrome and probable mechanisms of ischemia-reperfusion (IR) injury in rat liver transplantation.

METHODS: Sixty-six healthy male Wistar rats were randomly divided into three groups (11 donor/recipi-

ent pairs). In group II, organ preservation solution was lactated Ringer's solution with heparin 10 000/ μ L at 4 °C. In groups I and III, the preservation solution added, respectively, L-arginine or N^G-L-arginine methyl ester (L-NAME) (1 mmol/L) based on group II, and recipients were injected with L-arginine or L-NAME (50 mg/kg) in the anhepatic phase. Grafted livers in each group were stored for 6 h and implanted into recipients. Five rats were used for observation of postoperative survival in each group. The other six rats in each group were used to obtain tissue samples, and executed at 3 h and 24 h after transplantation. The levels of alanine aminotransferase (ALT), tumor necrosis factor (TNF)- α and NO metabolites (NOx) were detected, and expression of NO synthase, TNF- α and intercellular adhesion molecule 1 (ICAM-1) was examined by triphosphopyridine nucleotide diaphorase histochemical and immunohistochemical staining.

RESULTS: By supplementing L-arginine to strengthen the NO pathway, a high survival rate was achieved and hepatic function was improved. One-week survival rate of grafted liver recipients in group I was significantly increased (28.8 ± 36.6 d vs 4 ± 1.7 d, $P < 0.01$) as compared with groups II and III. Serum levels of ALT in group I were 2-7 times less than those in groups II and III ($P < 0.01$). The cyclic guanosine monophosphate (cGMP) levels in liver tissue and NOx in group I were 3-4 times higher than those of group II after 3 h and 24 h reperfusion, while in group III, they were significantly reduced as compared with those in group II ($P < 0.01$). The levels of TNF- α in group I were significantly lower than in group II after 3 h and 24 h reperfusion ($P < 0.01$), while being significantly higher in group III than group II ($P < 0.01$). Histopathology revealed more severe tissue damage in graft liver and lung tissues, and a more severe inflammatory response of the recipient after using NO synthase inhibitor, while the pathological damage to grafted liver and the recipient's lung tissues was signifi-

cantly reduced in group I after 3 h and 24 h reperfusion. A small amount of constitutive NO synthase (cNOS) was expressed in liver endothelial cells after 6 h cold storage, but there was no expression of inducible NO synthase (iNOS). Expression of cNOS was particularly significant in vascular endothelial cells and liver cells at 3 h and 24 h after reperfusion in group II, but expression of iNOS and ICAM-1 was low in group I. There was diffuse strong expression of ICAM-1 and TNF- α in group III at 3 h after reperfusion.

CONCLUSION: The NO/cGMP pathway may be critical in successful organ transplantation, especially in treating hepatopulmonary syndrome during cold IR injury in rat orthotopic liver transplantation.

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Key words: Nitric oxide; Nitric oxide synthase; Immunoregulatory; Hepatopulmonary syndrome; Ischemia-reperfusion injury; Orthotopic liver transplantation

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INTRODUCTION

Recent research has shown that nitric oxide (NO) plays an important physiological role as a messenger in an autocrine or paracrine form. NO can influence vascular permeability, regulate vascular tone, inhibit adhesion and aggregation of leukocytes and endothelial cells, regulate platelet function, and improve graft microcirculation, thus easing vasospasm to reduce the incidence of vascular crisis^[1-5]. Therefore, in this study, we established animal models of cold ischemia-reperfusion (IR) injury in rat orthotopic liver transplantation. We were able to investigate the effect of NO on hepatopulmonary syndrome and its possible immunomodulatory mechanisms during IR injury in rat orthotopic liver transplantation by enhancing or inhibiting the NO/Cyclic guanosine monophosphate (cGMP) pathway.

MATERIALS AND METHODS

Materials

Male Wistar rats weighing 180-260 g were purchased from Shanghai Experimental Animal Centre (Chinese Academy of Sciences). L-arginine and N^G-L-arginine methyl ester (L-NAME) were from Sigma, and intercellular

adhesion molecule 1 (ICAM-1) (AT-29), mouse anti-rat single cloned antibody, tumor necrosis factor (TNF)- α , polyclonal rabbit anti-rat antibody, and the avidin-biotin complex (ABC) immune staining kit were purchased from Beijing Zhongshan Biotechnology Company.

Experimental methods

Experimental design: The 66 healthy male Wistar rats were randomly divided into three groups (11 donor/recipient pairs). Liver grafts were placed in different organ preservation solutions at 4 °C and stored for 6 h, and then implanted into recipients. In group II, organ preservation solution was lactated Ringer's solution with heparin 10 000/ μ L at 4 °C. In groups I and III, the organ preservation solution added, respectively, L-arginine or L-NAME (1 mmol/L) based on group II, and recipients were injected with L-arginine or L-NAME (50 mg/kg) through the penis dorsal vein in the anhepatic phase. The levels of alanine aminotransferase (ALT), TNF- α , and NO metabolites (NO_x) in the serum of recipients were detected, and expression of NO synthase was examined. The expression of TNF- α and ICAM-1 was observed by triphosphopyridine nucleotide (NADPH) diaphorase histochemical and immunohistochemical staining.

Orthotopic liver transplantation: All rats were anesthetized with methoxyflurane gas. All procedures described below were approved by the Committee for Animal Care and Usage for Research. Orthotopic liver transplantation was performed according to Harihara's three-cuff technique with minor modifications, as previously reported^[6-11], in which the suprahepatic vena cava was reconstructed by the Cuff method, along with the infrahepatic vena cava (IVC) and the portal vein (PV). The bile ducts were internally stented with a polyethylene stent.

Specimen measurement: The blood samples were obtained postoperatively via the tail vein at 3 h and 24 h after perfusion, or via the PV or IVC in the recipients being killed or those undergoing liver biopsy, and then centrifuged at 3000 r/min at 4 °C for 10 min. The supernatant was rapidly frozen and immediately stored at -80 °C. NO_x were determined by the improved Griess method, and TNF- α was examined according to the MTT assay. Tissue levels of cGMP were detected by radioimmunoassay, protein concentration test with application of trichloroacetic acid solution and 200 g/L SDS protein precipitation method. Samples of liver and lung tissues from rats being killed or biopsied were immediately stored in liquid nitrogen, and kept frozen at -80 °C. Histopathological sections of the grafted liver were fixed in 100 mL/L formalin and prepared with hematoxylin and eosin staining for routine light microscopy.

Triphosphopyridine nucleotide diaphorase staining: The grafted liver or lung specimens were fixed in 40 g/L paraformaldehyde, 4 g/L picric and 0.1 mol/L sodium phosphate buffer (PBS), pH 7.4, for 4 h at 4 °C. Subse-

Table 1 Effects of serum alanine aminotransferase, guanosine monophosphate levels in grafted liver, serum nitric oxide metabolites, tumor necrosis factor- α activities and recipient survival on augmenting/inhibiting nitric oxide pathway¹

Group	Serum ALT (nkat/L)	cGMP protein levels in grafted (ng/L)	Serum NOx (nmol/g)	Serum TNF- α (ng/L)	Weekly survival rate (%); survival days (mean \pm SE)
I					80 ^b ; 28.8 \pm 36.6 ^b
3 h	837 \pm 15 ^{b,f}	2.1 \pm 0.1 ^{b,f}	69.4 \pm 6.7 ^b	35.8 \pm 9.7 ^f	
24 h	456 \pm 53 ^{b,f}	2.3 \pm 0.2 ^{b,f}	22.6 \pm 3.1 ^{d,b}	24.4 \pm 4.0 ^b	
II					20; 4.0 \pm 1.7
3 h	1708 \pm 72	0.7 \pm 0.1	17.3 \pm 3.4	77.2 \pm 7.0	
24 h	2338 \pm 140 ^d	0.8 \pm 0.1	14.3 \pm 2.5	55.3 \pm 6.8	
III					0; 1.9 \pm 1.2 ^a
3 h	2833 \pm 988 ^b	0.5 \pm 0.1 ^b	12.0 \pm 2.5 ^a	180.2 \pm 20.5 ^b	
24 h	3100 \pm 253 ^b	0.4 \pm 0.1 ^b	9.9 \pm 2.4 ^a	136.6 \pm 11.8 ^b	

¹During cold ischemia-reperfusion injury in the rat orthotopic liver transplantation. ^a $P < 0.05$, ^b $P < 0.01$ vs group II; ^d $P < 0.01$ vs 3 h; ^f $P < 0.01$ vs group III. cGMP: Cyclic guanosine monophosphate; ALT: Alanine aminotransferase; TNF- α : Tumor necrosis factor- α ; NOx: Nitric oxide metabolites.

quently, specimens were frozen at -80°C until section cutting. Cryostat sections were immersed for 10 min in 0.1 mol/L PBS, pH 8.0, and were incubated for 40 min at 37°C in prewarmed solution of 0.1 mol/L PBS, pH 8.0, 3 g/L Triton X-100, 0.5 mmol/L nitroblue tetrazolium, and 1.0 mol/L NADPH. After being washed in 0.1 mol/L PBS, pH 7.4, sections were dehydrated with graded alcohol. Slides were rinsed in PBS and counter stained with fast red for 2 min, and coverslips were mounted on microscopic glass slides. Areas with a positive reaction for NADPH diaphorase were stained dark blue in the cytoplasm and red in the nucleus.

Immunohistochemistry: Immunohistochemical methods were used to examine the expressions of ICAM-1 (AT-29) with mouse anti-rat single cloned antibody, and TNF- α with polyclonal rabbit anti-rat antibody by the Avidin-Biotin complex method using an ABC immunostaining kit^[12]. Areas with a positive reaction were stained pale brown.

Statistical analysis

Data are presented as mean \pm standard error (SE). Comparisons among different groups of samples were made by two-tailed χ^2 test and F test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Survival

One-week survival rate of grafted liver recipients in group I was significantly increased (group I vs group II, 28.8 ± 36.6 d vs 4 ± 1.7 d, 80% vs 20%, $P < 0.01$), while the survival rate in group III recipients was significantly lower, compared with that of group II (group II vs group III, 0% vs 20%, $P < 0.05$).

Biochemical parameters

Following orthotopic liver transplantation, serum and tissue samples were assayed for ALT, NOx, TNF- α and cGMP at 3 h and 24 h after reperfusion (Table 1). The serum levels of ALT in group I were 2-7 times

less than in groups II and III ($P < 0.01$). The levels of cGMP and NOx in liver tissue in group I were 3-4 times higher than in group II at 3 h and 24 h after reperfusion, while in group III, they were significantly reduced compared with group II ($P < 0.01$). The levels of TNF- α in group I were significantly lower than those in group II at 3 h and 24 h after reperfusion ($P < 0.01$), while there were significantly higher in group III than group II ($P < 0.01$, Table 1).

Histopathology

The pathological damage in grafted liver and recipient lung tissues was significantly reduced in group I, which revealed almost normal liver sinusoidal lobular architecture, and mild degeneration of grafted liver cells at 3 h and 24 h after reperfusion (Figure 1A and C). In group III, which received NOS inhibitor, there was extensive cloudy swelling of hepatocytes, vacuolar degeneration, nuclear shrinkage, chromatin concentration, and marginalized or fragmented apoptotic bodies. We sometimes saw coagulation necrosis at the center of the central vein, small areas of spotty distribution, periportal inflammatory cell infiltration, and intrahepatic vascular thrombosis (Figure 1B). Severe structural damage to the recipient's lung tissues, a large amount of inflammatory cell infiltration, and intravascular thrombosis were seen at the same time (Figure 1D).

Triphosphopyridine nucleotide diaphorase histochemistry

Cells that were positive for NOS were mainly expressed in the liver and endothelial cells, and were stained dark blue in the arteries, veins and capillaries (Figure 1E).

Immunohistochemistry

A small amount of cNOS was expressed in endothelial cells in normal liver tissue after 6 h cold storage, but there was no expression of inducible NO synthase (iNOS). Expression of cNOS was particularly significant in vascular endothelial cells and hepatocytes at 3 h and 24 h after reperfusion in group II (Figure 1F), but expression of iNOS and ICAM-1 was low in group I (Table 2, Figure 1G and H). There was diffuse strong expression of

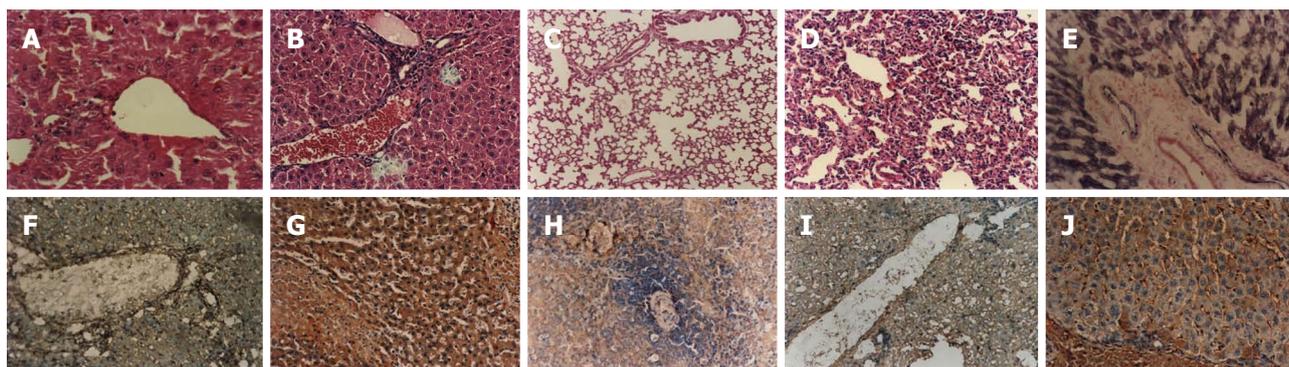


Figure 1 Hematoxylin and eosin staining and triphosphopyridine nucleotide-d staining and immunohistochemical staining for grafted liver and recipient's lung tissues. A, C: Grafted liver tissues and the recipient's lung tissues from group I of L-arginine revealed that the pathological damages of grafted liver and lung tissues significantly reduced after reperfusion 3 h (HE \times 132, \times 66); B, D: Grafted liver tissues and the recipient's lung tissues from group III of the NOS inhibitor after reperfusion 3 h showed extensive cloudy swelling of hepatocytes, vacuolar degeneration, nuclear shrinkage, chromatin concentration, and marginalized or fragmented apoptotic body and severe structural damage to the recipient's lung tissues, a large number of inflammatory cell infiltration, and intravascular thrombosis (HE \times 33, \times 66); E: NADPH diaphorase histochemistry staining of NO synthase and immunohistochemistry staining of cNOS for grafted liver tissues from group I after reperfusion 24 h (NADPH-d, \times 66); F: Expressions of cNOS in grafted liver tissues were particularly significant in vascular endothelial cells and hepatocytes after 24 h in group II [immunohistochemical staining (IH), \times 66]; G, H: Expressions of iNOS and ICAM-1 in grafted liver tissues were little in group I of L-arginine after reperfusion 24 h (IH \times 66, \times 66); I, J: Expression of ICAM-1 and TNF- α in grafted liver tissues were diffuse strong in group III of the NOS inhibitor after reperfusion 3 h (IH \times 66, \times 66). TNF- α : Tumor necrosis factor- α ; NO: Nitric oxide; NOS: Nitric oxide synthase; NADPH: Triphosphopyridine nucleotide; ICAM-1: Intercellular adhesion molecule 1; cNOS: Constitutive nitric oxide synthase; iNOS: Inducible nitric oxide synthase; HE: Hematoxylin and eosin.

Table 2 Intercellular adhesion molecule 1 expression in normal, storage and grafted livers

Groups	Portal area			Sinusoidal area		
	VE	BE	IC	HC	SLC	IC
Normal liver	±	-	-	-	-	-
Storage liver (6 h)	±	-	-	-	-	-
I	+	-	-	+	+	-
II	+	-	-	++	++	-
III	+++	-	-	++++	++++	-

Immunohistochemical results: "-", no significant staining; "±", a few staining; "+", focally stained; "++", diffusely stained; "+++ /++++", strongly diffusely stained. VE: Vascular Endothelium; BE: Biliary epithelium; IC: Cellular infiltrates; HC: Hepato cytes; SLC: Sinusoidal lining cell.

ICAM-1 and TNF- α in group III at 3 h after reperfusion (Table 2, Figure 1I and J).

DISCUSSION

IR injury during liver transplantation is a complex, multifactorial process in which numerous mediators and a variety of cells interact, leading to tissue damage. IR injury includes cold preservation injury, warm ischemia injury and reperfusion injury. Cold storage and warm reperfusion after restoration of blood are unavoidable steps in liver transplantation and all grafts undergo some degree of IR injury, thus leading to primary liver graft non-function or dysfunction^[13]. It is a hot issue at present and a major challenge in liver transplantation. So, which mechanisms lead to primary liver graft non-function or dysfunction? What role does the NO pathway play? How to improve quality and reduce the severity of early graft donor liver during IR injury? These are problems that need to be resolved in liver transplant surgery^[14].

NO has a modulatory function and is one of many organ-specific gaseous biological messengers newly discovered in recent decades in a variety of tissues or cells. NO modulates synthesis and secretion of a variety of immune mediators such as TNF- α , prostaglandin E₂, interleukin and interferon. It affects specific and nonspecific immune functions by mediating a variety of physiological and pathological phenomena, including endothelium-dependent vasodilatation, specific inhibition of proliferation of T lymphocytes, and adhesive accumulation of platelets. It plays an important extensive regulatory role in mammals. The immune regulation of NO is still not very clear in the physiology and pathology of solid organ transplantation.

L-arginine is an amino acid from which NO is synthesized. The NO synthesis pathway mainly refers to conversion of L-arginine to NO and L-citrulline under the action of NADPH cofactor and NOS in the endothelium. NO diffuses into both the vessel lumen and wall, thereby activating soluble guanylate cyclase to produce cGMP from GTP. NOx from the cGMP and non-cGMP pathway, either closely or remotely, directly or indirectly, play the role of second messengers on intracellular or extracellular target molecules^[15].

Our results demonstrated that early reperfusion rapidly depletes serum arginine, because of the explosive release of large amounts of arginase, thus leading to injury of liver parenchymal cells. The deletion of arginine decreases availability of tissue arginine with subsequent downregulation of endothelial NOS (eNOS). In contrast, it can protect liver graft against IR injury and improve the histopathological damage following liver transplantation by enhancement of arginine availability through arginase blockade, or by exogenous pathways to supplement NO donors or NO inhalation, or by endog-

enous pathways to induce the endogenous source downstream effector of eNOS (pharmacological treatment or ischemic preconditioning)^[16-18].

The non-cGMP pathway mainly affects the function of the substrate by acting on an iron-sulfur center protein. NO as an immune regulator mainly affects immune function through the non-cGMP pathway. Our study confirmed that ICAM-1 and TNF- α are also involved in the pathogenesis during reperfusion injury in liver transplantation^[19]. Although our previous studies demonstrated that cellular localization of NO varies according to the immunological status of liver transplantation, there are protective effects of hepatocyte-derived NO in the hyporesponsive status, but hepatic injury is probably triggered by overexpression of iNOS of the central lobule in the hyper-responsive state. NO has neither harmful nor beneficial effects, thus, it acts as a double-edged sword, mainly depending on its source and the experimental conditions^[20]. It is generally accepted that eNOS-derived NO is cell-protective by mediating vasodilatation, whereas iNOS mediates liver graft injury after transplantation^[21,22]. We showed that a small amount of eNOS was expressed in liver endothelial cells in normal liver tissue and after 6 h cold storage, but there was no expression of iNOS. Expression of cNOS was particularly significant in vascular endothelial cells and hepatocytes at 3 h and 24 h after reperfusion in group II.

We confirmed that NO plays an important protective role in organ preservation solutions by supplementing sufficient NO donors to enhance the NO/cGMP pathway. It might ameliorate function of the grafted liver and recipient's lung in hepatopulmonary syndrome, and significantly prolong the survivals of recipients after rat orthotopic liver transplantation. The levels of cGMP and serum NOx were significantly reduced after cold storage reperfusion, but there was no loss of NOS activity, suggesting that the reduction of NOx was probably the main reason for the accelerated destructive mechanism. We confirmed that NO has a significant beneficial effect on vascular function in heart and lung transplantation by strengthening NO/cGMP pathways. Therefore, by supplementing NO donors to enhance NO/cGMP pathways, it helps to balance NO/O₂ in the tendency to generate NO. The mechanisms underlying this protection involve preservation of the sinusoidal structure and maintenance of blood flow through the hepatopulmonary microcirculation^[23-26]. Our previous results demonstrated that NO of hepatocytes in the grafted liver can vary the immune status of the transplanted cells, but it is likely to have important immune protective roles for NO of hepatocytes in the hyporesponsive state^[27-30]. TNF- α is a wide range of polypeptide cytokines produced by a function of the body cells. Exogenous NO can significantly inhibit the macrophages stimulated by lipopolysaccharide to produce TNF- α ^[31]. Strengthening NO pathways significantly inhibits production and expression of TNF- α , but NOS inhibitors can contribute to increase the expression of TNF- α synthesized.

Apoptosis or programmed cell death is an active process that is controlled by specific genes without cell dissolution. Cytotoxic T lymphocytes (CTLs) leading to apoptosis can injury the target cells for lymphocyte-mediated cytotoxicity through the perforin/granzyme B and Fas/Fas-L lytic pathways^[32,33]. Apoptosis as a mechanism of cell death exists in acute rejection of liver transplantation, and it is related closely to the expression of perforin, transforming growth factor- β 1 and Fas-L^[34]. Thus, detecting expression of CTL-associated perforin and granzyme B genes provides two valuable markers to judge the effect of immunosuppression in acute rejection of liver transplantation^[35]. After reperfusion, the recipient's lymphocyte traffic to the graft is activated, and some infiltrating lymphocytes, hepatocytes, biliary endothelial and vascular endothelial cells are induced to apoptosis. We demonstrated that it can significantly reduce the expression of ICAM-1 in transplanted liver to enhance the NO pathway, and NOS inhibitors can increase the expression of ICAM-1 during cold IR in orthotopic liver transplantation. The results indicate that it is likely related to the induction of TNF- α in the expression of ICAM-1 during ischemia reperfusion after reconstruction of portal blood flow. However, we demonstrated that it was probably thrombin-induced expression of ICAM-1 in early reperfusion after liver transplantation. We confirmed the expression of ICAM-1 in liver transplantation during IR injury. It is likely to provide a more effective method for treatment of primary grafted liver dysfunction by the application of ICAM-1 monoclonal antibody 1A29F (ab)²^[36].

Hepatopulmonary syndrome is a disorder characterized by intrapulmonary vascular dilatation, leading to gas exchange abnormalities in the setting of liver disease. Hepatopulmonary syndrome is usually a progressive disease with worsening hypoxemia developing over time^[24,25,37]. The competitive inhibitor of NOS with substrate analogs such as L-NAME can reverse the local vasodilatation associated through inhibition of NOS. Administration of L-NAME abrogates the protection furnished by preconditioning^[38]. Inhibition of NO synthesis by a substance such as L-NAME leads to hypotension and an effect similar to the cytotoxic effect of endotoxins. In our study, there was both the most prominent biochemical and histological deterioration of graft liver and recipient's lung tissues, and a much higher mortality rate in the L-NAME group (group III). We observed that high survival in the L-arginine group (group I) was significantly increased, and 1-wk survival rate was 80% ($P < 0.01$), and one case showed long-term survival and was sacrificed on day 94. Survival of the recipients in group III was significantly reduced. One of the most important lethal causes in hepatopulmonary syndrome is hypoxia or hypoxemia, acute respiratory distress syndrome, or acute respiratory failure. This illustrated the remote protective effects of the NO cGMP synthesis pathway on the recipient's lungs.

In conclusion, we demonstrated the novel immuno-

modulation of NO and a crucial role of the NO/cGMP pathway during IR injury in rat orthotopic liver transplantation and the protective mechanisms of grafted liver and recipient's lung in hepatopulmonary syndrome. It is likely to be an effective way to improve postoperative antithrombotic therapy and the efficacy of liver transplantation by strengthening the NO/cGMP pathway.

COMMENTS

Background

Ischemia reperfusion (IR) injury during liver transplantation is a complex, multifactorial process in which numerous mediators and a variety of cells interact, leading to tissue damage. It is a hot issue at present and a major challenge in liver transplantation. In the field of solid organ transplantation, people do not know the immune suppression or immune stimulation of nitric oxide (NO) in acute rejection, chronic rejection, or the state of the immune response with low immune function.

Research frontiers

This research contributes to the theoretical basis of liver transplantation in clinical practice. The results improve the understanding of the molecular mechanisms of IR injury in liver transplantation.

Innovations and breakthroughs

Study results demonstrated novel immunoregulatory protective effects from IR injury in hepatopulmonary syndrome, involvement of intercellular adhesion molecule 1 and tumor necrosis factor- α in the pathogenesis after reperfusion injury in liver transplantation, and cellular localization of NO varies according to the immune status of the grafted liver. The study demonstrated the key role of the NO/cyclic guanosine monophosphate (cGMP) pathway during IR injury in liver transplantation. It is likely to be an effective way to provide a new pharmacological approach for preoperative antithrombotic therapy through strengthening the NO/cGMP pathway, which greatly improves the understanding of the molecular mechanisms of IR injury in liver transplantation.

Applications

Since 2003, the center has performed 97 cases of clinical liver transplantation. Eight cases complicated by hepatorenal syndrome were successfully reversed through enhancing the NO synthesis pathway by supplementing with an NO donor (L-arginine or alprostadil) in the early preoperative period. The authors suggest that acute renal failure might be successfully reversed to avoid further deterioration of multiorgan failure by the timely and decisive use of alprostadil, or by the repeated and sustained application of high-dose furosemide.

Terminology

IR injury is a complex, multifactorial process in which numerous mediators and a variety of cells interact, leading to tissue damage during liver transplantation. Hepatopulmonary syndrome is a disorder characterized by intrapulmonary vascular dilatation, leading to gas exchange abnormalities in the setting of liver disease.

Peer review

This is a good descriptive study in which authors evaluate immunological protections of NO in hepatopulmonary syndrome and the probable mechanisms of IR injury in rat liver transplantation. The results are interesting and suggest that the NO/cGMP pathway may be critical for successful organ transplantation, especially in treating hepatopulmonary syndrome during cold IR injury in rat orthotopic liver transplantation.

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Lymphogranuloma venereum proctosigmoiditis is a mimicker of inflammatory bowel disease

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to be an inflammatory bowel disease and subsequently identified as *C. trachomatis* proctosigmoiditis.

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Key words: Lymphogranuloma venereum; *Chlamydia trachomatis*; Proctitis; Proctosigmoiditis; Men who have sex with men; Crohn's disease; Inflammatory bowel disease

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Abstract

There has been an increasing prevalence of lymphogranuloma venereum (LGV) or *Chlamydia trachomatis* (*C. trachomatis*) cases among the men who have sex with men (MSM) population, particularly in Europe and North America. These cases may present with an incomplete or undisclosed history and proctosigmoiditis without characteristic adenopathy syndrome. During the initial evaluation and colonoscopy, there is a strong clinical and endoscopic suspicion of inflammatory bowel disease (IBD) by virtue of presentation and endoscopic and histological findings. The diagnosis of IBD is subsequently modified to LGV proctosigmoiditis when one or more of the following transpire: (1) there is failure of response to IBD therapy; (2) additional components of history (MSM/travel) may be identified; (3) return of initially performed *Chlamydia* antibody test is positive; and (4) response to antibiotics effective against *Chlamydia*. We describe three such cases initially suspected

INTRODUCTION

Lymphogranuloma venereum (LGV), an ulcerative sexually transmitted infection caused by *Chlamydia trachomatis* (*C. trachomatis*), has gained recent attention as a cause of hemorrhagic proctitis among men who have sex with men (MSM) in North America, Australia, United Kingdom, and the rest of Europe^[1,2]. This infection is found most frequently in tropical and subtropical areas in the world but it is rarely reported in developed countries. However, since 2003, outbreaks have been increasingly reported in Europe and the United States in MSM^[3-5]. Human immunodeficiency virus (HIV)-1 infection is the strongest risk factor for LGV infection and it presents in more than 70% of the subjects^[6]. The patients usually have high rates of promiscuity and high risk sexual behavior, often involving international sexual networks,

which correlates with the high rate of concomitant sexually transmitted diseases (STDs) observed^[2,5]. The atypical clinical presentation, the unawareness of physicians and patients in regards to the disease, and the lack of a routine diagnostic tests for LGV serovars of *C. trachomatis* have contributed to a delay in diagnosis and have caused misdiagnosis^[5]. We report three cases of LGV proctitis in MSM patients, initially misdiagnosed or suspected to be inflammatory bowel disease (IBD).

CASE REPORT

Case 1

A 30-year-old male presented with a three week history of passing bloody mucus per rectum several times a day. A stool exam was negative for ova and parasites. A limited sigmoidoscopy showed a cobble-stoned and granular mucosa with several ulcers in the distal 5-7 cm of the rectum (Figure 1A). Rectal biopsies showed patchy severely active colitis with distorted and mucodepleted crypts and lymphohistiocytic cryptitis reminiscent of Crohn's disease (Figure 1B). Immunostains for cytomegalovirus (CMV) and herpes simplex virus and special stains for fungi and acid-fast bacilli, performed retrospectively, were negative. The patient was given mesalazine 2.4 g/d which failed to produce any response after 1 wk. Due to the lack of response to IBD therapy and the clinical need to assess the remainder of the colon, a repeat full colonoscopy was performed. Colonoscopy showed similar findings in the rectum as seen previously, but the cecum showed a bright red nodule (Figure 1C). Biopsies from the cecal nodule showed classic histological features of Kaposi's sarcoma with spindle cells, vascular proliferation and extravasated erythrocytes (Figure 1D). Immunohistochemically, the cells were positive for CD31 and HHV-8. Random colonic biopsies away from the rectum and cecum were also performed which showed incidental intestinal spirochetosis. Additional clinical history revealed that the patient had unprotected anal intercourse while traveling through Europe three months prior. Microbiological studies were then performed which confirmed the presence of *C. trachomatis* by DNA probe and serologic testing. The specimen was sent the Center for Disease Control which detected *C. trachomatis* LGV DNA by sequencing of the outer membrane protein (*omp A*). The patient was subsequently found to be HIV positive (HIV RNA: 24 177 cop/mL) and was started on antiretroviral therapy. Doxycycline 100 mg twice a day for 21 d was given for his LGV proctitis and he had resolution of symptoms after 4 d.

Case 2

A 28-year-old HIV positive male presented with rectal bleeding and dyschezia. An anoscopy revealed an ulcer at the anal verge. Biopsies showed fragments of anal skin with ulceration, lymphoid tissue, and inflammatory granulation tissue histologically suspicious for an inflammatory bowel disease. Sigmoidoscopy was recommended to

assess for Crohn's disease and it showed an erythematous mucosa in the distal rectum with small areas of white exudates and heaped folds (Figure 2A). These biopsies showed severe lymphohistiocytic proctitis and cryptitis similar to Case 1 and again were suspicious for Crohn's disease (Figure 2B). Given the history of HIV positivity, LGV was also a clinical consideration and DNA probes and serologic testing were subsequently performed and were positive for *C. trachomatis*. Retrospectively, the proctitis and anal ulceration was deemed to be LGV-related and not Crohn's disease.

Case 3

A 42-year-old male with HIV and prior anal condyloma acuminatum presented with a 3 wk history of bright red blood per rectum. Colonoscopy was performed and showed moderate colitis in the rectum with friable and erythematous mucosa (Figure 3A). The biopsies showed moderately active proctitis with focal crypt distortion and cryptitis reminiscent of Crohn's-type IBD (Figure 3B). However, the clinical profile and the short duration of symptoms raised the possibility of LGV proctitis. Serologically, IgG antibodies against *C. trachomatis* were positive and the patient responded to antibiotic therapy for LGV.

DISCUSSION

Rectal bleeding or diarrhea may be caused by a variety of etiological factors including infections, diverticular disease, inflammatory bowel disease, ischemia, and neoplasia. Acute symptoms in young patients are generally linked to infectious colitis and often associated with travel, food, antibiotic intake, or sexual transmission. Common infectious agents include bacteria such as *Escherichia coli*, *Clostridium difficile*, *Salmonella*, *Shigella* and *Campylobacter*, viruses and parasites. When an infectious agent cannot be identified and/or symptoms persist, IBD is a clinical consideration.

Patients with LGV proctitis typically present with a rectal syndrome (> 90%) with moderate to severe ulcerative proctitis or proctocolitis with mucopurulent discharge, constipation, and tenesmus. Systemic symptoms like fever, malaise, and weight loss are also relatively common^[5]. LGV is typically a disease of lymphatic tissue. When the primary inoculation is in the penis or vagina, inguinal or femoral lymphadenitis develops, with a severe inflammatory reaction that suppurates and extends to adjacent structures. However, when the rectum is the site of primary inoculation, proctitis is the most common reaction and enlarged inguinal lymphadenopathies are absent^[6]. Simultaneous penile lesions are rarely seen. The ulcerative nature of LGV proctitis could enhance the transmission and acquisition of HIV and other STDs, as well as other blood-borne diseases like Hepatitis C^[5].

LGV proctitis must be routinely ruled out in MSM with unspecific anorectal complaints. A high index of suspicion is the mainstay to its early diagnosis in order

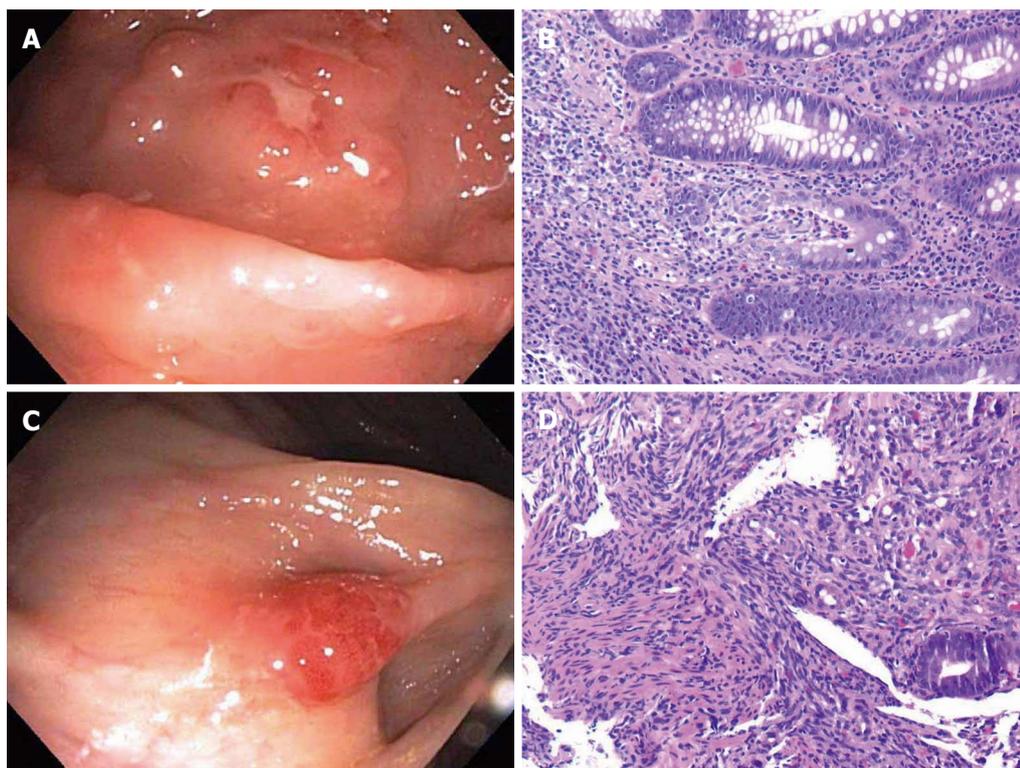


Figure 1 Endoscopic and microscopic findings (case 1).

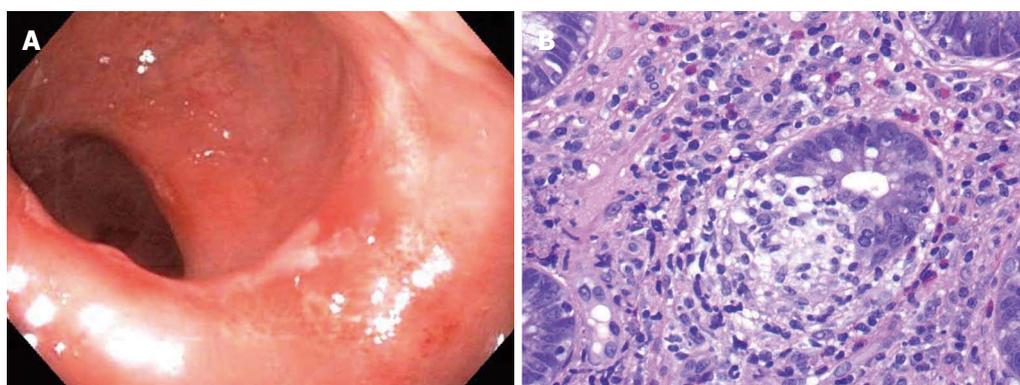


Figure 2 Endoscopic and microscopic findings (case 2).

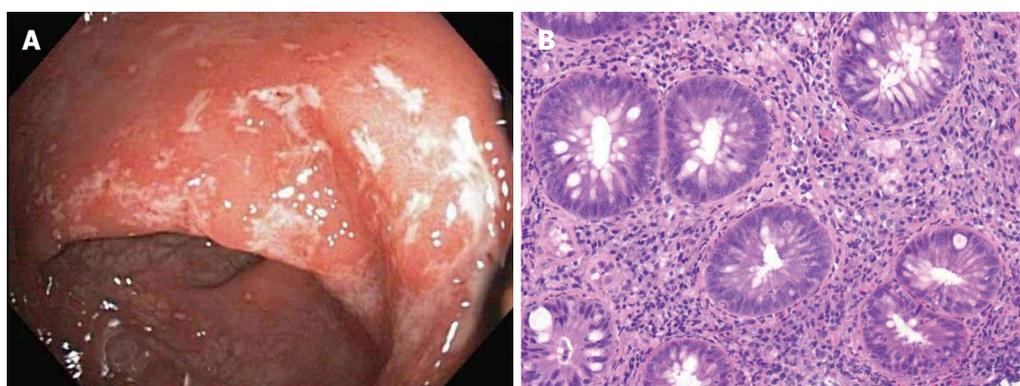


Figure 3 Endoscopic and microscopic findings (case 3).

to prevent avoidable complications. If left untreated, the disease evolves to a late stage, which is marked by fibrosis and strictures in the anogenital tract. Late complications include chronic genital ulcerations, anal fistulae, infertility, and disfiguring conditions such as genital elephantiasis and esthiomene^[4,6].

The endoscopic findings include mucopurulent exudates with a hyperemic and friable mucosa, multiple ulcers and erosions, and granulation tissue in the rectum. However, in some reports, up to 40% of men with LGV proctitis showed no anoscopic abnormalities^[5]. Also, in rare cases there were normal endoscopic findings^[7]. Hence, the endoscopic findings in LGV are nonspecific and can be easily misdiagnosed^[5].

Microscopically, the main changes are inflammatory changes, which can be easily misinterpreted even by experienced pathologists. Findings include lymphohistiocytic colitis, lymphoplasmacytic infiltrates, cryptitis, crypt abscesses, and crypt distortion.

These changes are often interpreted as IBD-type (particularly Crohn's-like) or described as non-specific inflammatory changes^[7,8].

LGV proctitis is caused by serovars L1, L2, and L3 of the obligate intracellular bacterium *C. trachomatis*, which is classified into 15 serovars based on immunogenic epitope analysis of the major outer membrane protein. Serovars A, B, Ba, and C are associated with ocular trachoma and strains D-K with genital tract disease and with inclusion conjunctivitis. Only serovars L1, L2, and L3 cause LGV, probably due to their tropism for the lymphatic system, in contrast to serovars A-K, which affect mucocutaneous tissue. The L2 serovar can be further classified into L2, L', L2a, and L2b, and the recently described L2c-L2g according to amino acid differences. The most common serovar causing LGV proctitis to date is L2b^[4,5].

A wide variation in the diagnosis and management of chlamydial infection in MSM currently exists which needs to be standardized^[9]. Historically, the laboratory diagnosis of LGV was based on culture, but this method is very tedious, has a low sensitivity (50%-80%), and cannot differentiate between different strains, therefore other detection methods have been applied. Serologic tests (complement fixation or immunofluorescence) have been used; however, these tests lack standardization, do not distinguish the different serotypes, nor do they differentiate past from present infections. Nucleic acid testing of clinical specimens has emerged as a promising technique that is both sensitive and convenient. Specialized laboratories such as the Center for Disease Control can further amplify sequences in the major outer-membrane protein and reliably distinguish among lymphogranuloma venereum serotypes^[5,6,10].

The diagnosis of LGV is challenging to clinicians because the clinical presentation may be nonspecific and specific laboratory procedures for its diagnosis require a high index of suspicion. The main pathological differential diagnosis of LGV proctitis includes other forms of infectious colitis and inflammatory bowel disease^[6].

Herpes, CMV, syphilis, and *Neisseria gonorrhoea* infections can also cause proctocolitis. Patients generally present with similar general symptoms such as anal discharge, pain, diarrhea, constipation, bloody stool, and tenesmus. Proctoscopic findings are again nonspecific and range from normal to erythema, mucosal friability, and surface erosions^[11]. Virally infected cells with herpes are histologically identified with molding and multinucleation of cells and the peripheralization of the chromatin. CMV infections have large cells with identifiable nuclear and cytoplasmic inclusions. These two viral infections are seen in a background of ulcerations with associated acute and chronic inflammation and granulation tissue and immunohistochemical markers are also helpful with identification. Syphilis is associated with a chronic inflammatory infiltrate rich in plasma cells and may also show granulomatous inflammation. With *Neisseria gonorrhoea*, as with *Chlamydia*, one may see evidence of cryptitis, crypt abscesses, and reactive epithelial changes^[12]. Since there are overlapping histological features with these two infectious processes, microbiologic studies become necessary.

There is considerable mimicry between IBD and LGV histologically^[8]. In IBD, crypt architectural distortion, the presence of a prominent increase in the cellularity of the lamina propria, and the development of well-formed granulomas can be seen. However, some of these pathologic features are often also seen in LGV proctitis. Furthermore, with more advanced LGV infection, transmural inflammation can ensue, further resembling Crohn's disease^[12]. There are no pathognomonic features of LGV, and this is why clinical suspicion along with microbiological identification in a patient with risk factors is crucial.

According to the current European and United States STD guidelines, the recommended treatment for LGV is doxycycline (100 mg orally twice a day for 21 d or as long as anorectal symptoms persist) in contrast to infection with other *Chlamydia* serovars, where only 1 wk (or a single azithromycin dose) is required. This cures the infection and prevents further tissue damage. An alternative regimen is erythromycin (500 mg orally four times daily for 21 d). However, this recommendation is not supported by randomized evidence in clinical trials, but by empirical data. Patients should be followed up until symptoms have disappeared and clinically reviewed at 3-6 wk. Likewise, sexual partners who have had unprotected contact with the patient within the previous 60 d of onset of clinical symptoms should be screened or empirically treated with an LGV regimen^[5].

In the absence of laboratory confirmation of L serovars, physicians are advised to treat possible cases presumptively for LGV and provide medical management of sexual partners^[1]. Patients should receive risk education counseling and be routinely screened for other STDs, especially HIV and hepatitis B and C^[5].

In conclusion, patients with *C. trachomatis* who present without classical history or symptoms and have isolated proctosigmoiditis may be confused clinically, endoscopically and histologically with inflammatory

bowel disease. When there is lack of response to IBD therapy and/or the clinical profile raises the potential for LGV, serological and DNA probe studies for *Chlamydia* are recommended. Awareness of its mimicry to IBD and prompt identification of *C. trachomatis* are crucial for treatment and prevention of further complications from this disease.

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Diagnosis in bile acid-CoA: Amino acid N-acyltransferase deficiency

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Abstract

Cholate-CoA ligase (CCL) and bile acid-CoA: amino acid N-acyltransferase (BAAT) sequentially mediate bile-acid amidation. Defects can cause intrahepatic cholestasis. Distinction has required gene sequencing. We assessed potential clinical utility of immunostaining of liver for CCL and BAAT. Using commercially available antibodies against BAAT and CCL, we immunostained liver from an infant with jaundice, deficiency of amidated bile acids, and transcription-terminating mutation in BAAT. CCL was normally expressed. BAAT expression was not detected. Immunostaining may facilitate diagnosis in bile-acid amidation defects.

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Key words: Amidation; Bile acid-CoA; Amino acid N-ac-

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INTRODUCTION

Conjugated bile acids are detergents essential in facilitating the enteric absorption of fats and of fat-soluble substances, among them several vitamins. They are synthesised in hepatocytes, which expel them into the lumen of the bile canaliculus *via* the transport protein bile salt export pump (BSEP). Conjugated bile acids are partially deconjugated during passage through the intestine. Bile acids are recovered from chyme and returned to the liver in portal venous blood; unconjugated bile acids are re-conjugated with glycine or taurine within hepatocytes before re-export into canalicular bile^[1]. That errors in synthesis or transport of bile acids may lead to intrahepatic cholestasis is well known^[1,2]. Errors in conjugation of bile acids with amino acids ("amidation"), a two-step process, also may cause usual cholestasis, with jaundice, but can also produce anicteric cholestasis manifest as malabsorption of fat-soluble vitamins, growth retardation, and pruritus. Few descriptions are published of children with bile-acid amidation defects and of mutations in genes that encode the enzymes responsible for amidation. Di-

agnosis can be elusive^[3]. We present our correlation of immunohistochemical, clinical-biochemistry, and genetic findings in a child with a bile-acid amidation defect and suggest approaches to diagnosis.

CASE REPORT

The first child of consanguine British parents of Pakistani heritage was born at term after an uncomplicated pregnancy. She was well until age 7 wk, when jaundice and pale stools were noted. Her liver and biliary tract were sonographically unremarkable. Clinical-laboratory findings included conjugated hyperbilirubinemia (total: direct bilirubin 106:67 $\mu\text{mol/L}$) with elevated serum alanine aminotransferase activity (378 IU/L), normal-range gamma-glutamyl transpeptidase (GGT) activity (30 IU/L), and hypercholanemia (total serum bile acids 282 $\mu\text{mol/L}$, expected < 14). Liver biopsy at age 8 wk found hepatocellular and canalicular cholestasis, with slight portal-tract fibrosis and a “neonatal hepatitis” pattern of focal giant-cell change of hepatocytes, with haemopoiesis. Canalicular-margin expression of BSEP and of ectoenzymes (alanine aminopeptidase, carcinoembryonic antigen, GGT) was normal. Transmission electron microscopy found changes of hepatocellular and canalicular cholestasis, without “Byler bile” or other abnormalities. Urine and plasma analysed by electrospray ionisation-mass spectroscopy^[1,3] contained di- and tri-hydroxy C24 bile acids, without amidated forms (Figure 1). A defect in cholate-CoA ligase (CCL) or bile acid-CoA: amino acid N-acyltransferase (glycine N-choleoyltransferase; BAAT) was hypothesised.

BAAT, encoding BAAT, was sequenced by routine techniques using DNA from peripheral-blood leucocytes^[4]. The results indicated that the patient was homozygous for the previously undescribed mutation c.415C > T/p.R139X in BAAT. Solute carrier family 27 (fatty acid transporter), member 5 (*SLC27A5*), encoding CCL, was not sequenced. Immunostaining with antibodies against CCL (HPA007292; Sigma, St Louis, MO, United States) and BAAT (ab97455; Abcam, Cambridge, United Kingdom), conducted using manufacturer-recommended protocols, found good expression of CCL and no expression of BAAT (Figure 2); controls, including liver of age-matched infants with cholestasis due to extrahepatic biliary atresia and to failure of BSEP expression, marked appropriately (reaction conditions described on request). The patient, now aged 7 years, receives ursodeoxycholic acid and fat-soluble vitamin preparations and despite slight hypercholanemia (total serum bile acids 77 $\mu\text{mol/L}$) is clinically well (height and weight 25th percentile for age, with good progress in school). Her only sibling, a sister, is without signs or symptoms of liver disease. The parents have declined genetic study of the sibling.

DISCUSSION

Bile salts within the biliary-tract and small-bowel lumina

exist principally as glyceryl or tauryl conjugates of primary bile acids. Bacteria within chyme and faeces deconjugate and dehydroxylate bile salts. The resulting secondary bile acids are taken up by the bowel and passed into venous effluent. They are then absorbed by hepatocytes and again conjugated with glycine or taurine in the process of amidation; newly synthesised bile acids also undergo amidation in the liver^[1].

Two enzymes, CCL [International Union of Biochemistry and Molecular Biology European Commission (EC) 6.2.1.7] in cytoplasm and BAAT (EC 2.3.1.65) in peroxisomes, act sequentially to amidate bile acids^[1,3]. Experimental data conflict on whether non-amidated bile acids are poorer substrates for BSEP, the protein that transports bile salts against a concentration gradient from hepatocyte cytoplasm into bile-canalicular lumen, than are amidated bile acids^[5,6]. Non-conjugated bile acids may back-diffuse from canalicular lumen into hepatocytes and into the space of Disse, impeding secretory efficiency, and excess of bile acids within hepatocytes may cause injury^[4]. Data on back-diffusion of non-amidated bile acids from bile-duct lumen into cholangiolar venous effluent, with drainage into portal-vein radicles and presentation to the liver lobule of such bile acids in excess (“cholehepatic shunting”), are not available. However, this process may conduce to hepatocellular overload with bile acids, and hence to hepatocellular injury.

Intrahepatic cholestasis has been described in association with amidation defects. Anicteric cholestasis, manifest as failure to thrive with malabsorption and fat-soluble vitamin deficiency, was found in Amish children homozygous for the mutation c.226A > G/p.M76V in BAAT^[4]. A preliminary description of children with three additional different mutations in BAAT, all in homozygous form, has appeared^[7]. Homozygous mutation in *SLC27A5* (c.1012C > T/p.H338Y) also has been associated with intrahepatic cholestasis in a prematurely born infant^[3]. To what extent exogenous factors - ontogenetic immaturity, parenteral alimentation, various drugs, or a genetic background that includes mutation in other cholestasis-associated genes^[3] - conduce to jaundice with amidation defects, expected *per se* to result in anicteric cholestasis^[8], remains to be determined. Our patient, who developed icterus, was born at term, was not subjected to parenteral alimentation, and received no drug treatment recognised as capable of precipitating cholestasis. Were intrahepatocytic concentrations of bile acids elevated (4), leading to damage to organelles and to cellular processes, with jaundice a non-specific sequela? The factors that predisposed to icterus in our patient must remain matter for speculation.

As reported in patients with defects of bile-acid amidation^[3,4], cholestasis in our patient was not associated with elevations in serum GGT activity even when conjugated hyperbilirubinemia was present. Normal-range serum GGT activity is a feature of disorders of bile-acid synthesis as well as of bile-acid amidation. It also characterises certain forms of intrahepatic cholestasis

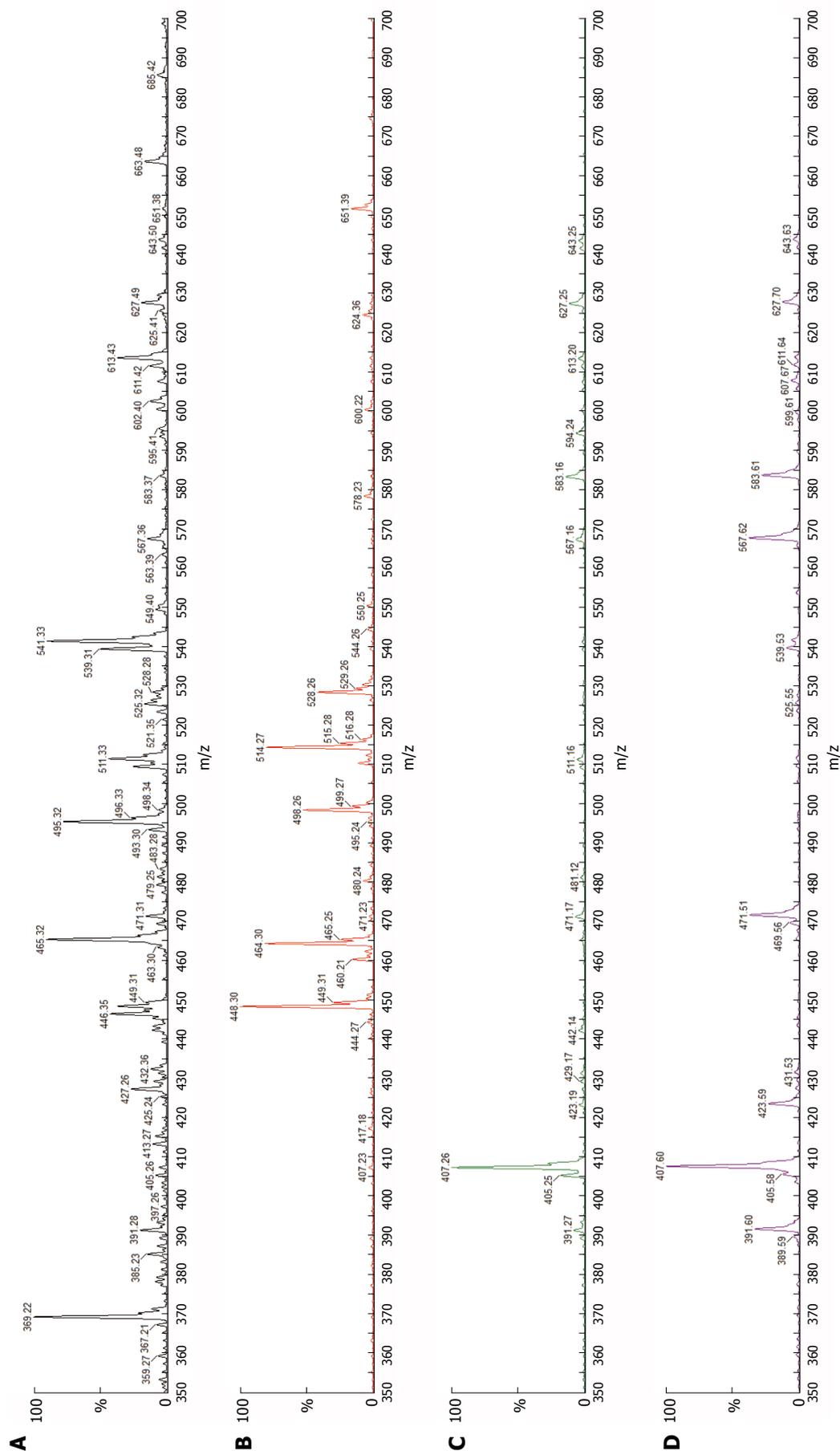


Figure 1 Bile acid mass spectra, urine. A: Normal (neither icterus nor known amidation defect); B: Deficiency of bile salt export pump (BSEP) (intrahepatic cholestasis with icterus); C: Deficiency of cholate-CoA ligase (described elsewhere³³); D: Deficiency of bile acid-CoA: amino acid N-acyltransferase (patient). Abscissa, mass-charge ratio (m/z); ordinate, intensity of ion current as percentage of most abundant peak in spectrum (%). From left to right, principal peak sites and the species represented are: 391: Unamidated chenodeoxycholic acid (CDCA); 407: Unamidated chenodeoxycholic acid (CDCA); 448: Glycine-conjugated CA; 471: Unamidated CDCA sulphate; 487: Unamidated CA sulphate; 498: Taurine-conjugated CA; 514: Taurine-conjugated CA; 528: Glycine-conjugated CDCA sulphate; 567: Unamidated CDCA glucuronide; 583: Unamidated CA glucuronide; 613: 27-Nor-cholestane pentol glucuronide; 643: Cholestane hexol glucuronide. In normal urine (A), bile-acid and bile-alcohol peaks are just detectable. In urine from a patient with BSEP deficiency (B), increased excretion of glycine- and taurine-conjugated bile acids is apparent. Large quantities of non-conjugated species are present in both cholate-CoA ligase deficiency (C) and BAAT deficiency (D); differences between the two in size and distribution of this and other peaks are not apparent.

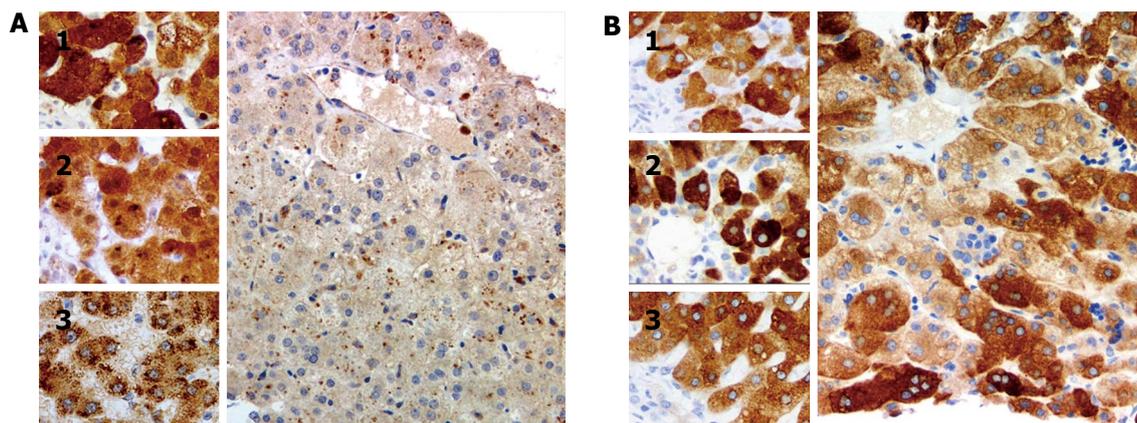


Figure 2 Photomicrographs of liver immunostained for enzymes that subserve bile-acid amidation. A: Main image: Core needle biopsy specimen, patient liver (age 57 d); inset, control livers (1, infant, matched for age with patient, with cholestasis and extrahepatic biliary atresia; 2, infant, matched for age with patient, with intrahepatic cholestasis in setting of absence of bile salt export pump (BSEP) expression; and 3, adult without cholestasis). The patient liver lacks immunohistochemically demonstrable bile acid-CoA: amino acid N-acyltransferase (BAAT). Granular cytoplasmic marking, suggestive of peroxisomal location, is found in (3); marking is more diffuse in the livers of age-matched infants; B: Tissue specimens as in (A). Diffuse cytoplasmic marking for cholate-CoA ligase (CCL) is seen in patient liver (main image) and in control livers (1, infant, matched for age with patient, with cholestasis and extrahepatic biliary atresia; 2, infant, matched for age with patient, with intrahepatic cholestasis in setting of absence of BSEP expression; and 3, adult without cholestasis). A, anti-BAAT antibody, and B, anti-CCL antibody (see principal text for sources); in all images, reaction development with EnVision proprietary technique (Dako UK, Ely, United Kingdom), haematoxylin counterstain, original magnification 200 ×.

ascribed to deficiency of BSEP expression or function, abnormalities in canalicular-membrane composition, and abnormalities of intracellular trafficking of canalicular-membrane components^[2]. Evidence indicating such disorders, either clinical or histopathologic, was not identified in our patient.

Little histopathologic information is available concerning hepatobiliary disease in bile-acid amidation deficiency. Whether the appearances of BAAT deficiency differ from those of CCL deficiency in routinely stained sections, for example, is still an open question. The same is true for differences in ultrastructural features; too few instances have been described to allow discrimination. As urine and plasma bile-acid spectra and chromatograms in amidation-deficiency disease do not vary between CCL deficiency and BAAT deficiency (personal observations, PTC; *cf.* Figure 1), screening of both *SLC27A5* and *BAAT* for variations from canonical sequence is at present necessary to distinguish between forms of amidation defect.

Our findings demonstrate that immunohistochemical study can detect absence of BAAT expression. They correlated well with genetic findings that suggested that BAAT synthesis was not to be expected in our patient. Lack of BAAT expression thus may constitute a strong indication for evaluation of *BAAT* for mutation.

We believe that immunohistochemical screening of liver tissue for CCL and BAAT expression may identify instances of bile-acid amidation deficiency disease in patients with normal-range serum GGT activity and either icteric or anicteric cholestasis. To do so admittedly may not identify all such instances; defects in protein function without defects in protein expression are to be expected, particularly with mutations that substitute one amino-acid residue for another^[3]. However, immunostaining is rou-

tinely practiced, whereas only a few laboratories speciate bile acids in plasma and urine or offer analysis of genes that encode enzymes involved in bile-acid handling. In addition, liver biopsy continues to be an approach widely used in the evaluation of neonatal cholestasis, permitting repeated interrogation of liver-biopsy material as application of diagnostic algorithms suggests candidate disorders. Results on immunostaining of liver-biopsy or hepatectomy specimens from patients with hepatobiliary disease and normal-range serum GGT activity thus may guide both clinical-biochemistry and genetic investigations, speeding definitive diagnosis.

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Giant choledocholithiasis treated by mechanical lithotripsy using a gastric bezoar basket

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Abstract

Mechanical lithotripsy (ML) is usually considered as a standard treatment option for large bile duct stones. However, it is impossible to retrieve oversized stones because the conventional lithotripsy basket may not be able to grasp the stone. However, there is no established endoscopic extraction method for such giant stone removal. We describe a case of successful extraction of a 4-cm large stone using a gastric bezoar basket. A 78-year-old woman had suffered from upper abdominal pain for 20 d. Contrast-enhanced computed tomogram revealed a 4-cm single stone in the distal common bile duct (CBD). Endoscopic stone retraction was decided upon and endoscopic papillary balloon dilation was performed using a large balloon. An attempt to capture the stone using a standard lithotripsy basket failed due to the large stone size. Subsequently, we used a gastric bezoar basket to successfully capture

the stone. The stone was fragmented into small pieces and extracted. The stone was completely removed after two sessions of endoscopic retrograde cholangiopancreatography; each of which took 30 min. No complications occurred during or after the procedure. The patient was fully recovered and discharged on day 11 of hospitalization. ML using a gastric bezoar basket is a safe and effective retrieval method in select cases, and is considered as an alternative nonoperative option for the management of difficult CBD stones.

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Key words: Giant choledocholithiasis; Mechanical lithotripsy; Bezoar basket; Common bile duct stone; Endoscopic papillary balloon dilatation

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INTRODUCTION

Since endoscopic sphincterectomy (EST) was introduced for bile duct stone extraction, up to 90% of bile duct stones could be removed by using EST and standard endoscopic maneuvers^[1]. However, bile duct stone removal can be challenging in patients with large stones (> 12 mm), multiple stones, barrel-shaped stones, and tapering or tortuosity of the distal common bile duct (CBD)^[2]. Mechanical lithotripsy (ML) is an established alternative in

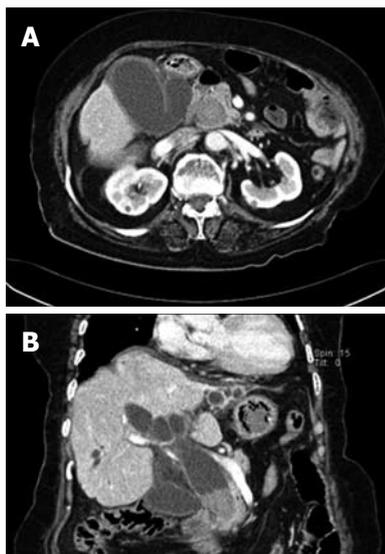


Figure 1 Computed tomogram revealed a huge stone in the distal common bile duct. Marked dilation of the gallbladder and bile duct was also seen. A: Axial view; B: Coronal view.

cases of large CBD stones that cannot be removed using standard endoscopic retrieval methods. Large and hard concretions, however, often make ML difficult. There are several nonoperative options available^[1-5], but there is no established technique for difficult large stones.

Here, we report a case of a giant CBD stone that was retrieved by ML using a gastric bezoar basket.

CASE REPORT

A 78-year-old woman was referred to our institution due to upper abdominal pain and vomiting that occurred 20 d prior to admission. Blood pressure was 100/50 mmHg, heart rate was 96 beats/min, and temperature was 36 °C. There was tenderness to palpation in the right upper quadrant of the abdomen, but rebound tenderness was not documented. Laboratory tests showed: white blood cells, 25 600/mm³; neutrophils, 97.2%; hemoglobin, 14.5 g/dL; total bilirubin, 5.5 mg/dL; aspartate aminotransferase, 104 IU/L; alanine aminotransferase, 87 IU/L; serum alkaline phosphatase, 510 IU/L; and γ -glutamyl transpeptidase, 365 IU/L.

Contrast-enhanced computed tomogram (CT) of the abdomen revealed a single 4-cm stone in the distal CBD, with marked and diffuse dilation of the entire biliary tree (Figure 1). Endoscopic retrograde cholangiopancreatography (ERCP) was urgently performed because the patient's mental state and vital signs had deteriorated on the day of admission. We identified an opening of a choledochoduodenal fistula near the ampulla of Vater where a large amount of pus was emitted. A 4.0 cm \times 3.2 cm filling defect was noted in the distal CBD on the cholangiogram (Figure 2). Nasobiliary drainage through the fistulous tract was performed endoscopically. After the first session, the signs of systemic infection improved as well as the patient's performance status, and the second session

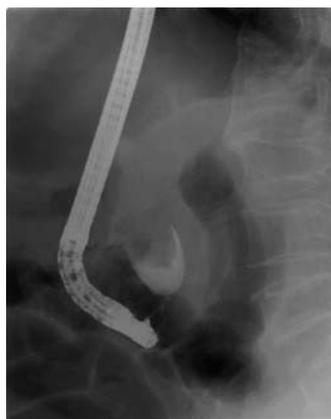


Figure 2 Endoscopic retrograde cholangiopancreatogram showed a large filling defect measuring 4.0 cm \times 3.2 cm. The proximal portion of bile duct was not opacified.



Figure 3 Basket for gastric bezoar (left) is larger than a conventional basket used for mechanical lithotripsy (right).

of ERCP was performed on day 3 in hospital, to retrieve the stone.

Instead of EST, endoscopic papillary balloon dilation was performed (for 60 s, one session) using a large balloon catheter, 15 mm in diameter (Controlled Radial Expansion balloon; CRE[®]; Boston Scientific, Natick, MA, United States) for esophageal dilation. ML using a usual lithotripter (Stonebuster[®], 40 mm-inner diameter; Medi-Globe GmbH, Achenmühle, Germany) was tried and failed to capture the stone due to its large size. Subsequently, we were able to capture the stone using a gastric bezoar basket (Stonebuster[®], 60 mm-inner diameter; Medi-Globe GmbH) that was larger than the usual basket for ML (Figure 3). The captured stone was broken into fragments with a mechanical lithotripter (Memory Eight Wire Basket 2 cm \times 4 cm (MB5-2X4-8[®]; Cook Medical Inc., Winston Salem, NC, United States) (Figure 4). Then, we performed endoscopic nasobiliary drainage to prevent cholangitis induced by the fragmented stones. On the 6th day in hospital, follow-up cholangiogram revealed multiple small filling defects within the CBD (Figure 5). All of the stone fragments were extracted with the basket, and we confirmed complete clearance of the CBD by a balloon-occluded cholangiogram (Figure 6). The patient

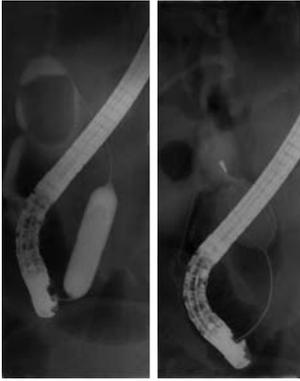


Figure 4 Endoscopic balloon dilation using a large balloon and mechanical lithotripsy. The balloon was inflated gradually to a diameter of 15 mm across the papilla and the ballooning was sustained for 60 s, and the bezoar basket captured the stone completely. The stone was fragmented by mechanical lithotripsy using a bezoar basket.



Figure 5 Follow-up endoscopic retrograde cholangiopancreatogram disclosed remnant stone fragments.



Figure 6 Remnant stone fragments were extracted by the basket and balloon occluded cholangiogram showed complete duct clearance.

was fully recovered and discharged on the 11 th day of hospitalization.

DISCUSSION

Since ML was first introduced by Riemann *et al*^[6] for the retrieval of CBD stones, ML has been the method of

choice in cases of large or impacted stones. Additionally, in cases with concomitant narrowing of the distal CBD from which it is impossible to remove stones by EST, ML is the simplest, most cost-effective, and safe procedure^[1-5]. For large stones > 1 cm, there are several therapeutic options other than ML. Endoluminal electrohydraulic lithotripsy (EHL) *via* the peroral or percutaneous route^[3,7,8], extracorporeal shockwave lithotripsy (ESWL)^[1,3], laser lithotripsy^[9], and stenting^[10,11] are nonoperative options, especially for elderly patients and patients at high surgical risk.

ESWL, EHL and laser lithotripsy yield similar success rates of 80%-95% and may be used in combination. These techniques are not available at all treatment facilities, thus, the selection of a specific treatment option may differ between institutions or physicians. There have been some studies of EHL using balloon catheters under fluoroscopy^[7] and single-operator duodenoscope-assisted cholangioscopy-guided EHL^[8]. These are complex and very labor-intensive procedures. ESWL usually requires multiple sessions and an additional endoscopic procedure for complete stone retrieval^[1]. The placement of biliary stents is used as an immediate management option to prevent cholangitis or stone impaction^[5], and for retrieval in choledocholithiasis patients who are intractable to the usual lithotripsy option, or have a high surgical risk^[6,11]. However, recent studies have shown high rates of late morbidity and mortality after long-term stenting. Temporary use of self-expandable metallic stents combined with EHL has been reported for avoiding long-term stent placement in patients with intractable CBD stones^[11].

Large stones (2.5 cm or 2.8 cm in diameter), or hard concretions, often make ML cumbersome or even impossible^[3]. Indeed, a stone size > 2.8 cm is associated with lithotripsy failure^[12]. Another study has shown that impacted stones, as well as stone size only, is a predictive factor for stone extraction failure, especially in cases where the bile duct is not dilated adequately to accommodate a large stone^[5]. In our case, the stone was oversized (4 cm) and dilation of the bile duct was not sufficient to capture the stone using a basket for ML. Other retrieval modalities, such as EHL, and laser lithotripsy, were not promptly available. Also, ESWL was not indicated because the stone was radiolucent. Therefore, we selected a 6-cm diameter bezoar basket to grasp the large stone completely for ML.

We performed endoscopic papillary large balloon dilation (EPLBD) without EST before ML for easy removal of the large stone. Several reports have shown the efficacy of EPLBD for treatment of large bile duct stones^[13-15]. Most studies have carried out EST followed by EPLBD, but some groups have attempted stone extraction by EPLBD alone without EST^[16]. The preceding EST has a lower risk of pancreatitis that the pancreatic orifice is separated from the biliary orifice after EST and it may result in balloon dilation forces away from the pancreatic duct^[15]. In our opinion, preceding EST before EPLBD may not play a role in the guidance of balloon

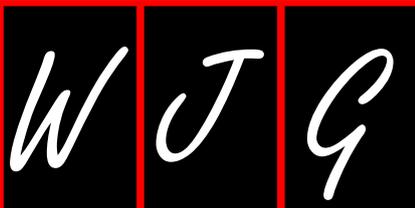
dilation because the direction of ballooning by the inflating catheter is always radial. Therefore, our case had LBS only without a previous EST.

In conclusion, ML using a gastric bezoar basket was safe and effective for the retrieval of a CBD stone, which was extraordinarily large. The bezoar basket is not a newly designed device, but an existing tool originally used for the removal of bezoars. This is believed to be the first published attempt to retrieve a giant and intractable bile duct stone and should be considered as an alternative nonoperative option for the management of difficult CBD stones.

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Asian Pacific <i>Helicobacter pylori</i> Meeting 2012
Kuala Lumpur, Malaysia</p> <p>January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States</p> <p>January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States</p> <p>January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States</p> <p>February 3, 2012
The Future of Obesity Treatment
London, United Kingdom</p> <p>February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom</p> <p>February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom</p> <p>February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada</p> <p>March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France</p> <p>March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States</p> | <p>March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States</p> <p>March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States</p> <p>March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States</p> <p>March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States</p> <p>March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States</p> <p>April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States</p> <p>April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore</p> <p>April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech</p> <p>April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain</p> <p>April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States</p> <p>April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia</p> <p>April 22-24, 2012
EUROSON 2012 EFSUMB Annual</p> | <p>Meeting
Madrid, Spain</p> <p>April 28, 2012
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Kiev, Ukraine</p> <p>May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
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Digestive Diseases Week
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2012 ASCRS Annual Meeting- American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States</p> <p>May 18-19, 2012
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SGNA: Society of Gastroenterology Nurses and Associates Annual Course
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American Society of Colon and Rectal Surgeons Annual Meeting
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Kiev, Ukraine</p> <p>September 20-22, 2012
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Prague, Czech</p> <p>October 19-24, 2012
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American Association for the Study of Liver Diseases
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Advances in Inflammatory Bowel Diseases
Hollywood, FL 33028, United States</p> |
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Acknowledgments

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

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

ic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

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

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 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

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

No author given

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

Volume with supplement

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

Issue with no volume

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

No volume or issue

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

Books

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary 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

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

Conference proceedings

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

Conference paper

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

Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious dis-

eases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

Patent (list all authors)

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

Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

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

Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose) 6.4 \pm 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 24.5 μ g/L; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantum numbers can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315223018.htm.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

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

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kbo I*, *Kpn I*, etc.

Biology: *H. pylori*, *E. coli*, etc.

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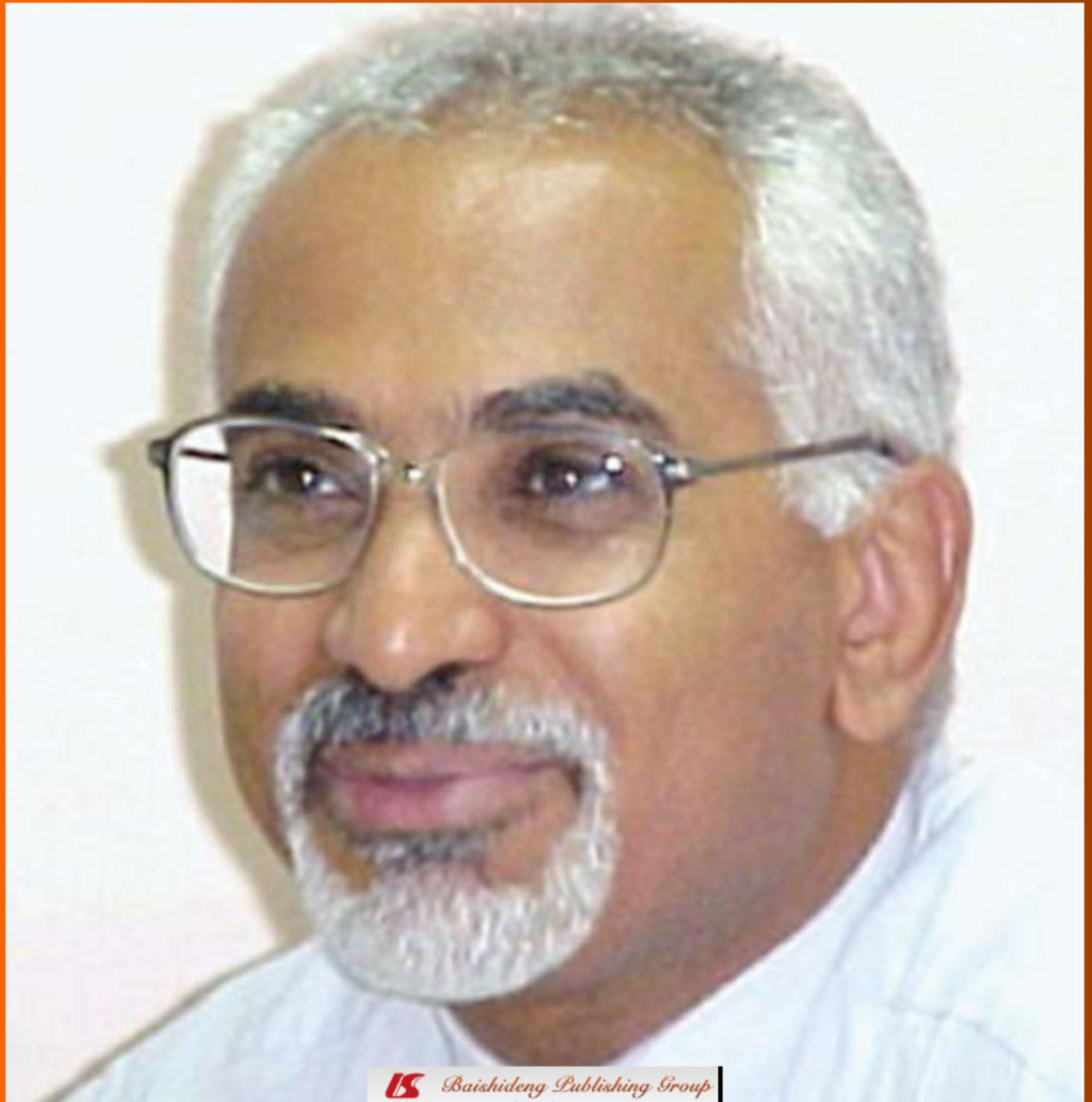
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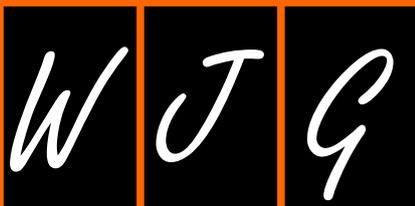
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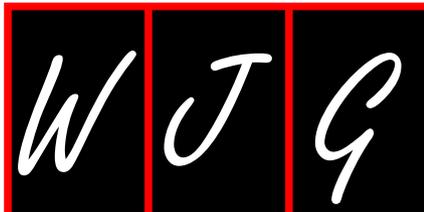
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Time to detoxify medical literature from guideline overdose

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overtreatment, inefficiency, and patient inconvenience. There is an urgent need to restrict articles with Guidelines and develop some strategy like have an intermediate stage of pre-guidelines and after 5-10 years of trials, a systematic launch of the Guidelines. There can be better ways than this for putting together guidelines as has been suggested by multiple authors and researchers.

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Abstract

The current financial turmoil in the United States has been attributed to multiple reasons including health-care expenditure. Health care spending has increased from 5.7 percent of the gross domestic product (GDP) in 1965 to 16 percent of the GDP in 2004. Healthcare is driven with a goal to provide best possible care available at that period of time. Guidelines are generally assumed to have the high level of certainty and security as conclusions generated by the conventional scientific method leading many clinicians to use guidelines as the final arbiters of care. To provide the standard of care, physicians follow guidelines, proposed by either groups of physicians or various medical societies or government organizations like National Comprehensive Cancer Network. This has led to multiple tests for the patient and has not survived the test of time. This independence leads to lacunae in the standardization of guidelines, hence flooding of literature with multiple guidelines and confusion to patients and physicians and eventually

INTRODUCTION

A well researched guideline will reduce financial burden in United States healthcare. Increasing and excessive health care budget is affecting major industries in the United States. We know it is not the sole cause, but is one of the major reasons for the crisis. A jaw dropping fact is that health care spending has increased from 5.7 percent of the gross domestic product (GDP) in 1965 to 16 percent of the GDP in 2004^[1]. It has been estimated that in 2015, we will be spending close to 20 percent of the GDP, or an estimated \$4 trillion, up from \$1.9 trillion in 2004, on health care^[2]. There is an obvious role of guidelines in reducing healthcare expenditure.

The initial step in systemic review was in 1970 with a perinatal trial^[3]. Although guidelines are intended to assist physicians, this functionality seems frequently lost among the sea of guidelines that are available. Indeed, at any

given point there are frequently multiple guidelines available with regard to a particular disease process, forcing physicians who wish to avoid accusations of undertreatment to order more than one diagnostic study or treatment (polypharmacy) referencing various guidelines. A well researched and tested guideline reduces healthcare cost and mismanaged malpractice lawsuits, and allows for better physician and patient understanding and better parameters for quality control in medical care. Most guidelines indicate that they are not a rule and should not be construed as establishing a legal standard of care.

MULTIPLE TESTING DUE TO MULTIPLE GUIDELINES

Guidelines are generally assumed to have the high level of certainty and security as conclusions generated by the conventional scientific method leading many clinicians to use guidelines as the final arbiters of care^[4] and sense of a shield to malpractice litigation. This has led to multiple tests and it has not survived the test of the time. Fuchs suggested greater expenditures do not necessarily result in better health outcomes^[5]. Guidelines may cause higher utilization of medical tests and procedures and, in some cases, have negative consequences for patients, as in the case of false-positive screening test results^[6].

GUIDELINES AND ITS USE IN MEDICAL MALPRACTICE

Another very compelling reason for its standardization is the use of guidelines in the malpractice lawsuit. Guidelines are frequently used as evidence by expert witness against a physician. It is very hard for judge and jury to understand the quality of evidence, but it easy for them to concur with evidence coming from an expert with a guidelines document.

GUIDELINES AS A TOOL FOR QUALITY MEASURE FOR HOSPITAL AND HEALTHCARE PROVIDER

Guidelines are used as a tool for quality measures. It seems immature to use consensus statements as performance measures or other similar tools to critique the quality of a physician's care. It could be better addressed if it is derived from high-quality guidelines based on the highest level of evidence and derived in a more thoughtful manner. Protocols for quality must include guidelines, but it must have safety and patient care at its core with efficiency also as an outcome parameter. Any project without safety and efficiency is going to fail; hence we much have safecient guidelines.

ROLE OF GUIDELINES IN PATIENT AND PHYSICIAN EDUCATION

Guidelines are the basis of evidence based medicine

(EBM). Guidelines are widely perceived as evidence based, not authority based, and therefore viewed as unbiased and valid. A systematic review uses preplanned scientific protocols and methods to summarize similar but separate studies to perform a scientific investigation for a specific question^[7]. Druss *et al*^[8] are of the opinion to rely more on guidelines due to increase in the quantity of relevant information. Evidence is a very strong word, but it's important to understand how the facts are studied, and realize the biases of studies and researchers. A major responsibility of an educator is to be meticulous with the data collection and near perfect interpretation, but it can, at times, be challenging due to the size of data and variables involved. This is primarily due to an unprecedented explosion of medical knowledge in the past 50 years secondary to breakthroughs in numerous areas of medical science and information technology.

The Institute of Medicine stresses on use of guidelines for medical care to ensure high quality patient care^[9]. Its needed as even the most sophisticated health care consumer has difficulty in deciding appropriate care for his or her condition^[10,11]. Guidelines will assure the patient of adequate and appropriate treatment. Institute of Medicine stresses a need for a strong pertinent guideline for any particular disease. The providers and patients are faced not only with incongruous but also conflicting research findings.

A major reason to stress their implementation is to reduce undesirable practice variation and reduce the use of services that are of minimal or questionable value. National Guidelines Clearinghouse (NGC, established in 1998) is a national body in the United States whose responsibility is to compile a bank of guidelines either by submission or active retrieval thru bodies like National Comprehensive Cancer Network (NCCN) and American Heart Association (AHA). It has established its own submission standards and substandard guidelines are not incorporated. Even then there are at least 500 new guidelines submitted or reviewed every year (Figure 1). Similarly, National Institute of Clinical Excellence (NICE) is established in the United Kingdom, with an objective to standardize the health care by issuing its own guidelines, after studying relevant literature and guidelines available at a given time in point. NICE uses term guidance in place of guidelines.

OVERUSE OF TERM GUIDELINES AND OVERLAPPING GUIDELINES

The term guideline has been drifting far from the original intent of the Institute of Medicine. Unfortunately a major share of current articles called "guidelines" is actually expert consensus reports^[12]. There are interestingly too many guidelines, often on the same topic. For example, clinicians do not need 10 different adult pharyngitis guidelines^[13]. Our information of total guidelines, compiled by NGC, shows there are close to 25% guidelines renewed every year and a disease as localized as breast has an average of more than 30 new guidelines

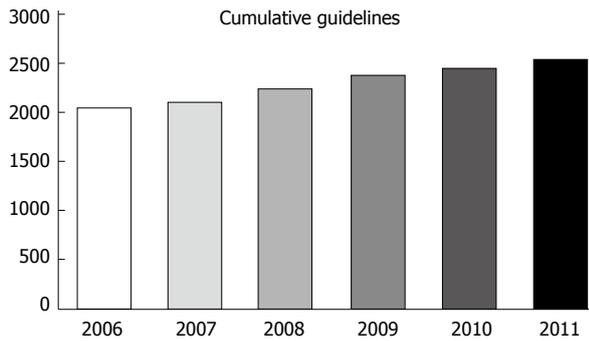


Figure 1 Cumulative number of all guideline summaries published on National Guidelines Clearing House. (Includes prior years) (Information taken from National Guidelines Clearing Annual Reports to Agency for Healthcare Research and Quality) (Source: NGC House, AHQR.com).

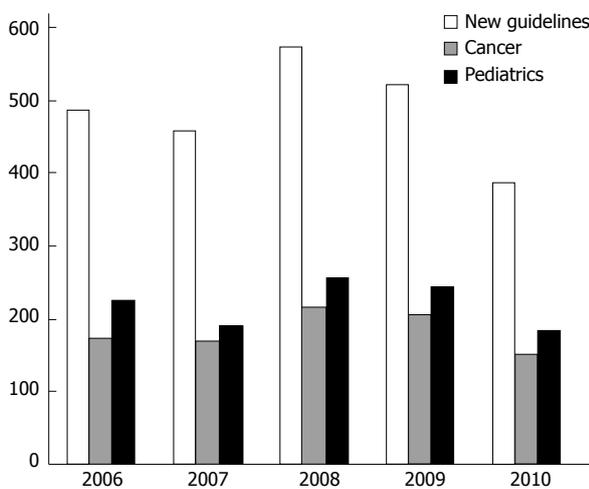


Figure 2 Total number of all new/revise, cancer and pediatrics guideline summaries organized by year of publication/revision. (Includes only that year) (Source: National Guidelines Clearing House, AHQR.com).

every year for last 5 years (Figures 1-3) (source: NGC). In 1999, after 1 year of NGC incorporation, there were total of 290 guidelines, with 82 cancers, 86 pediatrics and 21 and 7 breast and colon guidelines. The total number of guidelines, when this article is published has gone up to 2573 from 2060 in 2006 (source: NGC). This is according to the available information, the best compiled database of guidelines, by any organization internationally. According to their sources, it is possible a large number of guidelines are not even part of this pool.

CONFLICT OF INTEREST AND FACTORS INFLUENCING GUIDELINES

Personal bias has been studied by multiple researchers. In Choudhary's article, eighty-seven percent of authors had some form of interaction with the pharmaceutical industry with only seven percent admitting its influence on their inferences. Fifty-eight percent received financial support to perform research and fifty-five percent of respondents indicated that the guideline process had

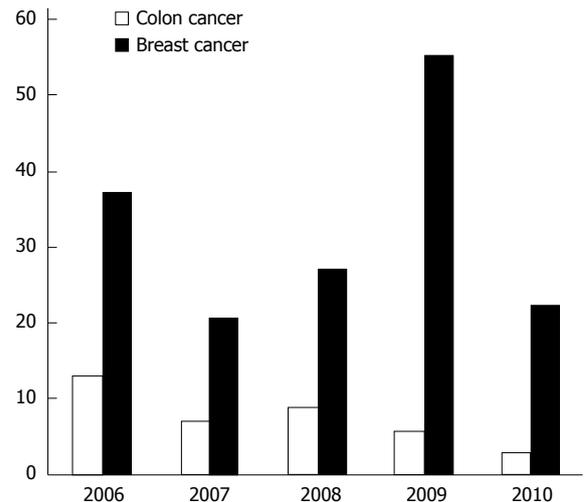


Figure 3 Total number of colon and breast cancer guideline summaries organized by year of publication/revision. (Includes only that year) (Source: National Guidelines Clearing House, AHQR.com).

no formal process for declaring these relationships^[14]. Unfortunately, all guideline committees begin with implicit biases and values affecting their worth^[15]. A very relevant question arises: affect of sponsorship by organizations, personal biases, and absence of restriction by journals, organizations and publishing bodies in using term guideline.

Another reason for the shortcomings in the guidelines is the need for resources to conduct a rigorous systematic review. The resource crunch might push guideline developers to revert to short-cuts or processes centered on expert opinion^[16]. On the contrary, a substantial investment in evidence gathering does not guarantee a sound guideline/evidence for a disease^[17]. Guideline authors often rely on research that are non rigorous, and yield conflicting results^[18]. This leads to pressure to rely more heavily on professional opinion which is problematic.

MULTIPLE GUIDELINES WITH CONFLICTING RECOMMENDATIONS AND QUALITY ISSUES

The exponential increase in guidelines is due to increasing number of research groups, societies, and associations performing analysis of a section of innumerable publications. It constitutes 40%-50% of indexed articles average annual volume. A large section of this growth is in articles on randomized trials and other types of clinical research used to guide evidence-based practice. Unfortunately, we are not seeing consistency in the guidelines issued on major areas of medicine. Contrast the guidelines with the decisions of any court of appeal in which some judgments are issued unanimously but most are not. Although unanimity is the rule in individual guidelines, it can be strikingly absent when different guidelines are compared.

Table 1 A simplistic presentation of conflicting statements in mammography and clinical breast examination by multiple authorities in last few years

Yr	Age for first mammogram	Recommending authority	Frequency	Clinical breast exam
2001	50-69 yr	Canadian Task Force	1-2 yr	1-3 yr
2003	40-49 yr (1-2 yr) > 50 yr (annually)	ACOG	As in column 2	Annually after 20 yr
2007	40-49 yr (individualize) 50-74 yr (1-2 yr)	ACP	As in column 2	No statement
2009	40-49 yr (individualize) 50-74 yr (2 yr)	USPSTF	2 yr	Insufficient evidence
2010	40 yr and above	American Cancer Society	Annually	20-40 yr every 3 yr and then annually
2011		NCCN		
2010	40 yr	NCI	1-2 yr	No statement
2011	47-73 yr	NHS, United Kingdom	3 yr	No statement

ACOG: American College of Obstetrics and Gynecology; ACP: American College of Physicians; USPSTF: United States Preventive Services Task Force; NCCN: National Comprehensive Cancer Network; NCI: National Cancer Institute; NHS: National Health Services.

EXAMPLES OF MEDICAL PRACTICE WITHOUT VALIDATION

It seems like we have come a full circle. The role of evidence-based practice, medical textbooks and editorials is to filter the literature filled with thousands of statements (based solely on the belief of the author or at best a consensus of experts)^[19].

In an article on guidelines on management of pharyngitis, review of 4 North American and 6 European guidelines were included, and recommendations were found to go tangential to each other with regard to the use of a rapid antigen test and throat culture and the indication for antibiotics^[13]. The reason for conflict is use of different data sets by different organizations. It has been suggested that guidelines are not necessary for every disease but are needed for diseases having significant practice variability and for which a valid evidence base can guide recommendations. They also suggested prioritizing individual recommendations^[12].

A simple example is commonly used guidelines issued by cardiology organization, American College of Cardiology (ACC)/AHA guidelines. It is largely developed from lower levels of evidence or expert opinion^[20]. It is flawed as it does not appear to follow the Institute of Medicine recommendations to separate the systematic review process from guideline formulation. According to the author, less than twenty percent of recommendations advocating a particular procedure or treatment in ACC/AHA practice guidelines are based on level A evidence. In another study of quality of recommendation, less than 45% recommendations in guidelines are based on high quality evidence^[21]. These studies bring a very valid question to the rational of application of evidence based medicine, when evidence itself is weak.

Another example of rapid turnover and too early update is due to the pressure of incorporating the development in technology to clinical arena without due research is inclusion of immunohistochemistry results in a sentinel lymph node specimen in cancer. This was used in tumor node metastasis staging and was withdrawn after

a short time. Situations like this make us wonder if we should make a pre-guideline of the results for a period of time and evaluate the data for its implication before incorporating the results for mass application. A similar example is withdrawal of hormone replacement therapy (HRT) in menopausal females. HRT was initially thought (1995) to have more benefits and no absolute contraindications or major risks, but after its aggressive introduction and urgency for compliance, increased incidence of hormone related side effects like lethal breast cancer, ovarian cancer and stroke persuaded clinical bodies to withdraw the major recommendations of guidelines for HRT therapy. Another example is a simplistic tabulation of guidelines on mammography. There are five guidelines from the leading medical organizations in last two years with highly conflicting recommendations, except for almost identical recommendations from NCCN and American Cancer Society. Similarly, the guidelines for colonoscopy have huge conflicts, except the frequency of every 10 years. This has not been challenged for last 40 years, even though this is based on a very flimsy theory. Clinicians and researchers are still struggling to identify appropriate intervals for colonoscopy in low risk patients, although there is some progress in identifying patients needing early colonoscopy (Table 1).

SOME OPTIONS TO MAKE GUIDELINES MORE ROBUST

Differentiating effective and ineffective health resource utilization is an important health policy objective. There are multiple articles suggesting their opinion of the subject. This awkward situation can be partially resolved by training physicians in biostatistics, a necessary tool to interpret the findings of published clinical research^[22]. At this point in time, the processes underlying guideline development is often vulnerable to bias and conflict of interest leading to sometimes poor quality. Grimshaw and group suggested a standardized and more diverse panel of physicians with competing interest with minimal bias^[23]. Another group suggested two separate grading

systems: (1) Quality of the evidence; and (2) Recommendations^[24]. We don't believe this will fix the state of affairs, but can definitely be one of the constructive steps. Another reason for streamlining the process is health quality measure reliance on guidelines. The NGC has thousands of guidelines produced by close to 300 organizations^[25] underlining the need for urgent streamlining.

A more radical thought is to reject them unless there is evidence of appropriate changes in the guideline process. Physicians will make better clinical decisions based on valid primary data^[12]. Another option is to update guideline after every 7-10 years or more if not much new data present and in areas of active research supplementing literature with pre-guidelines every 3-4 years. Also there can be a journal on guidelines where researchers can submit their opinion at the time of issues due date. And all the facts are incorporated and a final suggestion is given by the panel selected from the contributors and other established experts.

In summary, EBM is complex and incomplete. Guidelines must be directed only to the interests of patients and not to those who profit from them^[4].

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Recent advances in small bowel diseases: Part I

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Abstract

As is the case in all parts of gastroenterology and hepatology, there have been many advances in our knowledge and understanding of small intestinal diseases. Over 1000 publications were reviewed for 2008 and 2009, and the important advances in basic science as well as clinical applications were considered. In Part I of this Editorial Review, seven topics are considered: intestinal development; proliferation and repair; intestinal permeability; microbionics, infectious diarrhea and probiotics; diarrhea; salt and water absorption; necrotizing enterocolitis; and immunology/allergy. These topics were chosen because of their importance to the practicing physician.

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Key words: Diarrhea; Infectious diarrhea; Intestinal de-

velopment; Intestinal proliferation and repair; Intestinal permeability; Microbionics; Necrotizing enterocolitis; Probiotics

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INTESTINAL DEVELOPMENT

The development of the small intestine and its implications for nutritional support has been reviewed previously^[1]. The mucosa of the small intestine has many functions other than nutrient absorption and fluid secretion. These functions include host defense and immune responses. All of these small intestinal functions may be influenced by diet as well as by the intestinal microbionics, and may also be developmentally regulated.

At birth, there is rapid colonization of the intestinal lumen of the neonate, both with bacteria from the mother's milk, such as Gram-positive lactobacilli and bifidobacteria, and colonization with Gram-negative anaerobic bacteria. These bacteria are essential for growth and development of the gastrointestinal (GI) tract. In response to luminal microorganisms, intestinal Paneth cells produce microbicidal peptides, such as α -defensin. Paneth cells differentiate while migrating towards the crypt base, where these cells are eventually cleared by phagocytosis. In addition to the importance of Paneth cells in the innate immune defense against microorganisms, they are also involved in intestinal angiogenesis.

There is a complex interaction between bacteria in the intestinal lumen, the epithelial surface, and the underlying matrix, immune cells and enteric nervous system.

Enterocytes sense pathogenic bacteria, occupy the Toll-like receptors (TLRs) in response to their activation *via* pathogen-associated molecular patterns (PAMPs), leading to nuclear factor (NF)- κ B activation. Pattern recognition molecules (PRMs) detect any conserved bacterial products. PRMs, such as TLRs and nucleotide-binding oligomerization domain containing (NOD) receptors, are further important regulators of the intestinal barrier. TLRs are pattern-recognition receptors (PRRs) expressed by immune and nonimmune cells that signal in response to PAMPs expressed by bacteria in the intestinal lumen. Most TLR molecules signal through the adapter molecule MyD88 to interleukin (IL)-1-receptor-associated kinase. MyD88 activates the NF- κ B pathway, which is a critical regulator of tumor necrosis factor (TNF)- α and IL-1 β . Deficiency in MyD88 increases susceptibility to bowel inflammation, presumably by increasing brush border membrane (BBM) permeability. TLR ligands alter the properties of the intestinal barrier by redistributing zonula occludens (ZO)-1 and β -defensin 2. Cyclooxygenase-2-derived prostaglandin (PG)E₂ is important in TLR mucosal repair^[2].

When a child is born prematurely, its intestine is also premature, and is potentially ill-prepared for the extrauterine environment and contact with food bacteria. Microbial manipulation with antibiotic treatment or exposure of the pregnant rat dam to *Escherichia coli* (*E. coli*) results in long-lasting and potentially adverse effects on postnatal microbionics of the offspring^[3]. PAMPs produce an intestinal immune response through interaction with PRRs, including the TLRs. Transmembrane signaling may occur through TLRs^[4]. TLRs trigger signaling pathways which lead to NF- κ B activation and propagation of inflammatory responses. In addition to exogenous inflammatory mediators, TNF- α as well as IL-1 β trigger cytokine production through activation of this NF- κ B pathway. The NF- κ B proteins activate transcription, resulting in immune and inflammatory responses.

Intestinal alkaline phosphatase (AP) detoxifies lipopolysaccharide (LPS) by hydrolyzing it so that it can no longer activate TLR-dependent inflammatory responses^[5,6]. AP is involved in maintenance of homeostasis between the luminal bacterial microbionics and the body^[5,6]. About two-thirds of persons taking short or long courses of nonsteroidal anti-inflammatory drugs (NSAIDs) develop small intestinal damage. The NSAID diclofenac may induce enteropathy by way of the c-Jun-N-terminal kinase (JNK) pathway^[7]. This concept of stress-related changes in intestinal barrier and secretory function has therapeutic implications, as does the potential of JNK inhibitors to protect the GI tract from the damage of NSAIDs. For example, the NSAID indomethacin may enter the small intestine through a TLR4/MyD88-dependent pathway^[8]. In animals with stress as a result of immobilization, there will be an increase in TNF- α along with an increase in tight junction (TJ) permeability. These permeability changes are associated with alteration in the TJ proteins ZO-1, claudin-2, claudin-4, claudin-5, and β -catenin^[9].

IL-8 secretion in response to bacteria, IL-1 β and TNF- α are increased more in human fetal small intestinal epithelial cells than in older children or adults. This increased activation of NF- κ B DNA binding and transcriptional activity is due to reduced inhibition of signaling, resulting in increased phosphorylation, ubiquitination and degradation of the inhibitor of NF- κ B, in conjunction with decreased baseline expression and delayed resynthesis of this inhibitor^[10]. This represents a mechanism explaining the enhanced inflammatory response occurring in immature intestinal tissue.

The intestinal epithelial stem cells (ISCs) reside in the crypts of Lieberkuhn, giving rise to five daughter cell lines: Paneth cells, enterocytes, goblet cells, enteroendocrine cells, and M cells. In the upper third of the intestinal crypt, daughter cells differentiate into enterocytes, goblet cells, enterochromaffin cells, and Paneth cells. The Hedgehog platelet-derived growth factor and bone morphogenetic protein (BMP) signaling pathway are key mediators in morphogenesis of the intestinal villi and crypts, and maintain tenancy of ISCs. The ISCs are of two types: the fast cycling "crypt base columnar" (CBC) cells residing among the Paneth cells at the base of the crypt, and the slow cycling ISCs above the Paneth cells (+4ISC). Only one clonogenic cell is needed to regenerate a crypt and to maintain the appropriate number of crypts after injury. It is the +4ISCs that are highly sensitive to modulation and to chemotherapeutic agents such as doxorubicin^[11]. The ISCs produce progenitor cells, which form the enterocytes, goblet cells, enteroendocrine cells, and Paneth cells. Paneth cells are a major component of the innate immunity in the intestine, releasing granules containing β -defensins, matrix metalloproteinase-7, synovial phospholipase A₂, and lysozyme. Colony stimulating factor (CSF)1 acts in a direct juxtacrine/paracrine fashion, or acts indirectly through macrophages in the lamina propria, to regulate Paneth cell development^[12]. Fibroblast growth factor receptor (FGFR)-3 acts on the ISCs in the lower portion of the crypts to signal through both the β -catenin/Tcf-4 dependent and independent pathways, the expansion of the ISCs, expanding ISCs and crypt macrophages, including Paneth cells^[13]. The behavior of ISCs is regulated by canonical Wnt signals. Inhibition of Wnt signaling reduces epithelial proliferation in adult mouse intestine. In the developing intestine, the relationships among Wnt signaling, epithelial proliferation, and tissue differentiation are reversed^[14].

The intestinal epithelium is renewed through a series of programmed developmental transitions in the form and function of the intestine. For example, lactase-phlorizin hydrolase (LPH), fatty acid binding protein (Fabp1), and sucrase-isomaltase (SI) are intestinal proteins that are important for nutrition during different stages of development. LPH hydrolyzes milk lactose, SI hydrolyzes α -saccharidases, and Fabp1 is a cytoplasmic protein important for intracellular lipid transport. A member of the zinc finger transcription factor family (Gata4) and the hepatocyte nuclear factor α 1 (Hnf1 α) are essential for

LPH and Fabp1 gene expression but they do not mediate the glucocorticoid-induced precocious maturation of the intestine. Rather, specific intestinal genes have differential requirements for Gata4 and Hnf1 α , and these genes are dependent on the developmental time-frame in which each gene is expressed^[15].

Protective factors for the intestinal epithelium include secretion of mucins, as well as IgA and defensin peptides that are produced from epithelial cells. Epithelial cells also produce cytoprotective stress proteins, such as heat shock proteins (HSPs). N-formylmethionyl-leucyl-phenylalanine (fMLP) is a tripeptide produced by many enterobacteria as a by-product of protein synthesis. fMLP is a chemotactic agent from neutrophils and may be proinflammatory in nature. fMLP induces expression of HSPs and inhibits activation of the proinflammatory transcription factor NF- κ B^[16]. This finding needs to be translated to a clinical application.

Amniotic fluid and breast milk contain intestinal trophic factors that stimulate enterocyte development, and enhance various intestinal defense processes. Hydrocortisone is a trophic factor that is present in large amounts in human breast milk. Hydrocortisone reduces the cholera toxin (CT)-induced secretory response observed in fetal human small intestinal epithelial T84 cells, by inducing a pathway change from a clathrin-mediated to a caveolae-mediated endocytic process^[17]. Hydrocortisone also increases ganglioside/lipid raft association, which may also reduce intestinal secretion.

Dietary fat in mother's milk is a major modifiable environmental factor that influences intestinal growth and development of the baby. The n-6 fatty acid, linoleic acid, is the metabolic precursor for synthesis of arachidonic acid (ARA). α -linolenic acid is a precursor for synthesis of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Eicosanoids derived from ARA are proinflammatory, whereas the n-3 fatty-acid-derived metabolites are anti-inflammatory. EPA and DHA have direct effects on intestinal cells through gene expression and ion channels, as well as on G-coupled protein receptor activities. As a result of the potential for dietary substances to affect normal growth and development of neonatal intestine, the consumption of early-life non-maternal milk products must be undertaken with the greatest of care.

In adult animals, glucagon-like peptide (GLP)-2 has a trophic effect on the intestine, and enhances nutrient absorption. In contrast, in young animals, there is malabsorption of several lipids with GLP-2 or GLP-2 plus dexamethasone, but not with dexamethasone itself^[18]. The loss of body weight and jejunal atrophy induced by dexamethasone is prevented by giving GLP-2. Giving dexamethasone or GLP-2 and dexamethasone to lactating rat dams enhances glucose and lipid uptake in their suckling offspring^[19].

The premature human neonate has higher fasting levels of GLP-1 and GLP-2 compared to adults, and feeding increases these levels even further^[20]. GLP-2 levels are correlated with residual small intestinal length in the

infant with nutrient malabsorption following intestinal surgery^[21]. GLP-2 levels are directly correlated with absorption of fat, carbohydrate, and protein.

In suckling rats, GLP-2 plus dexamethasone increased the maximal transport rate (V_{MAX}) for jejunal glucose uptake, and the combination of these may be useful in stimulating glucose uptake in the developing intestine^[22]. In suckling animals, dexamethasone reduces jejunal lipid uptake to levels similar to those seen in weanling animals. Giving dams GLP-2 or dexamethasone during pregnancy and lactation reduces lipid uptake in the offspring, and this reduction persists for at least 1 mo^[23]. The impact this may have on the nutritional wellbeing of the animal in later life is unknown. This important consideration needs to be explored in terms of its long-term implications if nursing mother or child were to be exposed to steroids or GLP-2.

PROLIFERATION AND REPAIR

Homeostasis of the intestine is balanced between the process of proliferation, migration, differentiation, mitosis and apoptosis. Remodeling of the epithelium results from cell-matrix and cell-cell contacts. The proliferating regions are usually maintained by Wnt/ β -catenin signaling, whereas differentiation is regulated by BMP, transforming growth factor (TGF)- β , and Hedgehog signaling. These interact within the stem and progenitor cells in the lower regions of the intestinal crypts, and regulate the expansion and cell fate decision of the interstitial epithelial cells (IECs). Several proteins involved in the contact between cells act as the source (i.e., Notch signaling, which regulates the development of enterocytes)^[24] or the downstream effectors of signaling pathways such as E-cadherin/ β -catenin. Notch signaling regulates the formation of the Epcrin-B2-positive proliferating crypt cells, as well as the Ephrin-B1-positive differentiated cells^[25].

Homeostasis of the intestinal epithelium is a dynamic process created by a balance between proliferation, differentiation and apoptosis. This process is regulated by luminal nutrients, hormones, growth factors and cytokines. When acute damage is done to the GI tract, the usual repair process does not involve proliferation, but rather involves mucosal restitution. Restitution occurs by epithelial cell migration to reseal the superficial wound. Polyamines are involved in stimulation of intestinal epithelial cell migration during restitution. These polyamines (including spermidine, spermine, and precursor, putrescine) regulate calcium homeostasis by inducing alterations in cytosolic free Ca²⁺ concentration. Phospholipase C (PLC)- γ 1 modulates calcium store mobilization and calcium influx, thereby promoting intestinal epithelial restitution after wounding^[26].

Especially early in life, the BBM of the enterocyte is involved in the process of endocytosis, in which a tiny portion of membrane forms a bud around a luminal constituent, and breaks away from the rest of the BBM. This

hole in the remaining BBM must be quickly repaired. The subapical terminal web cytoskeleton inhibits movement of endosomes, and thereby helps to maintain permeability characteristics of the epithelium by keeping the endocytic activity occurring at the BBM under regulated control^[27].

Differential display and microarray analyses have been used to identify genes that may be involved in molecular mechanisms regulating proliferation and differentiation of the intestinal epithelium^[28]. Examples of transcription factors include the BMP expressed in the mesenchyme, Wnt which is essential for regulation of crypt stem cell proliferation and maintenance, and Notch signaling within the epithelium influencing differentiation of enterocytes, goblet cells, Paneth cells and enteroendocrine cells.

Glutamine is an amino acid that is the preferred fuel source for enterocytes. Glutamine activates extracellular signal-regulated kinase (ERK) and inhibits phosphorylated Akt, suggesting that ERK has an important role in the glutamine-mediated intestinal homeostasis^[29]. Activation of phosphoinositide 3-kinase (PI3K)/Akt during periods of glutamine deprivation limits apoptosis, and thereby may have a protective role. Glutamine-response genes influence the expression of a number of signaling pathways such as NF- κ B^[30] and peroxisome proliferator-activated receptor (PPAR) α ^[31]. This potential basis for protection with glutamine, for example, in necrotizing enterocolitis, remains to be tested.

The Notch signaling pathway is essential for the development of enterocytes, and the Wnt cascade is required for the development of Paneth, goblet and enteroendocrine cells (secretory lineage). The commitment of crypt stem cells to differentiate into enterocytes reduces the differentiation of these secretory cells. This interaction by Notch to activate the Hes1 transcription factor, which then blocks the MATH1 transcription factor, influences the Ets-domain transcription factor Spdef, thereby blocking steps in the differentiation of the stem cells into the secretory lineage^[32].

Notch, Wnt and Hedgehog signaling pathways are involved in epithelial cell proliferation and differentiation. Indian hedgehog (Ihh) is a signal sent by mature Paneth cells to their stem cell precursors, negatively regulating their differentiation. PPAR β is a nuclear hormone receptor activated by fatty acids in the intestinal lumen. PPAR β acts on Paneth cell homeostasis by downregulating expression of Ihh^[33]. Hedgehog proteins are members of a family of secreted signaling factors that orchestrate development of many organs and tissues, including those of the GI tract^[34]. In addition to epithelial differentiation being influenced by this variety of signaling cascades, amino acids and carbohydrates regulate gene expression and cellular function. For example, coenzyme A (CoA)-thioester derivatives contribute to cellular renewal along the crypt-villus axis in human small intestine^[35].

The Wnt/ β -catenin pathway involves molecules secreted by the mesenchyme, and controls proliferation and migration of enterocytes and Paneth cells. Transcription

regulators such as the CDX2 and CDX3 homeoproteins, members of the homeobox gene family of transcription factors, activate several intestine-specific genes such as SI and LPH, as well as the adhesion proteins L1-cadherin and claudin-2. Furin is a calcium-dependent serine protease which processes many proteins such as BMP-4, the insulin receptor, the Notch1 receptor, and the cell adhesion protein E-cadherin. CDX2 regulates furin expression in intestinal epithelial cells^[36]. Hepatocyte growth factor-1 α cooperates with CDX2, and regulates transcription of SI, LPH, claudin-2, calbindin 3 and liver Fabp1 gene^[37]. The crosstalk between lamina propria lymphocytes and intestinal epithelial cells may also be mediated by keratinocyte growth factor (KGF), such that lymphoepithelial interactions may both promote barrier function as well as regulate mucosal immune responses^[38].

Mitogen-activated protein kinases (MAPKs) and PI3K are activated in a cascade-like fashion by various growth factors. Three of the MAPKs that have been identified include ERK, JNK and p38 MAPK. Rac1 also mediates apoptosis irrespective of ERK1/2 and Akt activation^[39]. Signaling cascades such as the NF- κ B and MAPK pathways provide transcriptional activation of inflammatory mediators such as chemokines, adhesion molecules and antibacterial peptides. In addition, reactive oxygen species (ROS) are important mediators in cellular signal transduction cascades regulating proliferation, apoptosis and migration. TNF- α is an inducer of apoptosis that requires the small GTPase Rac1, which has a role in the control of ROS production^[40]. Protein-tyrosine kinase 6 (PTK6) is a stress-reduced kinase that promotes differentiation, and is a target of the serine threonine kinase AKT. After DNA damage, PTK6 is induced to promote apoptosis^[11].

The intestinal mucosa is constantly being deformed by peristalsis, movement of the villi, and passage of chyme along the surface of the intestine. This strain by a variety of signals stimulates the migration of epithelial cells across fibronectin [such as Scn, focal adhesion kinase (FAK), and ERKs]^[41].

INTESTINAL PERMEABILITY

The intestinal BBM of enterocytes constitutes the small intestinal epithelial barrier, which helps to protect the body against invasion by millions of luminal bacteria. This barrier function is a balance between the bacterial microbionota, and homeostasis provided by the preservation of the integrity of the BBM. TJs, innate immune cell function, and microbial peptides are important in establishing and maintaining this barrier integrity.

Modifications in NOD2 alter intestinal homeostasis, and may be associated in some individuals with chronic intestinal inflammation. PRMs, TLRs and NOD receptors are regulators of the intestinal epithelial barrier, which is essential along with innate immune cells and antimicrobial peptides, to maintain normal intestinal permeability^[42].

TJs between the intestinal epithelial cells provide a dynamic structure, altering cellular permeability. TJs are

formed by organization of a number of specific proteins including occludin, ZO-1, ZO-2, ZO-3, claudins, and junctional adhesion molecules. Mucosal hydration occurs through activation of ion channels and transporters, as well as through secretion of mucous and antibacterial protein. These are additional defense pathways against pathogens^[43]. Under some circumstances such as intestinal ischemia, there may be increased mucosal penetration and translocation of bacteria, as well as transcytotic movement of bacteria through intestinal epithelium by way of lipid rafts. This movement of bacteria across the disrupted intestinal barrier may precede cytokine-induced disruption of TJs^[44].

Adherens junctions (AJs) are a major part of the junctional complexes. The main transmembrane protein of AJ is E-cadherin. E-cadherin mediates adhesion or anchorage of the enterocytes to the matrix by a process that is Ca²⁺-dependent. Epidermal growth factor receptor (EGFR) is induced in the process by which there is deprivation of the E-cadherin-dependent junctions, and enterocytes lose their interactions with the basal lamina. This leads to anoikis, a form of apoptosis caused by the loss of cell anchorage^[45]. Symplekin is also a component of the TJ cytoskeletal plaques. Symplekin co-operates with the transcription factor ZONAB to regulate negatively the differentiation of goblet cells in the intestine^[46].

Mast cells (MCs) in the lamina propria contain both preformed bioactive mediators such as tryptases and histamine, as well as synthesized mediators like prostanoids and leukotrienes. MCs express corticotrophin-releasing factor receptor 1 (CRFr1) and CRF receptor 2 (CRFr2), G-protein-coupled corticotrophin-releasing factor receptors^[47]. In pigs, when CRFr1 is activated during early weaning stress in pigs, the epithelial barrier becomes leaky, whereas CRFr2 may be protective.

When the serotonin reuptake transporter (SERT) is lost, there is enhanced intestinal translocation of bacterial endotoxin in a murine model of chronic increased intake of fructose^[48].

The permeability of the intestinal mucosa is determined by the relative balance of uptake from the intestinal lumen, across the BBM and into the cytosol of the enterocyte, and efflux from the cytosol across the BBM and into the lumen, through transporters such as P-glycoprotein (P-gp; ABCB1), multidrug resistance-associated protein 2 (MRP2; ABCG2), and breast cancer resistance protein (BCRP; ABCG2). Changes in the activity of these efflux transporters will modify net drug absorption. For example, sulfasalazine uptake across the BBM is adequate, but sulfasalazine may be transported out of the enterocytes (efflux) by MRP2 and BCRP, contributing to the apparent low permeability of the small intestine to this drug^[49].

The transport and barrier properties of the intestinal epithelium are regulated, influenced by food and the microbionics that colonize mucus overlying the epithelium. Epithelial transport mechanisms are present to regulate the long-term adaptation of intestinal function. Specific

molecular components of TJs decrease or increase transepithelial resistance^[50]. In enterocytes in the upper portion of intestinal crypts, there are cell cycle promoting and inhibiting genes, as well as transcription/translation related genes. In the enterocytes, in the middle of the villi, there are metabolism-related genes, and vesicle/transport-related genes^[51].

Stressful events will increase intestinal permeability to macromolecules, deplete intestinal mucus, and alter interaction of bacteria in the intestinal lumen with the intestinal epithelial cells. Stress may even lead to inflammation, bacterial invasion of the mucosa, as well as T-cell activation. The disturbance of epithelial cell kinetics depends on the duration of the stress. The stress-induced increase of intestinal permeability may be related to an increased number of apoptotic cells in the epithelium^[52]. Perfusion of a jejunal segment in persons with low *vs* moderate background stress (called “pain stress”), results in an increase in chloride-related peak secretory response, as well as enhanced permeability to albumin^[53].

In addition to glucocorticoid-induced precocious maturation of the intestine, “stress” may also play a role in disease susceptibility through its actions on CRF, and subsequent activation of CRF receptors expressed locally in the intestine. It is predominantly the CRF-r1 activation that alters intestinal function in times of stress. One such type of stress in the developing animal is weaning, when the increased intestinal permeability and secretory activity are mediated by the intestinal CRF receptors. This is possibly through activation of enteric neural and PG synthesis pathways in weaned intestinal tissues^[54].

In humans, the increased intestinal permeability due to the acute effects of radiotherapy may be reflected by increased fecal excretion of DHA and calprotectin^[55]. From 50% to 70% of NSAID users have damage to the GI tract beyond the stomach. When capsule endoscopy, fecal calprotectin measurement, and urinary recovery of orally administered lactulose and mannitol are used, about half of NSAID users will have defects in the small bowel^[56].

The TJ (also known as the zonula occludens) has both a gate function (regulating the passage of molecules through the paracellular pathway), and a fence function. In some intestinal disorders, the permeability of the intestine may increase (“leaky” TJs), and endogenous PPAR β ligands may reduce the degree of TJ permeability, thereby reducing the degree of leakiness^[56]. This raises the therapeutic potential of using PPAR β ligands to correct the “leaky gut”. Adrenomedullin (AM) may also prove to be useful to reduce intestinal permeability. AM is a 52-amino-acid peptide formed in the mucosa. AM increases cAMP, activates protein kinase (PK)A, and stabilizes the barrier function of the intestine (i.e., prevents increased permeability and decreases leakiness^[57]). EGF binds to its receptor, and improves epithelial barrier function (i.e., decreases intestinal permeability). During sepsis there is barrier dysfunction, increased apoptosis, and higher levels of cytokines. Potentially all of these may lead to multiple

organ failure. Systemic EGF prevents peritonitis-induced intestinal barrier dysfunction^[58], and may thereby reduce mortality from noninfectious inflammation and intestinal injury. It remains to be established whether this modification of intestinal permeability will have any therapeutic benefits in humans.

MICROBIOTICA, INFECTIOUS DIARRHEA AND PROBIOTICS

Probiotics

The World Health Organization defines probiotics as “live microorganisms which, when consumed in adequate amounts as part of food, confer a health benefit on the host”. Probiotic therapy has been used for traveler’s diarrhea, to prevent antibiotic-associated diarrhea, and to prevent relapse of pouchitis (after colectomy and ileoanal pouch formation). *Lactobacillus rhamnosus* GG (LGG) is a probiotic bacteria that prevents cytokine-induced apoptosis by way of two LGG-produced viable proteins p75 and p40. These activate Akt and regulate intestinal epithelial cells as well as antiapoptotic responses by a PKC-MAPK-dependent mechanism^[59]. The clinically tested VSL#3 probiotic formula and its secreted components may augment the protective mucus layer through MUC2 gene expression^[60]. Treating infectious diarrhea in children with *L. rhamnosus* shortens the duration of diarrhea, and also reduces the duration of the need for parental rehydration^[61].

A meta-analysis of five randomized controlled trials of *Saccharomyces boulardii* has shown moderate effectiveness in preventing antibiotic-associated diarrhea (AAD) in children and adults. *S. boulardii* reduced the risk of AAD from 17% to 7%, with the number needed to treat to prevent one case of AAD being only 10^[62].

The probiotic bacterium *Lactobacillus casei* strain Shirota (LCS) suppresses the LPS/TLR4 signaling pathway and prevents indomethacin-induced intestinal injury, possibly by way of the production of lactic acid^[63]. The nonpathogenic yeast *S. baoulardii* inhibits EGFR and thereby also inhibits the downstream ERK1/2 MAPK pathway^[64]. This raises the possibility that *S. baoulardii* may have both anti-inflammatory and antineoplastic therapeutic potential.

It will soon be possible to target the human microbiome with antibiotics, probiotics and prebiotics. “Culture-independent strategies such as high-throughput parallel sequencing and comparative genomes, metabolic proliferating and functional genomes, fluorescence *in situ* hybridization, and phylogenetic microarrays will provide new insights into the composition, architecture and functional roles of the human microbiota”^[65].

A therapeutic approach to enteric infections is to express molecular mimics of toxin receptors on the surface of harmless bacteria, so that the toxin binds to the bacterium rather than to its natural target, the intestinal mucosa. This strategy using molecular mimicry has been used to develop recombinant probiotics for treatment and prevention of diarrheal disease caused by enterotoxigenic

E. coli^[66], and for prophylaxis and treatment of cholera^[67].

Infections

The protozoon *Giardia lamblia* is a human GI parasite that is common in Western Europe and in North America. Some 15% of infected individuals develop chronic symptomatic disease. *G. lamblia* infection increases intestinal permeability due to downregulation of TJ occludin 1, reduced sodium-dependant glucose absorption, and by increased epithelial apoptosis, as well as increased active electrogenic anion secretion^[68]. Exposure to DDT, organochlorine, reduces the efficacy of treatment of mice with experimental giardiasis^[69].

Cyclospora responds variably to albendazole^[70]. Nitazoxanide is effective in treating diarrhea and enteritis caused by *Cryptosporidium* in immunodeficient as well as non-immunodeficient patients aged ≥ 12 years^[71].

The synthesis of NO is increased in infectious diarrhea, but its physiological role is unknown. Inducible NO synthase (iNOS) is constitutively expressed by the intestinal villous cells. After acute injury of the intestinal epithelium rapidly induces iNOS and synthesizes NO. The induction of iNOS in response to neonatal piglet *Cryptosporidium parvum* is a nonspecific response^[72].

Campylobacter spp. are a common cause of diarrhea in developing countries. They causes mild to severe bloody diarrhea, chronic relapsing infection, and invasion of the mucosa. This may be associated with extraintestinal complications such as neuropathy in the peripheral nervous system, (e.g., Guillian Barré syndrome). Norepinephrine (NE) is present in high concentrations in the noradrenergic neurons in the intestine. Exposure to NE increases the virulence-associated properties of *Campylobacter*^[73]. This suggests that microorganisms may have evolved a “sense and respond” system. The evidence linking inflammatory bowel disease (IBD) and *Campylobacter jejuni* infection has been reviewed^[74].

Diarrhea in the traveler may be termed “traveler’s diarrhea,” therefore, detection of bacterial enteropathogens is not uniform. When diarrhea occurs in travelers, ciprofloxacin (500 mg *bid* for 3 d), levofloxacin (500 mg *qid* for 3 d), rifaximin (200 mg *qid* for 3 d), or azithromycin (1000 mg, single dose) should be taken in conjunction with an anti-diarrheal agent^[75]. Indeed, there is an advantage of adding loperamide to fluoroquinolone, rifaximin, and azithromycin treatment of traveler’s diarrhea. Two new antisecretory drugs that have been shown to be useful for reducing stool frequency of traveler’s diarrhea including crofelemer, a chloride channel blocker^[76], and zaldaride, a calmodulin inhibitor^[77]. Racecadotil, an enkephalinase inhibitor, may also be useful for the treatment of acute diarrhea^[78-80].

Vibrio cholerae secretes CT, which binds to mucosal enterochromaffin cells, and release large amounts of 5-hydroxytryptamine (5-HT). The sustained hyperexcitability of CT on the submucosal secretomotor neuronal pathway depends on 5-HT₃, and nicotinic receptors^[81]. The small GTP-binding proteins increase the CT-catalyzed ADP-ribosylation of the heterotrimeric guanine nucleo-

tide-binding (G) protein Gs β . There is increased susceptibility of neonates to infectious diarrhea. For example, there is increased uptake of CT by a developmentally regulated clathrin-endocytic pathway. Immature human enterocytes, DRF1, play a role in clathrin-mediated CT trafficking through the endoplasmic reticulum and Golgi, and ARF6 enhances Gs β activation by CT^[82].

Shiga toxin (Stx) 1 and 2 are virulence factors for noninvasive enterohemorrhagic *E. coli* (EHEC) infection, such as by *E. coli* 0157:H7 infection. Stx is taken up by microendocytosis in cells that lack the Stx1 receptor, mediated by Gc3 on the BBM^[83].

Clostridium difficile accounts for 15%-25% of episodes of AAD. Methicillin-resistant *Staphylococcus aureus* (MRSA), and particularly the enterotoxin-producing strains, may also cause nosocomial AAD^[84].

Rotavirus is the major cause of life-threatening diarrheal disease in infants and young children. Rotavirus-induced diarrhea can be prevented by use of a live-attenuated vaccine^[85]. Following acute mucosal injury such as from rotavirus infection, there is enterocyte migration that is proliferation-independent. This restitution or migration of cells requires NO, PGs and polyamines. Acute regulation of protein synthesis is achieved through changes in the rates of translation of mRNA, by way of alterations in peptide chain initiation. This chain initiation process is influenced by mammalian target of rapamycin (mTOR). mTOR is an amino acid-sensing mechanism that targets ribosomal p70 S6 kinase (p70^{S6K}). p70^{S6K} is activated during an active state of mucosal regeneration produced by a piglet rotavirus enteritis^[86].

The etiologic agent of Whipple's disease (WD) is *Tropheryma whipplei*, a PAS-positive, rod-shaped actinomycete. Some healthy persons harbor *T. whipplei* in their bowel, and there is no correlation between *T. whipplei* genotypes and clinical manifestations. The cumulative odds ratio (OR) for disease are 2.23 for the HLA-DRB1 X 13 allele, and 2.25 for the DBQ1*06 allele^[87]. A randomized controlled trial of 40 persons with previously untreated WD showed that daily infusions of ceftriaxone 1 \times 2 g or meropenem 3 \times 1 g for 14 d, followed by 12 mg oral trimethoprim-sulfamethoxazole, both gave an OR of 0.95 for remission for at least 3 years^[88]. One patient with asymptomatic cerebrospinal fluid infection with WD was resistant to ceftriaxone and to meropenem, but responded to chloroquine and minocycline. This compares favorably to retrospective case series with treatment of *T. whipplei* with tetracycline, penicillin, streptomycin and low trimethoprim-sulfamethoxazole, in which only about three quarters of WB patients had an initial response, and approximately a quarter of those who had an initial response subsequently relapsed.

Both Crohn's disease (CD) and intestinal tuberculosis (TB) (caused mainly by *Mycobacterium tuberculosis* or *Mycobacterium bovis*) have a predilection for the tumor ileum. An algorithm has been developed for the investigation of persons in whom the diagnosis includes CD and TB^[89].

Diarrhea is the most common GI symptom in persons

with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS), affecting 50%-90% of those with a low CD4 lymphocyte count. However, in some 30%-40% of cases of HIV/AIDS, enteric pathogens cannot be identified. Conceptually, HIV enteropathy has been supported by the detection of HIV proteins in the intestinal epithelial cells as well in gut lymphocytes and macrophages. In a case report, a 33-year-old woman with HIV enteropathy was described with structural changes in the duodenal mucosa, regressing after triple antiviral therapy^[90].

About half of the HIV-infected persons in the United States have diarrhea. The intestinal immune system is central to the pathogenesis of AIDS (transmission, viral complication, CD4⁺ T-cell destruction), with the progression of AIDS due to a decrease in the expression of genes that maintain the mucosal barrier, and HIV translocation^[91]. Endoscopy and biopsy of the upper and lower GI tract has identified pathogens in about half of those HIV-infected persons who were initially thought to have an idiopathic disorder or AIDS enteropathy. Viral replication occurs early in HIV in the gut-associated lymphoid tissue CD4⁺ T cells and macrophages. AIDS enteropathy is "an idiopathic, pathogen-negative diarrhea, and treatment includes highly active antiretroviral therapy (HAART), fluid and electrolyte balance, and nutritional support^[92]". The opportunistic infections can still occur in HIV-positive persons taking HAART, especially in those with a high viral load and a low T-cell count. Also note that these opportunistic disorders occur even if the HIV load is low and the CD4⁺ T-cell count is near normal.

In a young patient with chronic diarrhea, bacterial rDNA (16S rDNA) has been extracted from the duodenal biopsy, and was thought to be due to *Stenotrophomonas maltophilia*^[93]. Such genomic techniques may be used for the diagnosis of chronic infections, including those with HIV enteropathy.

Matrix metalloproteinases (MMPs) are a group of enzymes that are capable of degrading all components of the extracellular matrix. Fibroblasts are the major source of MMPs in the human intestine. Fibroblasts are a potential target of IL-21, a T cell-derived cytokine that acts on the IL-21 receptor, and then interacts with the common γ chain receptor IL-21 that controls MMP secretion by fibroblasts^[94].

Enteropathogenic *E. coli* (EPEC) is a leading cause of infantile diarrhea in developing countries. The EPEC-induced inflammation is a balance between pro- and anti-inflammatory proteins^[95]. Nosocomial infections with *Pseudomonas aeruginosa* are associated with a high fatality rate. A virulence circuitry of *P. aeruginosa* is activated by both soluble and contact-mediated elements of the intestinal epithelium^[96]. *P. aeruginosa* metabolizes adenosine to inosine, which enhances virulence of the organism^[97].

Malnutrition may be associated with prolonged episodes of EPEC infection. In the human epithelial Caco-2 cell model, EPEC inhibits uptake of thiamine^[98]. EPEC also inhibits SERT, the serotonin inhibitor; reduction in

SERT by EPEC may represent an additional and new mechanism of EPEC-mediated diarrhea^[99].

Severe acute respiratory syndrome (SARS) is characterized clinically by fever, nonproductive cough, dyspnea, lymphopenia, and rapidly progressing changes on chest X-ray, however, it is not uncommon for SARS patients to have diarrhea, nausea and vomiting, as well as abdominal pain. This may be associated with atrophy of the mucosal lymphoid tissue, staining of intestinal epithelial cells and lymphocytes for corona virus (*via in situ* hybridization), and demonstration by electron microscopy of SARS-coronal virus-like particles in the mucosal epithelial cells^[100].

The total microbial population within the GI tract exceeds the total number of mammalian cells in the body by at least an order of magnitude. There is a complex environment of microbes that interfaces with the mucosal lining of the GI tract. This coexistence between eukaryotes and prokaryotes has both beneficial and unfortunately harmful interactions^[101]. The intestinal mucosa downregulates proinflammatory signaling pathways, expresses antimicrobial proteins, and initiates epithelial repair after mucosal injury. Indeed, commensal bacteria are actively involved in shaping the very barriers that confine them to the gut lumen^[102].

Lipid rafts are cholesterol-sphingolipid-rich microdomains in the enterocyte BBM. These lipid rafts contain digestion and transport enzymes that are important in trafficking and signaling of various proteins. For example, Cx43 is present on enterocyte lipid rafts, and interferon (IFN) inhibits enterocyte migration by displacing Cx43 from these lipid rafts in enterocytes^[103]. Some pathogens recognize these lipid-rich specialized areas, and modify function of the nutrient digestive and transport proteins. Anti-glycosyl antibodies are induced by a glycosyl antigen, and are associated with lipid rafts. These anti-glycosyl antibodies act as competitors to pathogens, having a protective role on the lipid rafts by preventing lactic acid pathogens from gaining access to the intestinal BBM^[104].

SMALL INTESTINAL BACTERIAL OVERGROWTH

Small intestinal bacterial overgrowth (SIBO) occurs as a result of a reduction in gastric acid secretion, a failure of mucosal and systemic barrier functions, as well as a failure of immune responses. SIBO also occurs in the presence of anatomical abnormalities such as intestinal fistulae or multiple small intestinal (not colonic) diverticulae. Gram-positive and Gram-negative bacteria, including both aerobes and anaerobes, contribute to the diarrhea and malabsorption of nutrients. While the prevalence of SIBO is increased in persons with irritable bowel syndrome (IBS), especially those with diarrhea-predominant IBS, the exact rates depend upon the method used to diagnose SIBO, with low prevalence rates of 4% using the gold standard test of a positive jejunal aspirate and culture, and a prevalence of 54% using the lactulose or glucose hydrogen breath test^[105]. The crux of this association of IBS and

SIBO is whether treatment of the positive test for SIBO will improve the patient's symptoms.

SIBO may recur after it has been successfully treated with appropriate antibiotics (e.g., metronidazole or ciprofloxacin). The recurrence rates after initially successfully treated SIBO are about 13% at 3 mo, 28% at 6 mo and 44% at 9 mo^[106]. SIBO recurrence is more likely in older persons, those treated chronically with proton pump inhibitors, and curiously, those with a previous history of appendectomy. Retreatment with the same or different antibiotics will be necessary, and some persons with recurrent SIBO require maintenance use of antibiotics.

The absorbable antibiotic metronidazole is more effective than the nonabsorbable antibiotic rifaximin for treatment of SIBO in persons with blind loop syndrome^[107]. Rifaximin may safely prevent traveler's diarrhea: in a study in travelers to Mexico, rifaximin reduced the risk of diarrhea from 54% in those taking placebo to 15% in those taking rifaximin^[108].

In persons with chronic rosacea, 46% had SIBO, vs 5% of healthy controls. After eradication of the SIBO, the cutaneous rosacea lesions cleared in 71% and greatly improved in 21%, whereas those treated with placebo remained unchanged in 90% or worsened in 10%^[109].

DIARRHEA, SALT AND WATER ABSORPTION

The topic of diagnosis and treatment of acute or persistent diarrhea has been reviewed^[110]. Host genetic polymorphisms may influence susceptibility to traveler's diarrhea. The topic of chronic diarrhea has been reviewed and can be accessed for a tutorial (BMJ Learning <http://gut.dmj.com/tutorials/collection.dtl>)^[111].

Na⁺/H⁺ exchange (NHE) is the principal exchanger isoform responsible for transepithelial Na⁺ absorption at the BBM of the intestinal epithelium. Coupled activity includes both Na⁺/H⁺ and Cl⁻/HCO₃⁻ exchangers. The Cl⁻/HCO₃⁻ exchanger, SLC26A3 [also known as down-regulated in adenoma (DRA)], also exchanges sulfate and formate. Electroneutral NaCl absorption is mediated through NHE₃ as well as DRA as the major Cl⁻/HCO₃⁻ exchanger^[112]. NHE is highly regulated by a number of second messenger systems including cAMP-dependent protein kinase (PKA), cGMP dependent kinase, Ca²⁺, PKC, PI3K, and Akt. The phosphorylation of NHE by PKA alters the clathrin-mediated cAMP-stimulated endocytosis of NHE₃, thereby reducing Na⁺ absorption. Synaptotagmin 1 (Syt1) directs NHE₃ to the adaptor protein 2-clathrin complex^[113].

There are two Cl⁻/OH⁻ exchangers, DRA (SLC26A3) and PAT-1 (putative anion transporter-1; SLC26A6). DRA is in the colon, whereas PAT-1 is in the human jejunum and ileum. Cytokines such as IL-1β, TNF-α and IFN-γ increase secretion in models of inflammatory diarrhea. IL-1β decreases DRA mRNA, as also does IFN-γ by means of the STAT1/JAK (Janus kinase) pathway^[114]. This opens the way for the development of potential antidiarrheal

agents for the treatment of persons with inflammatory bowel disease. For example, *Lactobacillus acidophilus* prevents enteroinvasive *E. coli*-mediated disruption of the BBM barrier in Caco 2 cells, and increases Cl/OH⁻ exchange by enhancing the mRNA and activity of DRA^[115].

Lysophosphatidic acid (LPA) is a glycerophospholipid present in abundance in foods such as soybeans and egg yolk. There are seven LPA receptors, and LPA^[116] acts through these receptors to inhibit the activity of the cystic fibrosis transmembrane conductance regulator (CFTR)-dependent Cl⁻ channel. LPA also stimulates BBM Cl⁻/OH⁻ exchange and levels of DRA^[117]. This combined mechanistic effect of LPA may explain its antidiarrheal effect. This raises the possibility of using LPA for the treatment of diarrhea.

Serotonin (5-HT) is a neurotransmitter and hormone that influences many physiological functions. Over 97% of the serotonin in the body is in the enterochromaffin cells. SERT is present in the human intestine^[118], and the enterochromaffin cells have apical microvilli that protrude into the intestinal lumen. Serotonin is secreted from granules at the basolateral pole of the enterochromaffin cells. Abnormalities in the release, reuptake and catabolism of serotonin may be responsible for altered secretion, motility and pain sensation in patients with IBS, inflammatory bowel disease, or in persons with diarrhea or constipation. Release of serotonin may be mediated through central and enteric nervous system regulation, or by way of the enterochromaffin cells acting as sensors in the gut luminal milieu, responding to tastants such as (caffeine, tyramine and octopamine), olfactants (thymol and eugenol), as well as glutamine, deoxycolate, 2-deoxyglucose, and the artificial sweetener, sucralose. There are 11 secretory-associated genes, including the vesicle docking inhibitor STXBP3, which may be upregulated in response to luminal glutamine or bile acids, whereas anti-sense knockdown reduces serotonin secretion^[119].

NHE₃ is regulated by cAMP, cGMP, intracellular calcium, PI3K, glucocorticoid kinase-1 and PKC. The acute regulation involves changes in both functional activity and membrane trafficking. This acute effect on NHE₃ may be mediated by the glucocorticoid receptor, which stimulates SGK-1. Membrane insertion of NHE₃ may be caused by Na⁺-K⁺-ATPase-induced changes in A⁺ translocator Na⁺ flux^[120].

NHEs help to maintain intracellular pH homeostasis, cell volume regulation, and electroneutral sodium chloride (NaCl) absorption. NHE₂, NHE₃ and NHE₈ are present in the apical membrane of enterocytes and colonocytes. Sp3 is the major activator of NHE2 gene transcription in the intestinal epithelial cells^[121]. NHE₃ is also regulated by certain growth factors, protein kinase, and IFN-γ. The Sp transcription factor is involved in cell cycle regulation, hormonal activation, and development patterning. NHE₃ facilitates intestinal neutral NaCl absorption, and thereby increases the net transmucosal absorption of bicarbonate in rat duodenal mucosa. This mechanism is similar to the proximal renal tubular bicarbonate absorption^[122].

The epithelial sodium channels (ENaCs) are a rate-limiting component for electrogenic sodium absorption in the distal colon. ENaC function is regulated by mineralocorticoid and glucocorticoid hormones. Steroid hormones act through transcriptional induction of β- and γ-ENaC subunits. In macroscopically noninflamed colon in persons with active CD, there is impaired ENaC-mediated Na⁺ absorption, which contributes to the diarrhea arising from the inflamed mucosa^[123].

Water movement across the intestinal epithelium is driven osmotically, and is therefore influenced by the balance between sodium absorption and chloride secretion. These processes, absorption and secretion, are regulated. Intestinal motor and sensory function is altered by the action of transmitters and by changes in ion channel function. CFTR is a cAMP-dependent Cl⁻ channel. CFTR is located in the intestinal epithelium, and is activated by PKA-mediated phosphorylation. A minor component of Cl⁻ transport occurs by way of intestinal and colonic epithelial cell transport, the type 2 Cl⁻ channel (ClC-2) volume-regulated channel. CFTR acts as an entry pathway for Cl⁻ into epithelial cells, in parallel with Na⁺ entry through apical ENaCs. ClC-2 promotes anion secretion, with little anion reabsorption. The properties of ClC-2 differ depending upon the conditions in the tissue in which the ClC-2 channels are studied^[124].

Diarrhea is common after pelvic radiotherapy, and this may be associated with modification in the diversity of luminal microbionota, as reflected by a drop in the similarity index of the enteric bacteria. Cluster analysis of the microbial profile 5 wk after radiotherapy displayed a dentogram which was different in patients who presented with or without diarrhea^[125]. It is not clear why there is this differential effect of radiation on the intestinal organisms.

Newer and even more effective antidiarrheal agents are needed. In the presence of malabsorption of Na⁺ and water in the small intestine, the ENaCs in the distal colon are upregulated in order to absorb more Na⁺/water, and hopefully thereby reduce diarrhea. ENaCs are responsive to mineral corticoids as well as to glucocorticoids. Glucocorticoids act through p38 MAPK to enhance the transcription of the B- and J- subunits of ENaCs, thereby rapidly increasing water absorption^[126].

DRA is a Cl⁻/HCO₃⁻ exchanger. When DRA is eliminated in mice, there is an approximately 50% reduction of basal and cAMP-stimulated HCO₃⁻ secretion^[127]. Microfluorimetry of villi shows that DRA is the major Cl⁻/HCO₃⁻ exchanger in the lower villous epithelium. In humans, loss-of-function mutations cause congenital Cl⁻ losing diarrhea^[128].

The development and control of the inflammatory reactions, which occur in food allergies, are mediated by T helper (Th)2 or regulatory responses, in which IL-4 is a factor. Allergic responses include IL-4 induction of the differentiation of committed effector Th2 lymphocytes for mRNA for TNFβ, Th1, IFNβ, IL-12p40, regulatory cytokines or Foxp3 (forkhead box P3). These may serve

as “rescue channels” for all Cl⁻ secretion, but lubiprostone activation of Cl⁻ secretion requires CFTR. The PPAR- β agonist rosiglitazone reduces cAMP-dependent Cl⁻ secretion as a result of reduced expression of the CFTR Cl⁻ channel, KCNQ, K⁺ channel, and Na⁺/K⁺/2Cl⁻ cotransporter proteins^[129].

IBS

About half of diarrhea-predominant IBS patients report an exacerbation of their symptoms within 90 min of eating. Normally, the duodenal and ileal breaks slow the passage of chyme along the intestine, and this helps to prevent exceeding the approximately 2-L composition of the colon to absorb water. Indeed, the small bowel water content initially falls, and then rises after a meal of liquids and solids^[130]. This could possibly be due to MC products being released from the small bowel mucosa, causing rapid transit of mill as intestinal fluid secretion.

IBS may aggregate in families, and twin studies demonstrate both a genetic as well as an environmental basis for IBS. Human intestinal smooth muscle cells and ICCs express SCN5 (gene encoded Na⁺ channel, expressed in interstitial cells). There may be an association between genetic defects in SCN5A and GI symptoms, and SCN5A may be a candidate gene in the pathophysiology of IBS^[131].

In a group of 62 patients with presumed idiopathic chronic watery diarrhea, HLA-DQ2/DQ8 genotyping, eHCAT abdominal retention test, small bowel follow-through, and hydrogen breath test were undertaken. In 45% of these patients, bile acid was presumed to be the cause of diarrhea. Sugar malabsorption was seen in 16%, gluten sensitive enteropathy in 16%, and both bile acid and sugar absorption in 3% of patients. It is important to note that not only was the cause of the chronic watery diarrhea not idiopathic in 81% of the patients, but it responded to specific treatment^[132].

NECROTIZING ENTEROCOLITIS

Intestinal mucus is one of the important factors protecting the intestinal epithelium. Without this well-formed gel layer, the underlying mucosa is more susceptible to attack by bacteria. Much of the oral intake of threonine is used for mucin production. A threonine-deficient diet will lead to a reduced number of acidic mucin-producing goblet cells in the small intestine. The abnormalities in mucus resulting from the impaired oral intake of threonine can be prevented through parenteral administration of this amino acid^[133].

The mucus layer over the intestinal epithelium acts as a barrier, retarding the access of bacteria and bacterial products to the BBM. Goblet cells secrete mucin 2 (MUC2), a glycoprotein with high density and viscoelasticity. MUC2 contains large amounts of threonine and proline. Studies determining the fractional synthetic rate of MUC2 in human neonates have shown rapid incorporation of systemic threonine into MUC2^[134].

Premature infants are at increased risk of developing necrotizing enterocolitis (NEC). The initial colonization of the intestine by bacteria, and the use of enteral feeds, contribute to the development of NEC. The maldigestion of carbohydrate may also be a risk factor^[135]. This is possibly because maternal diets are high in n-6/n-3 fatty acids. These dietary lipids are epigenetic factors that contribute to the increased incidence of ischemia by altering the composition of amniotic fluid and intestinal membrane structural lipid essential fatty acids^[136].

There is a wide range of mortality rates of infants with NEC, depending on the number of risk factors (e.g., prematurity, use of feeding formula, bacterial colonization of the GI tract), as well as the extent of the damage to the bowel (inflammation, necrosis, perforation, peritonitis, sepsis, and systemic inflammatory response). In the healthy child, there is high blood flow and low resting vascular resistance. One mechanism of development of NEC is reduced blood flow in the arteries penetrating the wall of the intestine, as well as the submucosal arterial plexus.

NEC is a major cause of death from GI disease in neonates. The mucosal damage and increased intestinal permeability lead to translocation of enterobacteria, activation of macrophages in the lamina propria, and initiation of a systemic inflammatory response. NO is released in a response to cytokine induction of the enzyme iNOS. Restitution is regulated in part by the Rho family of small-molecular-weight GTPases. NO inhibits enterocyte migration through protein tyrosine phosphatase (SHP-2)^[137].

Intestinal barrier function can also be altered as a result of impaired intestinal restitution. This restitution is decreased in, for example, hemorrhagic shock, sepsis, intestinal ischemia and NEC. The increase in intestinal permeability that occurs with stress and the presence of viable nonpathogenic *E. coli* is exaggerated by TNF- α release from macrophages^[138]. The proinflammatory mediators IL-18 and TNF- α are involved in the development of NEC. As noted by these authors, this raises the possibility that “...anti-TNF- α could be used as a potential therapy for human NEC”^[139]. The use of TNF- α modifiers may also be effective to normalize *E. coli*-associated increased intestinal permeability. It is unknown whether the inhibition of membrane or soluble TNF- α may be used clinically to prevent or to treat NEC.

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is produced by epithelial cells, where it decreases apoptosis, preserves the integrity of the cytoskeleton, and rebuilds depleted intracellular stores of ATP. In newborn rat pups, HB-EGF maintains the blood flow of the microcirculation that normally would be decreased in NEC^[140]. Histological abnormalities correlate with changes in blood flow, and less histological damage occurs with the use of HB-EGF. This may prove to be a useful preventive or therapeutic agent in newborn human infants who are at risk of developing NEC.

The probiotics lactobacilli and bifidobacteria may help to prevent the development of NEC. TLRs on the

intestinal BBM are PRRs that identify components of the usual intestinal microbiota. TLR4 recognizes the Gram-negative LPS, which is increased in NEC. The TLR changes occur before the histological changes, suggesting that the activator of the inflammatory response through TLRs may be causative in the development of NEC^[141].

As a result of the importance of the formation of the microbiota in the development of NEC, the possibility has been raised for the therapeutic potential of altering the bacterial contents by the use of probiotics^[142]. Feeding premature rats the probiotic *Bifidobacterium bifidum* reduced the prevalence of NEC from 57% to 17%. This dramatic protective effect was associated with reduced and indeed normalized levels of proinflammatory IL-6, trefoil factor 3 and mucin-3, as well as TJ and AJ proteins^[143].

The release of bacterial LPS from enterobacteria associated with NEC increases the enterocyte expression of integrin. Integrin leads to adhesion of the enterocyte to the underlying matrix and healing of the damaged mucosa by the migration process of restitution. Dystroglycans (DGs) are a component of the dystrophin-glycoprotein complex that are involved in integrin activation in the intestinal epithelium^[144]. With restitution, the migrating cells transiently adhere to the underlying matrix, reducing fibrillar adhesions containing a structural core of transmembrane integrins. These integrins provide anchors (adhesion complexes) and also sensors for mechanical events, as well as signal transducers. Removal of neurocytes requires disassembly of the integrin-based adhesions structures. β 1-Integrin is endocytosed *via* a dynamin-dependent lipid-raft-mediated pathway that helps to regulate intestinal epithelial cell migration and wound closure^[145].

The bacterial ligand activated TLRs signal intracellularly to upregulate both pro- and anti-inflammatory cytokines. There are several genes induced in the inflammatory process such as cytokines, chemokines and adhesion molecules. A factor that is central to the regulation of these various genes is NF- κ B. For example, NF- κ B is induced in NEC. Platelet activating factor (PAF) is an endogenous proinflammatory phospholipid that rapidly activates NF- κ B. PAF also stimulates neutrophils to produce the chemokine that used to be known as macrophage inflammatory protein (MIP)-2, now called CXCL₂. The bowel injury produced by PAF is mediated through the processing of the NF- κ B p105 into NF- κ B p50^[146].

PAF induces Cl⁻ channel activation associated with intracellular acidosis. The Cl⁻ channel activation occurs through caspase-3 activation and DNA fragmentation, as well as by apoptosis^[147]. In NEC, PAF and accelerated apoptosis of enterocytes results in tissue necrosis. Inhibition of caspase activation prevents the progression of disease in a neonatal rat model of NEC. Palmitoylation is a post-translational modification that is necessary for efficient signal transduction by many G protein-coupled receptors (GPCRs). Nutrients in the intestinal lumen may be sensed. For example, free fatty acids lead to release

of peptide YY. This affects GPCRs, thereby modifying cell proliferation and differentiation as well as hormonal secretion^[148]. Inhibition of the PI3K/Akt signaling pathway is the main mechanism of PAF-induced apoptosis in enterocytes. Polyunsaturated fatty acids block this mechanism early in the signaling cascade, probably by way of an effect on protein palmitoylation^[149].

After a period of bowel ischemia, the reperfusion of blood actually increases damage to the tissue. As oxygenation of the reperfused tissue occurs, free radicals are produced, MCs degranulate, leukocytes adhere to the endothelial wall, and thus ischemia/reperfusion (I/R) damages especially the mucosal cell layer and villous structure, as well as mucosal permeability and mitochondrial activity. 3,4-Dihydroxy-phenyl lactic acid reduces the production of peroxide and the expression of adhesion molecules in neutrophils and thereby minimizes the disturbance in the microcirculation^[150]. If the initial period of ischemia is short (about 15 min in mice), these adverse effects may reverse, but in longer periods of ischemia, the changes in structure and function are irreversible^[151].

During hypoxia-associated inflammation, endogenous adenosine is protective. In hypoxia, the levels of equilibrative nucleoside transporter-2 (ENT2) falls in human epithelial cells^[148], whereas hypoxia-inducible-factor-12-dependent repression of ENT2 increases mucosal adenosine signaling, thereby reducing the hypoxia-associated inflammation of the intestine. During hypoxia, the actin cytoskeleton is aggregated in endothelial, epithelial and vascular smooth muscle cells. This aggregation results from the disposition of IgM and complement (C3), causing further tissue injury^[152].

IMMUNOLOGY/ALLERGY

Immune-associated disease is of epidemic nature in postindustrial society. For example, adverse reactions to food are common, and true food allergies are believed to affect about 7% of children under the age of 10 years, and 2% of the adult population. These immune reactions are mediated by IgE-dependent or -independent mechanisms involving MCs, eosinophils, and other immune cells^[153].

The intestinal immune system includes Peyer's patches, isolated lymphoid follicles, mesentery lymph nodes, lamina propria mononuclear cells, and intraepithelial lymphocytes. The human intestine has unique natural killer (NK) cells derived from hemopoietic stem cells in the bone marrow. These NK cells contribute to maintenance of immune homeostasis in the intestine^[154].

Chemokines and their receptors control the influx of T and B cells into the Peyer's patches of the intestine. There is different expression of chemokines along the length of the small intestine^[155].

The M (microfold) cells are a clonal population derived from a single stem cell in the small intestinal crypts, within the follicle-associated epithelium of the Peyer's patches. M cells transport antigens and microorganisms

into the underlying lymphoid tissues. Mature enterocytes may convert to the M cell phenotype under the influence of either T and B lymphocytes or microorganisms^[156].

Macrophages in the intestinal tract are important to host defense by way of recognizing, phagocytizing and killing microorganisms. Classically, activated Th1 immune response macrophages are major effector cells in the Th1 immune responses. These mediate the production of NO by inducing NOS-2. Alternatively, activated macrophages are induced by T helper (Th)2 cytokines, including IL-4 and IL-13, as well as signal transducer and activator of transcription 6^[157].

Under normal circumstances, the gut immune system is poorly responsive to food allergens and to commensal bacteria. This hyporesponsiveness occurs through a process of immune tolerance. The Th2 phenotype is involved in development of allergic disease through release of IL-4, IL-5 and IL-13. These cytokines play a role in antigen-specific IgE production by B cells. IL-4, IL-5 and IL-13 are also important in mucous secretion, muscle contraction and eosinophilia.

IL-4 is the key Th2 cytokine involved in switching antibody responses to IgE. IL-4 induces the expression of IgE receptors on MCs, as well as on other cell types. IL-4 gene transfer to the small bowel serosa leads both to intestinal inflammation and smooth muscle hyperresponsiveness^[158]. Sensitization induces expression of CD23 on intestinal epithelial cells. This CD23 enhances IgE-dependant transepithelial antigen uptake by encoding a functional IgE receptor on human intestinal epithelial cells^[159].

A localized Th2 milieu has been observed in the intestine of subjects with food allergic disorders. A gut-homing phenotype is induced by mesenteric lymph node, with selective upregulation of the Th2 chemoattractants^[160]. TGF β is an immunoregulatory cytokine that is constitutively expressed in the intestine by epithelial cells, fibroblasts and lamina propria mononuclear cells. As noted by Di Sabatino *et al.*^[161], “one of the paradoxes of human mucosal T-cell responses in health is the dominant Th1 profile and interferon γ (IFN γ) secretion of normal human gut T cells”.

It is of interest that there is a link between intestinal immunology and motility. The Th2 cytokines are implicated in intestinal muscle dysfunction following tissue injury. Products of the innate and adaptive immune responses contribute to the activation and sensitization of primary sensory neurons. T lymphocytes are an important determinant of visceral nociception, and may provide an important opioid-mediated antinociceptive influence in the gut^[162].

Cell surface molecules are required for development of intestinal allergy^[163]. Disruption of the enteric glial cell (EGC) network is associated with intestinal and mesenteric T-cell infiltration, leading to Th1 cytokine-associated bowel inflammation. EGCs express major histocompatibility complex (MHC) class I, and regulate MHC class II in persons with bowel inflammation. CD8 and CD4 T

cells trigger autoimmunity. Direct presentation of antigen by lymph node stromal cells protects against CD8 T-cell-mediated intestinal autoimmunity^[164].

CD23 is a tight II intercoil membrane glycoprotein with a carboxy terminal C-type lectin head that binds its ligand, IgE, in a calcium-dependent manner. CD23a is expressed constitutively on mature activated B cells, whereas CD23b is induced by IL-4 or IL-23. CD23a is increased in food-allergic patients, and CD23a is expressed by primary human IECs. The increase in antigen uptake by specific IgE is through the process of diverting antigen from delivery to lysosomes. Delivery of antigen-IgE complexes across the intestinal epithelial barrier may induce deactivation of MCs, and contribute to food-induced pathophysiology of the GI tract^[165].

The *in vitro* treatment of smooth muscle tissue with IL-1 β decreases expression of CPI-17. Smooth muscle serine-threonine protein phosphorylases are endogenous inhibitory proteins. This TNF- α -mediated process inhibits muscle contraction in the small intestine^[166]. It is not yet known whether intestinal motility may be modulated for therapeutic purposes by immune alterations.

The vagus nerve regulates GI motor and digestive functions through the release of acetylcholine (ACh). This parasympathetic neurotransmitter activates mAChRs, the nicotinic ACh receptors. The afferent vagal nerve, working through the hypothalamic pituitary adrenal axis, acts to inhibit the activation of NF- κ B. This reduces proinflammatory cytokine secretion by macrophages, as well as modifying macrophage endocytosis and phagocytosis. Thus, “vagus nerve efferent activity may stimulate surveillance in the intestinal mucosa and peritoneal compartment^[167]. This role of the vagus nerve awaits study as a potential therapeutic target in persons with allergies.

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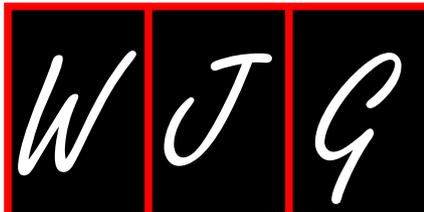
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Recent advances in small bowel diseases: Part II

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Abstract

As is the case in all areas of gastroenterology and hepatology, in 2009 and 2010 there were many advances in our knowledge and understanding of small intestinal diseases. Over 1000 publications were reviewed, and the important advances in basic science as well as clinical applications were considered. In Part II we review six topics: absorption, short bowel syndrome, smooth muscle function and intestinal motility, tumors, diagnostic imaging, and cystic fibrosis.

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ABSORPTION

Triglycerides

For triglycerides (TG), it has been traditionally considered that lipid uptake is by way of passive permeation through the lipophilic portion of the intestinal brush border membrane (BBM). However, enterocyte-binding or transport proteins have been identified as also being important in this process^[1]. Gata4 is a zinc-containing transcription factor, expressed in the epithelium of the upper small intestine, and functions to assist in fat and cholesterol absorption^[2]. Lipid micelles at the BBM modulate a large number of genes, and this transcriptome responding to dietary lipids has an impact on cell architecture, signaling and metabolism genes^[3]. Most lipids are in the enterocyte, and may be bound to the liver and the intestinal fatty acid (FA) binding proteins (L-FABP and I-FABP). L-/I-FABP function to translocate long chain FAs and monoacylglycerol from the BBM to the endoplasmic reticulum (ER). These long FAs are then used in the resynthesis of diacylglycerol and then triacylglycerol.

The absorption of dietary TG in the small intestine is accompanied by a rise of intestinal alkaline phosphatase (IAP) in the serum, and by the secretion of IAP-containing surfactant-like particles (SLPs) from the enterocytes. IAP is a membrane-bound protein that hydrolyses monophosphate esters at high pH optimum, and limits fat absorption by enterocytes by way of its action as a SLP^[4]. Translocation of IAP across the enterocyte BBM occurs within 5 min of lipid intake by way

of induction of endocytosis *via* clathrin-coated pits^[5]. After fat has been taken up into the enterocyte, IAP is incorporated into membranes surrounding intracellular lipid droplets, and is also incorporated into basolaterally secreted SLPs. IAP is not associated with chylomicron formation, but rather with chylomicron secretion. Serum IAP levels are correlated with levels of apolipoprotein B-48 (apoB48), a protein exclusive to intestinal chylomicrons in humans^[6].

After ingestion of a meal rich in TG, the small intestine continues to form very low density lipoprotein (VLDL), but the predominant TG-rich lipoprotein particles secreted in this postprandial condition are the larger chylomicron particles^[7]. In the liver, TG is synthesized and packaged with apoB100 to form VLDL particles, whereas chylomicrons produced by the human gut contain apoB48. ApoB48 provides efficient chylomicron formation and lipid absorption. Apolipoprotein A-IV synthesis in the small intestine is regulated by lipid absorption, and plays a role in the regulation of chylomicron assembly and secretion.

Hepatocyte nuclear factor-4 α (HNF-4 α) is a nuclear receptor that regulates gene expression during enterocyte differentiation. HNF-4 α is also involved with the transcription of genes involved in lipid metabolism, such as *Apo-IV*^[8]. In newborn swine intestine, dietary lipid causes ligand-independent transactivation of HNF-4 α to induce Apo-A IV and microsomal triglyceride transfer protein (MTP).

The uptake of FAs across the BBM may be partially passive and partially facilitated, mediated by the multiligand scavenger protein CD36. CD36 also participates in the orosensory detection of lipids and the production of the sensation of satiety. Thus, CD36 may play a role in lipid preferences and feeding behaviour^[9]. Monoglyceride and free FAs in the cytoplasm reform TG by the successive actions of monoacylglycerol acyltransferase and diacylglycerol acyltransferase at the membrane of the smooth ER. After transfer in the ER lumen, TG droplets associate with primordial lipoprotein comprising apoB48 and phospholipids through the actions of MTP, to form TG-rich lipoproteins (TRL). The lipid droplets fuse with apoB48 plus a resident ER chaperone, MTP. MTP-dependent fusion of lipid droplets with apoB48 in the ER is the crucial restriction point in the formation of chylomicrons. The lipoprotein particle enlarges as more TG is added to the droplet. The maturing lipoprotein particles (prechylomicrons) undergo vectorial vesicular transport through the Golgi membranes. Chylomicrons cross the basolateral membrane (BLM) and into the lacteals.

Glucagon-like peptide-2 (GLP-2) increases lipid absorption, but how does this occur, when enterocytes have no GLP-2 receptors? Perhaps the GLP-2 acts on the enteroendocrine L cells, releasing insulin-like growth factor (IGF-1). GLP-2 increases the glycosylation of CD36 and increases the number of chylomicrons.

After 1 wk of feeding with a high fat diet (HFD) in mice, there is repression of genes involved in FA syn-

thesis, and an increased expression of genes involved in lipoprotein assembly (*apoB*, *MTP*, *apoA-IV*). This process may be coordinated by an increase in the transcription factor SREBP-IC^[10]. The number of secreted chylomicrons falls, but there are larger chylomicrons containing increased associated TG, as well as increased amounts and activity of MTP. These changes result in postprandial hypertriglyceridemia, but normal fasting levels of TGs. This postprandial hypertriglyceridemia in the absence of changes in fasting levels may explain some of the risk factors for the development atherosclerosis and cardiovascular diseases.

Cholesterol

Dietary and biliary cholesterol are solubilized by bile acid micelles in the upper intestinal lumen. These are large negatively-charged unilamellar vesicles, smaller mixed micelles or monomeric bile acids. Bile acids promote cholesterol absorption and reduce cholesterol synthesis^[11].

It is now recognized that intestinal absorption of cholesterol is a complex process, involving both BBM permeation and cotransporters^[12,13]. Uptake of cholesterol from the intestinal lumen across the enterocyte BBM is also mediated by at least five proteins: Niemann-Pick C1-like 1 (NPC1L1), the scavenger receptor B-1 (SR-B1), CD36, the ATP-binding cassette protein 5 (ABCG5) and ATP-binding cassette protein 8 (ABCG8) ATP-binding cassette transporters^[14,15]. NPC1L1 protein is predominantly expressed in the liver and in the proximal intestine^[16]. Modulation of NPC1L1 expression is by cholesterol, as well as by the involvement of several nuclear receptors, such as liver X receptor (LXR), peroxisome proliferator-activated receptor (PPAR)- α , and by sterol regulatory element (SRE) binding proteins (SREBPs). SREBPs are transcription factors which regulate cholesterol synthesis and metabolism^[17]. SREBP-2 activates the NPC1L1 promoter, which has two sterol regulatory elements.

The ATP-binding cassette transporter ABCG1 promotes cholesterol efflux across the BLM and out of the enterocyte. In contrast, ABCG5/G8 facilitates cholesterol efflux back across the enterocyte BBM and into the intestinal lumen^[18]. The ATP-binding cassette transporters are target genes of the nuclear receptor LXR. Mice on a high-fat cholesterol free diet have reduced or downregulated NPC1L1, ABCA1, ABCG5, and ABC8, reduced fractional cholesterol absorption, and a posttranslational increase in 3-hydroxy-3methylgluteral-coenzyme A reductase activity. Downregulation of cholesterol transporters is independent of LXR A^[19].

NPC1L1 also occurs in intracellular compartments, and is involved as well in the absorption of dietary saturated FAs such as steric and palmitic acids^[20]. The drug ezetimibe binds NPC1L1, reduces intestinal absorption of cholesterol as well as saturated FAs, and reduces weight gain in animals fed a diabetogenic diet. In this way, the drug may protect against diet-induced hyperglycemia and insulin resistance^[20]. NPC1L1 and the FA translocase (FAT/CD36), as well as scavenger receptor class B type 1 (SR/B1)

transporter protein, have been shown to be influenced by 5 mmol/L glucose in the intestinal lumen; enhancing protein expression of NPC1L1 and CD36, decreasing SR/B1 protein, but having no effect on the protein expression of ABCA1 and ABCG8^[21]. Higher intraluminal glucose concentration increases 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity, increasing the transcription factors LXR- α and LXR- β , PPAR- β and PPAR- γ , as well as SREBP2. Thus, reducing the luminal concentration of glucose will also reduce uptake of cholesterol.

Aging enhances cholesterol absorption by suppressing expression of the sterol efflux transporters ABCG5/G8. In contrast, estrogen enhances cholesterol absorption due to upregulated expression of NPC1L1, ABCG5 and ABCG8^[22]. Cholesterol absorption is also enhanced in diabetes; medium levels of glucose concentration in Caco-2 cells in culture increase cholesterol uptake as well as the expression of NPC1L1 and CD36 proteins^[21].

Liver FA binding protein (L-FABP) increases FA uptake, intracellular transport, esterification, and oxidation in transfected transformed cells, and gene-ablated mice with no L-FABP show reductions in these steps of FA metabolism^[23]. L-FABP may also play a role in hepatic cholesterol metabolism^[24].

Phytosterols are cholesterol-like compounds found in plants, which reduce cholesterol absorption and plasma concentrations of low density lipoprotein cholesterol. Natural phytosterol glycosides purified from lecithin are bioactive in humans^[25].

Two Na⁺-coupled (SMIT1 and SMIT2) and one H⁺-coupled (HMIT) secondary active intestinal transporters for myo-inositol have been identified^[26].

One hypothesis suggests that cholesterol is absorbed by an energy independent passive diffusion process regulated *via* a concentration gradient^[14]. The second hypothesis proposes that cholesterol is absorbed through an energy-dependent, protein-mediated process^[27].

NPC1L1: NPC1L1 is the main cholesterol transporter in the jejunal BBM^[16]. NPC1L1 shares 42% amino acid homology with Niemann-Pick type C1 protein (NPC1), a protein involved in the intracellular transport of cholesterol^[28]. Post-translationally, NPC1L1 moves from internal membranes to the mucosal membrane during cellular cholesterol depletion, facilitating absorption^[29]. Other studies suggest that NPC1L1 is located at the BBM of enterocytes^[30]. NPC1L1 mRNA expression appears to be positively correlated with plasma apoB48 and chylomicron cholesterol content^[31].

Scavenger receptor B1: Scavenger receptor B1 (SRB1) is highly expressed in the BBM of the proximal small intestine^[32]. Intestinal SRB1 overexpression in transgenic mice has been associated with increased cholesterol absorption^[33]. Moreover, antibodies against SRB1 demonstrate abolishment of high affinity binding of cholesterol to BBM vesicles that would normally be observed in *NPC1L1*^{-/-} mice^[32]. SRB1 may play a role in the initial step of cho-

lesterol absorption by facilitating high affinity cholesterol binding to the mucosal BBM, but alternative cholesterol transporters may compensate for the absence of SRB1 in mediating cholesterol absorption in KO models^[32].

FAT/CD36: FAT/CD36 (translocase), a human analogue of SRB1, is expressed along the BBM of the duodenum and jejunum. CD36 deficiency correlates with abnormal lipid processing in enterocytes^[14].

ABCG5/8: ABCG5 and ABCG8 are located at the enterocyte BBM^[14]. Their expression is greatest in the duodenum and jejunum, where they work in tandem to efflux cholesterol (mainly plant sterols) from the enterocyte back into the lumen for excretion^[34]. A negative correlation exists between ABCG5/8 and chylomicron cholesterol content^[31]. Mutations of *ABCG5* and *ABCG8* in humans enhance intestinal cholesterol absorption, and predisposes these individuals to atherosclerosis^[35].

ATP-binding cassette protein 1: ATP-binding cassette protein 1 (ABCA1) not only mediates cholesterol efflux from the basolateral surface of enterocytes to high-density lipoprotein^[36], but it also contributes to the efflux of cholesterol out of the enterocyte and back into the intestinal lumen^[37].

Bile acids

Bile acids are synthesized from cholesterol in the liver, secreted into the bile ducts, stored in the gallbladder, and intermittently released into the duodenum in response to a meal, where bile acids solubilize lipids in the intestinal lumen by formation of micelles^[38]. Bile acids dissociate from the lipids which they stabilize prior to the uptake of the lipids across the BBM of the proximal intestine. The bile acids are absorbed passively along the length of the small intestine. In the ileum, enterocyte BBM sodium-dependent bile acid transporters (ASBTs) also mediate bile acid uptake and bile acids are returned to the portal circulation. This is known as the “enterohepatic” circulation of bile acids. ASBT is, in effect, a salvage mechanism for luminal bile acids, providing for maintenance of cholesterol homeostasis, as well as for efficient lipid absorption.

The apical ASBT in the lipid rafts of the ileal BBM functions in concert with hepatic bile acid efflux transporters to regulate hepatic bile acid synthesis from cholesterol. One of the green tea catechins decreases the maximal transport rate (V_{max}) of ASBT, without altering its content in the BBM. This reduction in V_{max} is achieved by moving the transporter out of the lipid rafts^[39]. This suggests a role for lipid rafts in the modulation of the function of this transporter, reducing the size of the bile acid pool, stimulating the hepatic synthesis of bile acids from cholesterol, and thereby reducing the serum concentration of cholesterol.

Initially, ASBT in the enterocyte cytosol undergoes vesicular trafficking to microdomains in the BBM. These

ASBT lipid rafts are enriched with sphingolipids and cholesterol. Alterations in cholesterol content of the BBM lead to rapid modulation of the activity of ASBT^[40]. Obstructive cholestasis leads to downregulation of ASBT mRNA expression. Thus, luminal bile acid levels may be involved in regulation of ASBT gene regulation^[41]. In patients with ileal inflammation, such as Crohn's disease, reduced bile acid transport may be due to diminished ASBT protein expression, as the result of ASBT inhibition by inflammatory cytokines *via* direct interactions of c-fos with the ASBT promoter^[42].

Once bile acids are in the ileal enterocytes, they bind to ileal bile acid-binding protein (I-BABP). Organic solute transporters (Ost) α and β are located in the BLM of the ileocytes. Ost α /Ost β expression is induced by bile acids through ligand-dependent transactivation of both *Ost* genes by the nuclear bile acid receptor/farnesoid X receptor (FXR)^[43]. "By coordinated control of Ost α /Ost β expression, bile acids adjust the rate of efflux from enterocytes in response to changes in intracellular bile acid levels". Ost α is a seven transmembrane domain protein, and Ost β is a single transmembrane domain polypeptide. Ost α -Ost β is the major BLM transporter of bile acids and conjugated steroids in the intestine, as well as in the kidney and liver^[44]. Ost α and Ost β promoters harbor both FXR and liver receptor homolog-1 (LRH-1) elements. FXR and LRH-1 mediate positive- and negative-feedback regulation, respectively^[45].

When the BBM uptake of bile acids is impaired, excess bile acids spill into the large intestine, where bile acids stimulate cAMP and cause a secretory diarrhea. The locally-acting steroid budesonide is beneficial for the symptoms of collagenous colitis, which in turn is associated with bile acid malabsorption. This clinical benefit may be due in part to stimulation of bile acid absorption, with decreased bile acids entering the colon, less stimulation of cAMP, and less secretory diarrhea^[46].

Glucose-dependent insulinotropic polypeptide (GIP) is a potent insulin secretagogue. GIP is an incretin, a gut factor released after intestinal transport of hexoses, long-chain FAs and TG, and GIP stimulates insulin secretion at physiological concentrations. GIP is secreted by enteroendocrine K cells in the proximal small intestine. Intestinal lymph contains high concentrations of GIP that respond to both enteral carbohydrate and to fat absorption. The combination of glucose and lipid has a potentiating effect on stimulation of GIP secretion in lymph fistula rats^[47].

Approximately 25% of individuals with irritable bowel syndrome (IBS) have a previous history of enteric infection, such as with *Campylobacter* or *Salmonella*. Persistent chronic diarrhea is more frequently associated with infectious IBS, and bile acid malabsorption may be observed in as many as a third of patients with diarrhea-predominant IBS. In a mouse model of IBS, it was shown that ileal uptake of bile acids was reduced. Surprisingly, this was associated not with a decrease but rather with an unexpected increase in expression of the

BBM Na⁺-dependent bile acid transporter (ASBT)^[48].

Bile acids act as detergents to solubilize lipids, but also act as signaling hormones: bile acids activate the G-protein-coupled receptor TGR5, resulting in changes in energy expenditure and glucose homeostasis, as well as having an anti-inflammatory role. Novel patent and selective bile acid derivatives are being developed as TGR5 agonists for possible therapeutic enhancers^[49].

Bile acids are synthesized from cholesterol. In the neutral pathway, the rate-limiting enzyme β hydroxylase (Cyp7a7) converts cholesterol to 7-hydroxycholesterol. In the attenuated acidic pathway in mitochondria, sterol 25-hydroxylase or 27-hydroxylase hydroxylates the cholesterol, and a 7 β hydroxyl group is added from catalysis by oxysterol 7 β hydroxylase (Cyp7b1). The ring structure is then modified, and the side chain is oxidized and shortened, and further hydroxylation occurs to form the primary bile acids, cholic and chenodeoxycholic (chenic) acid. Bile acids regulate their own synthesis by way of negative feedback on the transcription of the rate-limiting enzyme, Cyp7a1. When bile acid concentrations are high, there is activation of the nuclear FXR, which leads to increased transcription of short heterodimeric partner (SHP). Cyp7a1 is activated by the SHP-dependent as well as by the SHP-independent pathway.

The small size of the bile acid pool in neonates is increased as the result of elevated mRNA levels of FXR and SHP, and later by an increase in mRNA and protein levels of Cyp7a1^[50]. The increase in Cyp7a1 levels and therefore the increased synthesis of bile acids occurs independently of FXR and SHP, and is not influenced by the administration of sterols^[50].

Gangliosides

Gangliosides are sialic acid-containing glycosphingolipids which are found in lipid rafts in outer plasma membranes, such as the BBM of the small intestine. The oligosaccharide portion of the ganglioside faces the cell surface, whereas the lymphatic ceramide portion is anchored into the inner (cytosolic side) layer of the BBM. In the rat intestine, 34% of the glycosphingolipids are gangliosides. The amount of ganglioside in the membranes varies along the intestine, being higher distally than proximally. Gangliosides differ depending upon whether ingested in micelles or unilamellar vesicles. GM3 is localized on the BBM whereas GD3 is mainly localized on the BLM. GD3 uptake into Caco-2 cells is greater across BLM than BBM, and gangliosides taken up by the BLM are largely metabolized by these enterocyte-like cells^[51]. In contrast, GD3 uptake across the BBM is time- and concentration-dependent, reaches a plateau, and the GD3 is metabolized, stored, or transported out of the cell across the BLM. GD3 is found in milk and colostrum, and feeding GD3 increases its content in the intestinal lipid rafts, and in the blood membrane: the main ganglioside in the BBM is GM3, whereas GD3 is the main ganglioside in the BLM. This raises the possibility of the oral use of gangliosides to modify or to enhance some of their functions,

such as regulating cell signaling, protein functions, as well as the recognition of microbes and macromolecules.

Sugars

SGLT1, the Na⁺-glucose cotransporter in the enterocyte BBM, is a secondary active transport process which requires a favorable intracellular Na⁺ gradient. This gradient is provided by Na⁺-K⁺-ATPase on the BLM of enterocytes. Constitutive nitric oxide (cNO) has opposite effects on the two primary Na⁺-absorptive pathways in the mammalian small intestine: reducing cNO inhibits SGLT1 and stimulates the Na⁺/H⁺ hydrogen exchanger NHE3^[52]. cNO also regulates mucosal blood flow, mucous secretion, and intestinal motility. The glucocorticoid-inducible kinase-1 (SGK1) stimulates SGLT1 as well as NHE3. The effects of glucocorticoid on SGLT1 are fully dependant on SGK1, whereas for NHE3 the effects of glucocorticoids also involve some additional processes^[53].

During chronic intestinal inflammation, there is a transcription-mediated decrease in the number of glucose transporters. This is possibly due to altered binding of Sp1 and Hnf1, transcription binding sites for the SGLT1 promoter regions^[54].

When glucose is taken by mouth, there is a fast rise in expression of SGLT1. Intestinal sugar uptake is increased in diabetes and in obesity. Roux-en-Y gastric bypass (RYGB) is a successful form of bariatric surgery. RYGB reduces glucose absorption in the Roux limb, as well as in the remaining intestine^[55].

Fructose is prevalent in the diet either as a free hexose, as the disaccharide sucrose, and in the polymerized form, fructans. About 50% of adults are unable to absorb a 25 g load of fructose. Fructans are neither hydrolyzed nor absorbed in the small intestine. This osmotic load may alter intestinal motility and change the microbiota by producing a mucosal biofilm. Restricting dietary intake free of fructose and/or fructan has symptomatic benefits in some persons with diarrhea and bloating^[56].

The revised SLC Transporter Gene Tables are available online at <http://www.bioparadigms.org/slc/intro.htm>.

Carbohydrate malabsorption, as assessed by hydrogen breath testing, is common in persons with Crohn's disease (CD) and celiac disease (CeD)^[57]. The absolute increase in the rate of fructose malabsorption is about 20% higher in Crohn's disease, and lactose malabsorption is 30% higher.

The BBM hydrolysis of carbohydrates takes place by the BBM-bound glycoproteins sucrase-isomaltase (SI), maltase-glucoamylase, and lactase-phlorizin hydrolase (LPH). The pro-SI passes from the ER to the Golgi apparatus. With glycosylation it becomes targeted to the BBM, where it is cleaved by trypsin to form sucrase and isomaltase. Compound heterozygous mutation defects in the protein folding, the direct interaction between sucrase and isomaltase, and an intermolecular chaperone included in the intracellular transport of SI, all have a role in the development of congenital sucrase-isomaltase

deficiency^[58]. Congenital lactase deficiency results from mutations in the coding region of LPH, with misfolding of LPH which prevents the mutant protein from exiting the ER^[59].

Amino acids and proteins

The numerous BBM transporters for amino acids are differentiated functionally by their substrate specificity and driving forces. Neutral amino acids are transported by the system B0+ (Na⁺-dependent transporter for neutral and cationic amino acids), as well as by the ASC system (Na⁺-dependent transporter for mid-size neutral amino acids).

Glutamine comprises approximately 20% of the total amino acid content in the human blood stream, and as such is an important amino acid. Glutamine is the preferred substrate for enterocytes, and is also important for mucosal integrity and the intestinal permeability barrier. The Na⁺-glutamine cotransporter in the BBM of the enterocyte is B0AT1 (SLC6A19)^[60]. Glutamine is converted to citrulline in the enterocytes. A citrulline generation test has been developed to assess enterocyte function, and the value of the slope from baseline to peak plasma citrulline concentrations is reduced in persons with celiac disease^[61].

Under the influence of cholecystokinin (CCK), bile and pancreatic enzymes are secreted into the duodenal lumen where the pancreatic proteolytic enzymes (trypsin, chymotrypsin, elastase, carboxypeptidase A and carboxypeptidase D) digest proteins and polypeptides into peptides, which are usually 2-6 residues in length. Conjugated bile acids accelerate protein hydrolysis by pancreatic proteases^[62].

During chronic intestinal inflammation, there is a decrease in the activity of several transporters such as the short-chain FA-bicarbonate exchanger, H⁺-dipeptide cotransporter, Na⁺-amino acid transporter, Na⁺-glucose cotransporter 1 (SGLT-1), and Na⁺-bile acid transporter. There may be a decreased number of SGLT -1 transporters in villus cells (lowering the value of the maximal transport rate, Vmax), and decreased affinity of the cotransporter for Na⁺-neutral amino acid transport (increasing the value of the affinity constant, Km).

For amino acids, the reduction in transport during chronic inflammation arises from a decrease in the affinity of the transport systems, and may be mediated through an increase in leukotriene D4 (an eicosanoid pathway product), which is released in chronic inflammation^[63].

The proton-amino acid transporter 1 (PEPT1) transports small neutral amino acids as well as small peptides, through mediation of an inwardly directed H⁺ gradient across the enterocyte BBM. PEPT1 also transports drugs such as β -lactam and angiotensin-converting enzyme inhibitors. PEPT1 is under diurnal variation, relating to food intake. It is also influenced by transcription factors, such as Sp1, Cdx2, and PPAR- α . Leptin treatment increases enterocyte uptake of di- and tripeptides *via* the PepT1 transporter, through transcription activation of

the MAPK pathway as well as translational activation *via* ribosomal protein S6^[64].

The albumin D site-binding protein (DBP) expression is regulated in a circadian manner by oscillators called “circadian clocks”. These circadian clocks reside in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. This clock system consists of single-cell circadian oscillators that are composed of several clock genes. The expression of DBP is in phase with that of PEPT1. DBP binds to the DBP binding site in the distal promoter region of the *PEPT1* gene, and thereby induces transcriptional activity^[65].

The *SLC6A19* gene encodes the main sodium-independent BBM transporter for neutral amino acids, B0AT1. The expression of B0AT1 requires angiotensin-converting enzyme 2 (ACE2)^[66]. It is unknown whether the use of inhibitors of ACE2 in humans alters the protein homeostasis of the body by way of inhibiting the intestinal uptake of neutral amino acids in the small intestine (as well as in the proximal tubule of the kidney).

Small peptides are absorbed predominantly in the proximal small intestine, and free amino acids in the distal intestine. The uptake of sugars is increased by a high carbohydrate diet by upregulation of the Na⁺-dependent BBM glucose transporter SGLT, and the amino acid alanine also controls its own absorption, through capsaicin-sensitive primary afferent neuronal fibers as well as by the peptide calcitonin gene-related peptide, CGRP^[67].

Glutamines become conditionally essential during metabolic stress. Glutamine prevents apoptosis and also plays a role in regulating glucose metabolism. For example, during fasting as well as in diabetes, the intestine by way of its glucose-6 phosphatase (G6Pase) contributes about a quarter of endogenous glucose production through gluconeogenesis.

After a RYGB, used to treat severe obesity, the glucose sensing vagal afferents in the portal vein influence glucose homeostasis. After RYGB, the absorption of glutamine by B0AT is increased in both the biliopancreatic (3.8-fold increase) and the Roux limbs (1.4-fold increase), but not in the common channel. The levels of glutaminase are also increased, but the levels of GEPase (intestinal gluconeogenesis) and PEPCK-C (cytosolic phosphoenolpyruvate carboxykinase, a measure of glutamine metabolism) were not seen to be affected^[68].

Biotin

Biotin is a coenzyme for the “carboxylases” which catalyze essential steps in FA biosynthesis, gluconeogenesis, and catabolism of several amino acids and FAs. Biotin is essential for cellular functions including growth and development. The human intestine utilizes the sodium-dependent multivitamin transporter (hSMVT) for biotin uptake across the enterocyte BBM^[69]. The uptake process is adaptively regulated during biotin deficiency, by induction of protein and mRNA levels of hSMVT, mediated by transcriptional regulatory mechanisms.

Two other functionally unrelated nutrients, the water-

soluble vitamin pantothenic acid and the metabolically important antioxidant lipoate, share the biotin transport system (hSMVT).

Iron

Dietary non-heme ferric (Fe³⁺) iron in the intestinal lumen is reduced to the ferrous (Fe²⁺) form by cytochrome b reductase1 (Cybrd1) in the BBM of the mature villus enterocytes of the proximal small intestine. Iron is transported across the BBM by the divalent-metal ion transporter 1 (DMT1). DMT1 is also known as solute carrier family 11, member 2, (SLC11A2). Fe²⁺ is transported through the cytoplasm of the enterocyte, and is then transferred across the BLM of the enterocyte and into the body by the BLM exporter ferroportin (solute carrier family 40, 1, Slc40a1). There is coordinated expression of ferroportin in enterocytes as well as in tissue macrophages^[70].

Ferroxidase (hephaestin) in the BLM promotes the conversion of Fe²⁺ to Fe³⁺. Once the Fe³⁺ is in the vascular system, it binds to transferrin (Tf). There are two mechanisms by which mRNA levels of iron homeostasis-related genes are regulated; firstly, by post-transcriptional mechanisms mediated by the iron response element/iron regulatory protein system, and secondly, by mechanisms related to transcriptional regulation. In peripheral tissues, the Tf-Tf receptor (TfR) system delivers iron in the Tf-Fe loaded TfR by way of endocytosis. Unlike TfR1, TfR2 mRNA does not contain an Fe-responsive element, and TfR2 mRNA expression is not regulated by intracellular Fe levels. Instead, hepatic TfR2 protein is regulated post-translationally by diferric (Fe²⁺) transferrin. In this way, TfR2 is a sensor of body iron status, and regulates duodenal Fe²⁺ absorption and liver Fe³⁺ uptake^[71]. Hephaestin expression also occurs in gastric antrum, enteric nervous system and pancreatic β -cells^[72].

Fe²⁺ uptake across the enterocyte BBM responds to body iron stores, whereas transport across the BLM is regulated by the enterocyte iron status. When the enterocyte intracellular ferritin level is increased, iron will be transferred across the BLM and into the portal blood. In addition to a potential cytoplasmic route for iron across the enterocyte, there is evidence of vesicular transport or transcytosis of apotransferrin (apoTf). Approximately half of iron transfer across the enterocyte BLM is by way of apoTf and non-apoTf-dependent vesicular pathways^[73].

Factors that affect hepcidin have recently been reviewed and include body iron stores, rate of erythropoiesis, hypoxia and inflammation^[74]. The amount of iron absorbed is regulated by the hepatic synthesis of hepcidin. The most common inheritable form of iron overload is an autosomal recessive disorder caused by mutation in the *HFE* gene, HFE-associated hereditary hemochromatosis (HH). *HFE* codes for a major histocompatibility complex class I (MHC-I)-like molecule. HFE also needs to be associated with a β 2 microglobulin for its appropriate expression of the cell surface.

HFE modulates the expression of hepcidin in the liver. HFE may influence iron status by acting on hepatocytes and/or Kupffer cells, as well as on duodenocytes^[75]. Hepcidin inhibits cellular efflux of iron by binding to and inducing degradation of ferroportin^[76]. Hepcidin causes ferroportin on the BLM to be internalized and degraded^[77]. In macrophages, hepcidin inhibits iron export by inducing ferroportin degradation, whereas in enterocytes hepcidin inhibits DMT1 transcription and thereby reduces BBM iron uptake^[76,78]. Other critical regulators of systemic iron homeostasis are intestinal hypoxia-inducible transcription factors (HIFs)^[79]. HIFs (HIF-1 and HIF-2) are critical mediators of cellular adaptation to hypoxia. HIF-2 α , but not HIF-1 α , promotes iron absorption in mice^[80].

The normal decline in intestinal iron absorption which occurs from neonatal to adult animals is due to loss of the iron transporters (particularly ferroportin) from the distal small intestine and colon^[81]. Curiously, in iron deficiency there is altered intestinal lipid metabolism resulting in production of biologically active lipid molecules (12-HETE, 13-HODE and 13-HOTE), arising as a result of changes in arachidonate 12-lipoxygenase (Alox15)^[82]. It is unknown if this has any clinical significance.

The cytochrome b reductase in the BBM of the duodenal enterocytes (Dcyt6) reduces dietary iron from Fe³⁺ to Fe²⁺. Fe²⁺ is transported across the BBM by divalent metal transporter (DMT). The Fe²⁺ is transported into vesicles containing either ferroportin (FPN1) or hephaestin (Heph). These Fe²⁺-containing FPN1 and Heph-containing vesicles cross the enterocyte cytoplasm to the BLM. The Heph oxidizes the Fe²⁺ to Fe³⁺. Fe³⁺ binds to transferrin and is released into the circulation^[83]. Hepcidin is secreted from the liver in response to the body iron stores: increased body iron stores result in increased hepcidin, decreased FPN1 mRNA expression and increased FPN1 internalization and degradation. The end result of this repositioning of the FPN1 from the BLM is to reduce iron efflux from across the BLM of the duodenocyte, and thereby decrease iron absorption.

Heph, therefore, is a protein in the BLM of the duodenum which has ferroxidase activity to oxidize dietary Fe²⁺ to Fe³⁺. Heph is also found by immunocytochemistry to extend from the gastric antrum along the length of the entire GI tract, and to be present in both the submucosa and the myenteric plexi of the entire nervous system^[72].

In HH, the variable phenotypic expression of the homozygous HFE C282Y genotype has been attributed to possible disease-modifying genes which affect the iron transporters. In HH “expressors” and “nonexpressors”, there is a significant difference in the expression of DMT1 and DMT1 (IRE), such that HFE C282Y homozygotes without phenotypic expression do not have significantly decreased duodenal gene expression of non-transport genes compared with HH subjects with iron overload^[84]. Also, regardless of phenotype, “...there is coordinated regulation between duodenal expression of

FPN1 [ferroportin] and DMT1 [divalent metal transporter 1], FPN1 and DCYTB [ferriductase duodenal cytochrome b] and FPN1 and HEPH [ferroxidase hephaestin] and also DCYTB and HEPH...”.

Calcium

Canonical transient receptor potential (TRPC)1 acts as a calcium channel, with the total calcium effect being mediated by calcium influx through calcium-permeable channels in the plasma membrane, as well as calcium release from intracellular stores such as the ER and cytoplasmic reticulum^[85].

Much of our understanding of calcium (Ca²⁺) absorption has come from studies in animals. Ca²⁺ enters the enterocyte across the BBM using TRPV₆ (aka CAT, or ECAC₂), a Ca²⁺ channel. Intracellular Ca²⁺ is bound to calbindin-D9K, maintaining a low intracellular concentration of free Ca²⁺. PMCA (a Ca²⁺ ATPase) pumps Ca²⁺ across the BLM. The major storage form of vitamin D is 25-hydroxy vitamin D (25OHD). In humans, 25OHD is metabolized by the gene product of CYP27B1 [25-hydroxy vitamin D 1 α -hydroxylase (1 α OHase)] to the biologically active 1 α , 25-dihydroxycholecalciferol [1,25(OH)₂O₃]. 1 α OHase forms 25OHD, which increases the transcription of TRPV₆, PMCA, and CYP₂₄, thereby enhancing Ca²⁺ absorption^[86].

The active hormonal form of vitamin D is 1.25 dihydroxyvitamin D₃ [1,25(OH)₂D]. 1,25(OH)₂D activates the vitamin D receptor (VDR) which heterodimerizes with the retinoid X receptor to interact on response units such as the apical membrane Ca²⁺ channel, TRPV₆ (the transient receptor potential cation channel, subfamily V, member 6), and the Ca²⁺ binding protein calcium binding protein D9k (calbindin D9k). VDR and 1,25(OH)₂D, acting on TRDV₆ and calbindin D9k, maintain high rates of Ca²⁺ absorption^[87].

Copper

Copper is a mineral essential for normal growth and development. The level of copper in the body is regulated, because excessive amounts may be toxic. The copper transporter (Ctr1) is copper-specific; its transport function is energy-independent and saturable. Copper efflux from enterocytes across the BLM is mediated by ATP7A. The ability of suckling rat pups to tolerate varying amounts of dietary copper may be due to changes in copper transporters, Ctr1 and ATP7A, facilitated by transcriptional and post translational mechanisms^[88].

The Steap proteins on the BBM reduce copper to the cuprous form, which is then transported by Ctr1 across the BBM. In the enterocyte, copper is bound to chaperone Atox1, and reaches ATP7A for export across the BLM^[89]. When copper intake is high, Ctr1 is endocytosed into the enterocyte, where there is induction of the copper-binding protein metallothionein, and ATP7A moves to a more basal lateral location. Maturation of small intestinal copper transport occurs by way of increased abundance and local alteration of Ctr1, ATP7A

and ATP7B.

Zinc

The intestinal absorption of zinc is regulated to meet zinc requirements in the body. ZIP4 is a major intestinal zinc transporter; absorptive upregulation of ZIP4 enhances the uptake of zinc from the intestinal lumen, replenishing any deficiency. When the zinc content of the diet is low, there is induction of the transcription factor Kruppel-like factor 4 (KLF4)^[90], which leads to increased intestinal zinc uptake, thereby preventing disease manifestations of zinc deficiency such as acrodermatitis enteropathica.

SHORT BOWEL SYNDROME AND TRANSPLANTATION

“Intestinal failure” refers to a condition in which inadequate digestion and/or absorption of nutrients leads to malnutrition and/or dehydration. The most common condition resulting in intestinal failure is the short bowel syndrome (SBS). The SBS occurs following massive resection of the small intestine^[91]. SBS may be defined anatomically as less than 30% of the normal intestinal length (less than 200 cm in adults). In the United States, the estimated annual prevalence of SBS in patients who have non-malignant intestinal disease, and who require home parenteral nutrition, is at least 4 per 10⁵. The point prevalence is reported to be between 0.6 and 12.7 per 10⁵. The commonest cause of SBS is surgical resection of small intestine for Crohn’s disease. Other common causes include mesenteric infarction, congenital abnormalities, and multiple strictures due to adhesions or abdominal irradiation.

Early in the adaptive response after an intestinal resection, there is an increase in proliferation of intestinal epithelium, with increased depth of crypts, increased villous height, and increased microvillous surface area. The process of adaptation involves the presence of luminal nutrients, gastrointestinal secretions, the mesenchyme, as well as neuronal and hormonal factors. Expansion of the number of intestinal stem cells (ISC) occurs following intestinal resection. This increases the number of intestinal crypts, through the process of intestinal dilation^[92]. These ISCs are located deep in the crypts of Lieberkuhn. Isolation of ISCs has been simplified by the use of side population sorting of viable fractions of cell progenitor characteristics^[93].

Wnt proteins are regulators of cell proliferation, differentiation and adhesion. Mutation in mice of the adenomatous polyposis coli (*APC*) gene, together with augmented Wnt signaling in the intestine, results in an enhanced adaptive response to extensive small bowel resection^[94]. The increased mucosal surface area occurring following resection is due to sustained increases in crypt depth and villus height. This arises from resetting of proliferative responses, with increases in expression of mRNAs associated with proliferation (c-MYC) and dif-

ferentiation of goblet cells and Paneth cells^[95]. This raises the possibility that early expansion of intestinal secretory lineages within the epithelium may serve to amplify the signal(s) for initiating and sustaining intestinal adaptation. Further proof of concept studies are needed.

The Hedgehog (Hh) signaling pathway plays an important role in epithelial-mesenchymal interactions in gut morphogenesis and in epithelial cell proliferation. Hh proteins are produced in epithelial cells, and interact with underlying mesenchymal/stromal cell receptors. Blocking Hh signaling in the fetus or neonate leads to increased crypt cell proliferation, crypt-villus axis structural abnormalities, and alterations in enterocyte morphology. In Hh antibody-treated mice following intestinal resection, enterocyte migration from the crypt to the villus tip is increased, and apoptosis is also increased^[96].

The epidermal growth factor receptor (EGFR) is important in the pathogenesis of intestinal adaptation. This EGFR-mediated induction of enterocyte proliferation requires induced expression of the cyclin-dependent kinase inhibitor p21 to transcribe *waf1/cip1*, as well as mitogen-activated protein kinase (MAPK)^[97]. The cyclin-dependent kinase inhibitor (CDK1) p21*waf1/cip1* may be necessary for induction of enterocyte proliferation following initiation of intestinal adaptation^[98]. To maintain the new homeostasis achieved with adaptation, the high cell production rate must be matched by an equivalent rate of cell loss. EGFR signaling regulates specific Dcl-2 (Dax and Dcl-w) in the intestinal crypts, and this regulation of Dcl-2 influences apoptosis following extensive small bowel resection^[99].

The vascular endothelial growth factor (VEGF) enhances angiogenesis (the growth of new blood vessels from pre-existing blood channels). Angiogenesis is a requirement for successful healing or adaptation. As expected, VEGF is important in the intestinal adaptive response^[100].

The bcl-2 family of intracellular proteins has apoptotic properties. An increase in the ratio between pro- and anti-apoptotic members of these pathways occurs after massive small bowel resection, with upregulation of inducers of apoptosis including Fas and TNF- α by way of the death receptor pathway. Angiotensin converting enzyme (ACE) also promotes apoptosis in association with a reduced bax-bcl-2 protein ratio^[101]. Thus, ACE may play an important role in epithelial cell adaptive responses.

GLP-2 is released from the ileum and colon in response to nutrients in the intestinal lumen. GLP-2 enhances morphologic and proliferative indices of intestinal adaptation, and this adaptation is inhibited by GLP-2 immunoneutralization^[102]. GLP-2 administration enhances intestinal crypt cell proliferation and villus height, and increases expression of glucose transporters. Basal and postprandial GLP-2 levels are correlated with the magnitude of intestinal resection in experimental SBS^[103].

A number of hormones and peptides act on the intestinal tract^[104]. For example, glucagon-like peptide-1 (GLP-1) stimulates glucose-dependent insulin secretion, pancreatic B-cell proliferation, and reduces lipid absorp-

tion, food intake and the rate of gastric emptying^[105]. GLP-2 may reduce Ach release from the enteric nervous system, and thereby reduce neuronally evoked intestinal crypt epithelial Cl⁻ secretion^[106]. GLP-2 enhances the absorption of sugars and lipids^[107] and has a therapeutic potential in patients with the SBS^[108].

SBS patients with an end jejunostomy and no colon have limited meal-stimulated GLP-2 secretion. This is due to the removal of GLP-2 secreting L cells which are located primarily in the terminal ileum and colon. Teduglutide (ALX-0600), a dipeptidyl peptidase IV resistant GLP-2 analog, has been administered to enhance the adaptive process in patients with SBS, and to aid intestinal absorption^[109]. Thus, there is a therapeutic role for GLP-2 analogs in SBS.

Long-term parenteral nutritional (PN) support may be necessary in persons with SBS. Because of the potential complications of PN, such as infection or cholestatic liver disease, efforts have been undertaken to understand and to improve the intestinal adaptive process, and to thereby enhance nutrient absorption and to possibly reduce the need for PN^[110]. The success of surgical procedures designed to optimize intestinal absorptive function, such as bowel tapering or lengthening, has only been modest.

Small intestinal transplantation is an accepted treatment for severe intestinal failure. Over the past 50 years, more than a thousand children have received small bowel transplantation (SBT), alone or with liver and other organs. The one- and five-year graft survival routinely exceeds 90% and 80%, respectively^[111]. However, transplantation is usually used only in those persons who have SBS with complications from home PN. With good control of acute rejection and infections, patient and graft survival after small intestinal transplantation is approximately 77% and 65%, respectively. Patient and graft survivals of 60% and 59% are seen in those with combined liver and small bowel transplantation. Unfortunately, almost half of these transplanted patients require enteral nutrition again within two years after transplantation^[112]. Thus, small bowel transplantation has its risks and limitations for the SBS patient.

NOD2 is an intercellular microbial sensor present in macrophages, dendritic cells and Paneth cells. *NOD2* polymorphisms may be associated with impaired expression of certain Paneth cell-derived antimicrobial peptides. The likelihood of allograft failure is about one hundred-fold higher in small bowel transplantation recipients with mutant *NOD2* alleles, as compared with recipients with wild-type *NOD2* loci^[113].

While intestinal stem cell transplantation may play a role in refractory patients with Crohn's disease (CD) or celiac disease, the role of stem cells in treatment of other intestinal disorders remains at an early stage of consideration^[114,115].

Patients with short bowel syndrome from other non-CD causes were recently reported to develop CD in the residual intestine. The authors suggested that this shortened intestine may be a predisposing factor because of

alterations in the motility of the intestine as well as alterations in the intestinal flora^[116]. In persons with a short bowel syndrome, continuous tube feeding alone or with oral feeding enhances nutrient absorption, as compared with oral intake alone^[117]. "SBS results from surgical resection, congenital defect, a disease-associated loss of absorption and is characterized by the inability to maintain protein-energy, fluid, electrolyte, or micronutrient balances when on a conveniently accepted, normal diet"^[118].

SMOOTH MUSCLE FUNCTION AND INTESTINAL MOTILITY

Segmentation in the intestine consists of rhythmic contractions of the inner circular muscle and occurs after meals. These rhythmic contractions are regulated by slow waves, with the enteric nervous system (ENS) having a permissive role. These stationary contractions are independent of slow-wave activity, while simultaneously activating surrounding inhibitory motor neurons^[119].

The ENS "...coordinates the peristaltic and secretory activity of the gut and is also involved in the regulation of blood flow and modulation of the immune system"^[120]. ENS and enteric glial cells (EGCs) are in the submucosal plexus and the myenteric plexus. The ENS is composed of two ganglionated plexuses, the submucosal and the myenteric plexus, as well as the mucosal plexus. This extends within the lamina muscularis mucosae and the lamina propria mucosae beneath the epithelial lining of the intestine. Activation of human submucosal neurons decreases cellular permeability, and also decreases intestinal epithelial cell proliferation. Neurons respond to changes in intracellular calcium levels or to the expression of activation markers such as c-FOS. TTF- β 1 mRNA is expressed and TGF- β 1 is secreted by EGCs and they have anti-proliferative effects on intestinal epithelial cells^[121]. EGCs promote neuronal survival by regulating substrate supply, and thereby help to maintain neuronal homeostasis. EGCs also synthesize cytokines, and in inflammatory conditions may modulate glia proliferation. Glial disruption alters neurochemical coding of the enteric neurons, and leads to dysmotility^[122].

A method has been described for isolation and culture of primary enteric neurons. These cell lines have neuronal characteristics similar to those of primary enteric neurons^[123]. It is possible to isolate and expand enteric progenitor cells from human adult tissue^[120], thereby providing a potential future role for cell-based therapies for disorders of the ENS.

Patients with IBS may have abnormal colonic transit, as well as increased or decreased rectal sensation. The β 3-adrenoceptor (β 3-AR) is a member of the family of G-protein-coupled receptors. β 3-ARs are co-localized with choline acetyltransferase in human neurons in the cholinergic myenteric and submucosal plexus. Selective β 3-AR agonists inhibit cholinergic contractions, and enhance the release of somatostatin without altering carbachol-induced contractions. β 3-ARs inhibit cholinergic

contractions and inhibit spontaneous contraction of the human colon, as well as relaxing pre-contracted colonic longitudinal and circular muscle. Somatostatin may act as an endogenous analgesic substance, and a β -adrenergic agonist may modify visceral sensitivity. Solabegron is a β 3-AR agonist which has been studied in healthy human volunteers, where it is well tolerated^[124]. This raises the possibility that β 3-ARs may be useful for the pain suffered by persons with IBS.

The IGF system influences cell development, growth, and apoptosis. IGF-binding proteins (IGFBP-1 to -6) transport IGFs in the blood. IGFBP-3 to -5 are present in human smooth muscle, and modify the interaction of IGF-I with its receptor, IGF-IR. IGFBP-5 has a role in regulating smooth muscle growth independent of IGF-1, by activating the G protein G_i3 ^[125].

Acetylcholine (ACh) is released from cholinergic neurons in the myenteric plexus, and activates M2 and M3 receptors on the smooth muscles of the gastrointestinal tract. This results in smooth muscle contraction. The M2 and M3 receptors are expressed in the ratio of approximately 75%/25%, respectively, and are coupled to TRPC4 and TRPC6 (transient receptor potential channels), which depolarize the intestinal smooth muscle cells^[126]. Cholinergic nerves contain substance P, and there are also nitrergic nerves and nerves which release ATP as well as other mediators in the deep muscular plexus.

Substance P (SP)-mediated sustained contraction of the small intestine is negatively regulated at a pre-synaptic level by the M2 receptor, whereas the atropine-sensitive phasic contraction is positively regulated at the M2 receptor^[127]. Cytokines exert differential effects on the muscarinic receptors of intestinal longitudinal smooth muscle^[128]. Corticotropin-releasing-factor (CRF) acts through both central and peripheral mechanisms. CRF induces Ca^{2+} transients in myenteric neurons *via* a CRF-1 receptor-dependent mechanism^[129]. Calcium influx through voltage-operated Ca^{2+} channels, and in particular the R-type channels, causes the calcium transients necessary for muscle contraction.

Nonselective cationic channels in the smooth muscle cells generate muscarinic receptor-induced nonselective cation currents (MICAT), with increased Ca^{2+} influx by way of voltage-dependent Ca^{2+} channels. The Ca^{2+} leads to smooth muscle contraction, and peristalsis.

The gastrointestinal tract contains most of the serotonin (5-hydroxytryptamine, 5-HT) in the body, where it acts as a neurotransmitter, neuromodulator, and a paracrine factor. 5-HT is produced and released by enterochromaffin cells and by enteric nerves of the intestine. In addition to 5-HT having an effect on motility, it also regulates cell survival and proliferation. The 5-HT_{2D} receptors are expressed on the interstitial cells of Cajal (ICC). Exogenous 5-HT regulates the number of ICC through the 5-HT_{2B} receptor, in part by increasing ICC proliferation^[130].

The sensory intrinsic primary afferent neurons (IPANs) from an ENS network modify enteric reflexes,

which in turn alter gastrointestinal functions. The sensory terminals of IPANs are close to the enterochromaffin cells, which contain serotonin (5-HT). Extrinsic afferents (vagus and spinal afferents) have similar innervation territories, and both respond to chemical and to mechanical stimulation. In response to mechanical stimuli, extrinsic afferent neurons do not crosstalk with the IPANs. 5-HT activates the extrinsic afferents by a Ca^{2+} -dependent pathway which is different from the N-type Ca^{2+} channels in IPANs^[131].

The topic of the role of the ICC in gastrointestinal motility has been reviewed^[132]. It has become controversial with regard to the way nerves transmit their signals to regulate activity of intestinal smooth muscle^[133]: the c-kit receptor may be of importance^[134]. ICC help in maintaining the gradient of resting membrane potential, rather than by pacing the slow waves or assisting in their propagation^[135]. It is by volume transmission rather than wire transmission *via* the ICC that there is communication between the enteric neuronal varicosities and muscle cells. While the ICC may be impaired in numerous motility disorders, "...a cause-and-effect relationship between ICC impairment and motility dysfunction is not established". In the small intestine, ICC in the deep muscular plexus mediate neurotransmission, whereas ICC surrounding the myenteric plexus generate slow waves. The slow waves are transferred to the adjacent smooth muscles, and can be recorded as straight "...spontaneous, rhythmic, electrical oscillations of the resting membrane potential"^[136].

The ICC occur in the myenteric plexus, within either the circular (CM) or longitudinal (LM) muscles, contributing to pacing these muscle slow waves. ICC also line the septa (ICC-SEP) separating circular muscle bundles, and ICC-SEP form an important conduction pathway for spreading excitation deep into muscle bundles in the human jejunum^[137]. The $Na^+/K^+/2Cl^-$ cotransporter (NKCC1) is involved in generation of slow waves in the jejunal musculature. ICC at the deep muscular plexus (ICC-DMP) are closely associated with the enteric nerve endings. Varicosities of nitrergic and other nerves are found on ICC-DMP or on CD34-positive, c-kit-negative fibroblast-like cells. The gap junction coupling is necessary for pacing or nerve transmission to the circuit or muscle of the mouse intestine^[138].

The ICC act as pacemakers, producing slow waves of depolarization along the intestinal muscle. A transient outward K^+ current may moderate the uptake of the pacemaker potential, resulting in motility arising from the waves of depolarization^[139].

Bone morphogenetic protein 2 (BMP-2) is a regulatory molecule which induces the phosphorylation of the Smad1 signaling cascade, and thereby increases the differentiation of the neurons of the ENS^[140].

Endogenous adenosine (eADO) is a metabolite of ATP that acts on A1, A2A and A2B receptors on enteric neurons to suppress coordinated responses triggered by immune-histamine H₂ receptor activation^[141].

Extracellular adenosine levels control adenosine re-

ceptor signaling. Enzymes that produce CD73 or degrade adenosine deaminase (adenosine), and thereby alter activity of transport systems in the plasma membrane, influence these extracellular adenosine levels, thereby affecting adenosine receptor signaling, which in turn alters intestinal motility^[142]. β -Adrenoceptors are G-protein-coupled receptors which, when activated by an agonist, stimulate adenylyl cyclase to produce the second messenger, cAMP. cAMP activates cAMP-dependent protein kinase (PKA). There is compartmentalization of the process by which these proteins form an interaction.

Caveolae are non-clathrin-coded plasma membrane invaginations which are present in a variety of cells, including monocytes. These caveoli are present in microdomains, also known as lipid rafts, an area of the plasma membrane which is rich in cholesterol and sphingolipid. The caveolae are coated on their cytoplasmic side by caveolins, a family of integral membrane proteins including adenylyl cyclase, which are involved in signal transduction. In knockout mice depleted of caveolin-1, there is reduced PKA activity and thereby reduced function of the β -adrenoceptors^[143].

Adenosine is generally accepted to be the ligand for the P1 receptor, and ATP is the ligand for P2 receptors. Adenosine A2A receptors on neuronal cells are excitatory in nature, but A1 receptors in the submucosal plexus have inhibitory actions^[144]. In the murine enteric nervous system, adenosine "... suppresses synaptic transmission, efferent function of extrinsic capsaicin-sensitive sensory nerves, mucosal reflexes, neuroeffector transmission, and morphine withdrawal diarrhea". Purinergic signaling pathways are important in sensory signaling in enterochromaffin cells and secretomotor reflexes in the intestinal tract; purinergic modulation of synaptic transmission also occurs in human intestine^[145].

Intestinal motor activity and secretion are linked, and are changed cyclically in a rhythm called the migrating motor complex (MMC). Submucous neurons are both directly and indirectly mechanosensitive, and myenteric neurons can be activated by stretch. There are both rapid and slow components to the potential difference (PD) response to intestinal stretching or distension. The rapid component operates *via* nicotinic transmission and NK1 receptors; the slow component operates by way of VIP-ergic transmission and involves both NK1 and NK3 receptors^[146].

Intestinal inflammation causes hyperplasia of smooth muscle, and this thickening of the smooth muscle layer results in dysmotility. IL-1 β is a proinflammatory cytokine which results in production of PGE2 and NO from macrophages within the ileal smooth muscle tissue, and IL-1 β acts as an anti-proliferative mediator^[147]. Nematode infection in the small intestine induces a smooth muscle hypercontractility that depends on IL-4 and IL-3 (Th-2 cytokines) activation of the signal transducer and activator of transcription (STAT) 6. 5-HT_{2A} is one of the molecules downstream from STAT6 activation that mediates changes in smooth muscle function^[148].

Integrins are a family of transmembrane proteins, and the expression of integrins and their preferred ligands is tissue specific. In the small intestine, occupancy of a specific integrin receptor acts in concert with IGF-I-stimulated receptor tyrosine kinase activity on muscle cell growth^[149]. Both insulin and IGF-I prevent apoptosis through the activation of phosphatidylinositol 3-kinase (PIK3-kinase). Through the subsequent activation of the downstream protein serine/3 kinase, Akt IGF-I stimulates proliferation and inhibits apoptosis in intestinal smooth muscle^[150].

Mechanisms underlying the sustained tonic contraction of the intestinal smooth muscle include prolonged myosin-like chain phosphorylation, phosphorylation of cytoskeleton filaments and associated proteins, alterations in Ca²⁺ influx, and increased sensitivity of contractile elements to Ca²⁺^[151]. Muscarinic agonists acting through M3 receptors contract gastrointestinal smooth muscle by a protein kinase C (PKC)-dependent mechanism in the guinea pig ileum; this is thought to be through a novel PKC, PKC- δ ^[151].

Electrical stimulation may be synchronized with the intrinsic intestinal smooth muscle slow waves [synchronized intestinal electrical stimulation (SIES)]. SIES induces small intestinal contractions during phase I of the MMC in the fed state, and improves postprandial small intestinal hypomotility^[152]. SIES remains to be of proven clinical use.

There are olfactory receptors in human mucosal enterochromaffin cells. Odorants present in the luminal environment of the gut may stimulate serotonin release by way of olfactory receptors present in these EC cells^[153].

The "ileal break" describes the process by which high concentrations of lipids in the terminal ileum will slow gastric emptying and intestinal transit. High intra-ileal carbohydrate and lipid loads induce phase III motility, probably through release of neurohormonal mediators, glucagon-like peptide (GLP-1) and peptideYY (PYY). Physiological postprandial ileal lipid concentrations inhibit human digestive pancreatic protease and bile acid output, but do not influence intestinal motor activity^[154].

Acute radiation exposure of the abdomen is associated with accelerated small intestinal transit through involvement of cholinergic receptors. This raises the possibility that M3 receptors "...may provide specific therapeutic targets in acute radiation enteritis"^[155]. The mucosal damage in the small intestine produced by abdominal radiation may occur independently of intestinal dysmotility, and may result in diarrhea and nutrient malabsorption. Interestingly, the high molecular weight polyethylene glycol-based copolymer PEG 15-20 prevents radiation-induced intestinal injury in rats, prevents apoptosis and lethal sepsis due to *Pseudomonas aeruginosa* in mice, and protects cultured intestinal epithelial cells from apoptosis and microbial adherence, possibly by binding characteristic lipid raft coalescence during the development of intestinal radiation damage^[156].

The mechanisms of drug-associated changes in intes-

tinal motility have been reviewed^[157]. Chronic intestinal dysmotility or chronic intestinal pseudo-obstruction may be primary, or secondary to disorders such as diabetes mellitus or scleroderma. These disorders may affect isolated components of the GI tract, or the entire GI tract. There may be absence of the phase III component of the MMC, postprandial low amplitude contractions, bursts of sustained uncoordinated phasic contractions, and clusters of contractions. All of these mechanisms contribute to the pathophysiology and the high morbidity of these dysmotility syndromes^[158].

Cannabis (CB) and cannabinoid receptors inhibit intestinal motility. The CB1 receptor is present in the central and peripheral nervous system, including the enteric nervous system, as well as in non-neural tissues such as liver and adipose tissue. CB2 receptor expression is present in cells of the immune system as well as in the brain. Lipopolysaccharide (LPS) enhances intestinal transit, and this effect is reversed by cannabinoid CB2 receptor agonists^[159].

Lubiprostone is a bicyclic FA compound, a prostone derived from a metabolite of prostaglandin E1. Prostones have highly selective activity on ClC-2 chloride channels, enhancing intestinal fluid secretion, but also accelerating small intestinal and colonic transit^[160]. These compounds may be used clinically for the treatment of constipation.

Bowel inflammation may lead to abnormalities in intestinal motor and secretory pattern, through changes in enterochromaffin cell activity, as well as through changes in the excitability of primary afferent neurons of the enteric nervous system. Inflammation at one site of the intestine also alters the cellular components of enteric reflex circuits in non-inflamed regions^[161]. Intestinal inflammation is a key event in the pathogenesis of post-operative ileus, and in rats the degree of intestinal paralysis is directly proportional to the degree of intestinal handling and inflammation which occurs at the time of surgery. Intestinal handling triggers mast cell activation and prolongs post-operative ileus^[162]. The therapeutic role of this observation in preventing or treating post-operative ileus remains to be proven.

There are three endogenously-produced biologically-active gases, carbon monoxide, hydrogen sulfide and nitric oxide. Methane is also produced by enteric bacteria in one- to two-thirds of humans, and may slow intestinal transit by augmenting small bowel contractile activity^[163]. Excess methane production has been recognized in a proportion of persons with constipation-predominant IBS.

Substance P and neurokinin A are the main endogenous tachykinins in the enteric neurons. Stimulation of the NK3R receptor in the GI tract activates protein kinase C, then protein kinase D, leading to noncholinergic slow excitatory postsynaptic potentials in the myenteric intrinsic primary afferent neurons of the guinea pig ileum^[164].

The ICC generate pacemaker potentials which drive the electric slow waves that contribute to neuromuscular

signaling leading to motor neurotransmission in the GI tract. ICC express receptor tyrosine kinase c-kit. *Kit* gain-of-function mutations lead to hyperplasia of ICC, with maintenance of both pacemaker function and normal enteric neural responses^[165]. This is in contrast to the association between fewer ICC and the development of disturbances in GI motility. *Ano1* is part of a family of 10 gene products, and labels ICC around ganglia in the deep muscular plexus^[166]. Because *Ano1* does not label mast cells, it may prove to be a better marker than c-kit for ICC and for mesenchymal tumors.

C-kit immunohistochemistry is used to diagnose gastrointestinal stromal tumors (GIST), since about 94% of mesenchymal tumors are positive for c-kit receptors. Between 80% and 90% of GIST tumors have gain-of-function mutations in *Kit*.

Stimulation of the myenteric plexus of the ENS by food in the intestinal lumen or by stretching of the intestine activates mucosal pathways to produce three different types of slow excitatory post-synaptic potentials (EPSPs) which are mediated by tachykinin or purine nucleotide neurons^[167]. The predominant cell type in the ENS, the glial cells, provide functional purinergic neuron-glia communication in the ENS^[168].

Myofibroblasts are an intermediate cell type between smooth muscle cells and fibroblasts. In persons with Crohn's disease, there is disruption of the subepithelial myofibroblasts of the epithelial sheath, adding to the suggestion that myofibroblasts may be involved in the pathogenesis of inflammatory bowel disease^[169]. This role of myofibroblasts is likely because they serve as a component of the innate immune system, and respond to luminal bacterial adjuvants such as LPS^[170].

Manipulation of the intestine rapidly causes activation of the p38 nitrogen-activated protein kinase (MAPK), a stress-activated protein kinase. There is liberation of NO and prostaglandins from the macrophages in the muscularis external to the intestine, and the extravasation of immunocompetent white blood cells^[171]. In turn, this leads to postoperative ileus, which can be prevented by giving mice a single preoperative dose of semapimod, which inhibits p38-MAPK and NO production in macrophages. This is an exciting development for its possible future application to humans undergoing abdominal surgery.

An increase in the intracellular concentration of Ca^{2+} in the smooth muscle of the intestine results from the release of Ca^{2+} from intracellular stores, as well as from the entry of Ca^{2+} into the cell through L-type Ca^{2+} channels. With stretching of the wall of the intestine there develops tension in the plasmalemmal membrane. This tension is transmitted to the mechanosensitive L-type Ca^{2+} channels, thereby leading to increased intracellular Ca^{2+} and the possibility for smooth muscle contraction^[172].

The peristaltic reflex is mediated by IPANs (intestine sensory efferent neurons), interneurons, as well as excitatory and inhibitory motor neurons. The antipropulsive effect of cannabinoids on the small and large intestine result from the inhibition of the calcitonin gene-related

peptide (CGRP)-containing neurons that begin the peristaltic reflex, as well as the inhibition of both the excitatory cholinergic/tachykinergic and inhibitory VIPergic motor neurons responsible for ascending contraction and descending relaxation, respectively^[173].

The balance between myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) controls the overall phosphorylation of the 20-kDa regulatory light chains of myosin III. Ca²⁺-independent contraction of longitudinal ileal smooth muscle is potentiated by a zipper-interacting protein kinase pseudosubstrate peptide^[174]. This raises the possibility of developing synthetic peptides from the autoinhibitory region of the smooth muscle myosin light chain kinase to treat hypomotility disorders of the GI tract.

TUMORS

Small bowel cancers represent a heterogeneous group of rare tumors, and the prognosis depends upon the cell type. The standardized incidence rate for primary malignant small bowel cancer is 1.2/10⁵ men and 0.8/10⁵ women^[175]. The four main histological types are adenocarcinoma (40%), carcinoid (31%), lymphoma (20%) and sarcoma (9%). The five-year survival rate is about 37%, and varies between 57% for neuroendocrine tumors and 18% for sarcomas.

The gastrointestinal tract is the most common extranodal site for non-Hodgkin's lymphoma (NHL). GI NHL may be primary or secondary, the latter usually representing involvement from diffuse nodal disease. GI NHL used to be increased in the HIV-positive population, but with HAART therapy, GI NHL has virtually disappeared^[176]. One intestinal nuclear receptor map has been developed, which "...indicates that the localization pattern of a receptor in normal intestine (signature) predicts the modulation of its expression in tumors"^[177].

DIAGNOSTIC IMAGING

Imaging techniques for the small intestine include the classic small bowel series, enteroclysis, CT enterography, MR enterography, and more recently capsule endoscopy (CE), push enteroscopy (PE), and double balloon enteroscopy (DBE)^[178]. The topic of recent developments in CE has been reviewed^[179-182]. Optimal bowel preparation for CE is a PEG solution plus simethicone^[183]. Using duodenal histology as the gold standard, the performance characteristics of CE for the diagnosis of celiac disease are: sensitivity 88% (95% CI: 76%-99%), specificity 91% (95% CI: 81%-100%), positive predictive value 97% (95% CI: 90%-100%), negative predictive value 71% (95% CI: 56%-87%), as well as positive and negative likelihood ratios of 9.6 and 0.14, respectively^[184]. Of 43 celiac patients, 42% had mucosal changes extending beyond the duodenum, and in 7% the alterations involved the entire small intestine. Interobserver agreement for the diagnosis of celiac disease by CE ranges between 79% and 94%; and κ

values range between 0.6 and 0.9. Murray *et al.*^[185] reported a sensitivity of CE for the detection of small intestinal mucosal atrophy, as compared with upper endoscopy, to be 92% *vs* 55% ($P = 0.0005$), with a specificity of 100%. Other authors have agreed with this high sensitivity of CE (over 90%), but reported a much lower specificity of approximately 64%^[186].

The topic of small bowel enteroscopy has been reviewed^[187]. CE is contraindicated under a number of circumstances^[188]: (1) Swallowing disorder; (2) Known or suspected intestinal obstruction, strictures, or fistulae; (3) Pregnancy; (4) Children less than 10 years old; and (5) Persons with implanted electromedical devices.

In 120 persons on long-term NSAIDs or COX-2 selective agents, CE demonstrated that the 62% with abnormal CE had denuded areas (39%), mucosal breaks (29%), or reddened folds (13%)^[189]. CE demonstrated small intestinal polyps in 60% of subjects with familial adenomatous polyposis (FAP) and in 75% of subjects with Peutz-Jeghers Syndrome^[190].

A meta-analysis of nine studies compared CE *vs* other diagnostic methods for Crohn's disease. The diagnostic yield for CE *vs* barium radiography was 63% and 23%, respectively. The yield for CE *vs* ileoscopy was 61% and 46%, respectively, and the yield of CE compared to computed tomography (CT) enterography/CT enteroclysis was 69% and 30%, respectively. A meta-analysis of the yield of CE *vs* other modality examinations of the small intestine in patients with non-stricturing Crohn's disease showed that CE was superior to small bowel barium radiography, ileoscopy, CT enterography, CT enteroclysis and PE, as well as small bowel magnetic resonance imaging (MRI)^[191]. In patients with previous surgical resection for Crohn's disease, CE is inferior to ileocolonoscopy, but does detect about two-thirds of lesions outside the reach of the colonoscope^[192]. These authors suggest that CE "...cannot systematically replace ileocolonoscopy in the regular management of patients after surgery" (for Crohn's disease). The CE findings have an impact on patient management: physicians change post-CE diagnostic strategy in 61% of patients^[193]. Clearly, CE has proven its diagnostic potential.

There is an incremental diagnostic yield (yield of CE minus yield of comparative modality) of 38% comparing CE to PE, and 22% compared to small bowel MRI. As compared with PE, CE provides superior identification of obscure bleeding sites in the small intestine (50% *vs* 24%). While CE missed 8% of lesions, these sites were accessible to standard endoscopy; in contrast, PE missed lesions in 26% of patients^[194].

DBE may be used from the oral or anal route, or from both. The overall detection rate of small bowel diseases using CE is superior to that with DBE (72% *vs* 41%, respectively), and is also superior for the detection of small bowel diseases in patients with obscure gastrointestinal bleeding (88% *vs* 60%, respectively)^[195]. In another study, for detection of causes of obscure gastrointestinal bleeding, 80% of small bowel abnormalities were detected by

CE vs 60% with DBE^[196]. PE may be inferior to push-and-pull enteroscopy to find lesions in patients with suspected small bowel bleeding^[197]. DBE performance has also been evaluated in patients with refractory CD who had circumferential, discreet, or confluent ulcerations^[196].

When patients with acute intestinal symptoms after allogeneic stem cell transplantation underwent esophago-gastroduodenoscopy (EGD) with duodenal biopsies, as well as CE within 24 h of the onset of their symptoms, acute intestinal graft-vs-host disease (GVHD) was diagnosed by EGD with biopsies in 7 out of 13 patients, 3 of whom would have been missed by EGD alone but were detected by CE. In all 7 patients with histologically confirmed acute intestinal GVHD, CE revealed the typical lesions of GVHD^[198]. The authors concluded that CE showed comparable sensitivity with EGD plus biopsies, and CE also demonstrated a high negative predictive value for diagnosing acute intestinal GVHD.

After the formation of an ileal pouch anal anastomosis (IPAA) in patients having a colectomy for ulcerative colitis, development of pouchitis is common. It is unknown how frequently lesions occur elsewhere in the small intestine. At small bowel follow-through of persons with pouchitis, 13% showed a focal ectasia of the middle ileum and a stenosis of the pouch, whereas CE performed in patients with chronic pouchitis after IPAA showed diffuse lesions from the duodenum to the ileum in all evaluable patients^[199]. These lesions included aphthous ulcers, erosions, redness, atrophy, cobblestoning, and deep ulcers or fissures.

A retrospective analysis of the charts of 562 patients who underwent CE at the Mount Sinai Medical Centre (NYC) for a variety of indications showed small bowel tumors in 9%^[200]. In a report of the largest series of patients with small bowel tumors detected by CE, half were identified in the jejunum and approximately a quarter were in the ileum or in the mid to distal small bowel^[201]. The most common malignant small bowel tumors were adenocarcinoma, carcinoids, melanomas, lymphomas and sarcomas. The most common benign tumors were GIST, hemangiomas, hamartomas, adenomas, and granulation tissue polyps. In a three-center report of Australian experiences with CE, the usefulness of CE was also confirmed, and the authors suggested that “in many patients, detection of a tumor alters management and improves outcomes”^[202].

CE found a median of four small bowel polyps greater than 1 cm in size in persons with Peutz-Jeghers syndrome, while barium follow-through detected a median of only one polyp^[203]. In persons with known familial adenomatous polyposis (FAP), regular examination of the small intestine for small intestinal tumors is part of the recommended management. CE detected ileal or jejunal polyps in 30% of patients, and all polyps were less than 5 mm in size. Upper gastrointestinal endoscopy detected duodenal polyps in 11 of 23 patients, and only four of these patients were identified as having duodenal polyps on CE. Thus, CE underestimated the number of polyps

and did not visualize the ampulla of Vater. This suggests that CE is useful for detection of jejunal and ileal polyps in patients with FAP, but standard forward- and side-viewing endoscopic procedures are advised for detection of duodenal polyps^[204].

While duodenal biopsy represents the gold standard for the diagnosis of celiac disease, CE has shown that over a third of celiac patients have mucosal changes extending beyond the duodenum, and in approximately 7% the entire small bowel is involved^[184]. As compared with duodenal biopsy for detecting celiac disease, the sensitivity of CE was 88%, specificity 91%, positive predictive value 97%, and negative predictive value 71%.

Although CE provides excellent visualization of the small intestinal epithelium, if a small bowel lesion is identified, it cannot be biopsied. DBE is clinically useful to identify and biopsy such lesions^[205-210].

Because of the varying values of the sensitivity and specificity of the various diagnostic methods available to diagnose small bowel disease (such as capsule endoscopy, CT- or MRI- enteroscopy, ileocolonoscopy, small bowel follow-through), it is suggested that except for CE, “...a combination of two of the other available imaging methods is the best diagnostic option for small-bowel Crohn’s disease...”^[211,212]. While CE gives a diagnostic yield in about two-thirds of patients with obscure gastrointestinal bleeding, DBE (when used within 1 mo after the last episode of overt bleeding) reveals positive findings in 84% and provides a means to control bleeding in 64%^[213].

CYSTIC FIBROSIS

Meconium ileus occurs in approximately 20% of newborns with cystic fibrosis (CF). The distal intestinal obstruction syndrome (DIOS) occurs in about 25% of CF adults. Mucus accumulation in the CF intestine is partly due to the dehydrated, acidic environment, as well as to the altered glycosylation of mucins. Mucin glycoprotein levels are increased, due to reduced mucus clearance rather than an enhanced synthesis. This is suggested by the lack of increase in the levels of expression of the major intestinal mucin genes (*Muc2*, *Muc3*). Interestingly, *Muc1* is not a major component of the accumulated mucus of CF mice^[214]. Mucin binds bacteria, both by nonspecific trapping as well as by binding to specific glycans, which help to carry bacteria aborally for efficient clearance from the small intestine.

In human CF, there are mutations in the *CFTR* gene that result in little or no cystic fibrosis transmembrane conductance regulator (CFTR) activity. Some 30%-50% of CF patients have small intestinal bacterial overgrowth (SIBO), thought to be due to slow small intestinal transit^[215]. This SIBO may be due to impaired migrating motor complexes, which lead to less removal of mucus and bacteria from the small intestine. Laxatives and N-acetylcysteine (NAC) reduce bacterial overgrowth in the CF intestine of mice, and this eradication is associated with normalized intestinal transit and a reduction in the innate

immune response^[215].

Exogenous pancreatic replacement enzyme therapy improves, but does not normalize, steatorrhea in CF patients. In CF mice, the crypt-villus axis height is decreased, there are fewer apoptotic cells in the intestinal crypts; there is also goblet cell hyperplasia and inflammatory cell infiltration^[216]. In humans with CF, there is more than just defective lipolytic enzyme activity leading to the malabsorption of lipids. Indeed, there is evidence for abnormal enterocyte intracellular lipid processing in intestinal biopsies from CF subjects, such as decreased lipid esterification and lipid secretion, decreased output of triglyceride-rich lipoproteins, as well as diminished synthesis of apoB48 and apoA-1^[217].

Factors that may contribute to the incomplete normalization of fat malabsorption in CF patients who are compliant with their intake of adequate amounts of pancreatic replacement enzymes include the use of outdated or inactivated enzymes, incorrect timing of the intake of the supplements with regards to meals, excessive duodenal acid inactivation of the enzymes, or reduced duodenal and pancreatic bicarbonate secretion leading to high intraduodenal acid concentrations, impaired formation of mixed micelles, altered composition of the BBM [decreased linoleic acid (18:2n-6) and docosahexaenoic acid (26:6n-3), as well as increased arachidonic acid (20:4n-6) and elevated (20:3n-9) to (20:4n-6) ES] with changes in its permeability and absorptive function, impaired esterification of lipids in the enterocytes, reduced apolipoprotein synthesis, chylomicron formation or secretion across the BLM. CFTR knockdown in Caco-2 cells stimulates both the synthesis and transport of fat but not cholesterol^[218].

Approaches are needed to prevent the CF-associated increased viscosity of the intestinal luminal contents, and hopefully reduce the frequency of meconium ileus in CF newborns, or DIOS in CF adults. The activation of proteinase-activated receptors on the BLM of enterocytes by EGFR activation, MAP kinase signaling, Ca²⁺, PKA (and possibly PKC), causes chloride secretion. PKC enhances the activation of PKA, or increases BLM NKCCI, thereby enhancing the phosphorylation of CFTR, and thus Cl⁻ and water secretion. Basolateral PAR2-induced Cl⁻ secretion induces the activation of PKC β 1 and PKC δ *via* a phospholipase C mechanism, which results in the stimulation of cRaf and ERK 1/2 signaling^[219]. Reduction of NHE3-mediated Na⁺ and water absorption helps to increase the fluidity of the intestinal contents that would otherwise be very thick and dehydrated if NHE3 activity remained normal in the presence of reduced CFTR activity^[220]. Lubiprostone activates ClC-2 chloride channels, causing Cl⁻ and water secretion^[221].

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How can portal vein cavernous transformation cause chronic incomplete biliary obstruction?

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Abstract

Biliary disease in the setting of non-cirrhotic portal vein thrombosis (and similarly in portal vein cavernous transformation) can become a serious problem during the evolution of disease. This is mostly due to portal biliary ductopathy. There are several mechanisms that play a role in the development of portal biliary ductopathy, such as induction of fibrosis in the biliary tract (due to direct action of dilated peribiliary collaterals and/or recurrent cholangitis), loss of biliary motility, chronic cholestasis (due to fibrosis or choledocholithiasis) and increased formation of cholelithiasis (due to various factors). The management of cholelithiasis in cases with portal vein cavernous transformation merits special attention. Because of a heterogeneous clinical presentation and concomitant pathophysiological changes that take place in biliary anatomy, diagnosis and therapy can become very complicated. Due to increased incidence and complications of cholelithiasis, standard treatment modalities like sphincterotomy or balloon sweeping of bile ducts can cause serious problems. Cholangitis, biliary strictures and hemobilia are the most common complications that occur during management of these patients. In this review, we specifically discuss important issues about bile stones related to bile duct

obstruction in non-cirrhotic portal vein thrombosis and present evidence in the current literature.

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Key words: Portal vein cavernous transformation; Cholelithiasis; Hemobilia; Portal ductopathy; Portal biliopathy

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INTRODUCTION

The thrombosis occurring secondary to various causes results in acute and generally asymptomatic thrombosis in an extra-hepatic portion of portal vein transformation (PVT). Eventually, portal vein cavernomatous transformation (PVCT) forms as a compensatory reaction 3 wk to 3 mo after the initial event at the hilum of the liver. However, this salvage also results in inevitable anatomical or functional changes at surrounding structures including the biliary tree, gallbladder and pancreas^[1-4]. These changes are secondary to perfusion changes, neovascularization, fibrosis reaction or compression of ducts by newly formed but highly perfused vessels.

Recent terminology uses the term "portal biliopathy" to denote overall anatomical, morphological and functional changes in the biliary tree^[5]. The manufactured term "biliopathy" originates from a combination of the Latin word "bilis" (meaning bile) and the Greek word "pathos" (meaning disease) suggesting a disease of liq-

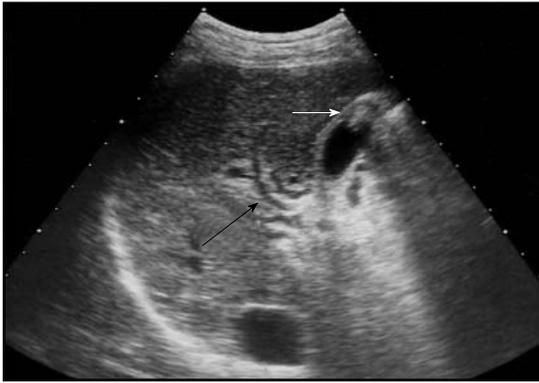


Figure 1 Liver ultrasound image showing stone (white arrow) and portal vein cavernomatous transformation (black arrow) in gallbladder.

uid bile or its constituents. However, current knowledge lacks compelling evidence of formation of diseased bile in patients with PVCT. Therefore in this review, the term “portal ductopathy” (PD) will be used instead of portal biliopathy as suggested by our group previously^[6].

The changes that take place in the biliary ducts during the course of PVCT can be summarized as strictures, dilatations and varicose veins located at the ductular walls and gallbladder. In the long term, these changes result in biliary stasis, jaundice, cholangitis and cholelithiasis adding more to morbidity and even resulting in mortality (Figures 1 and 2). The superimposition of cholelithiasis observed in the setting of PD is very important since these patients are prone to secondary biliary cirrhosis, a complication observed in 4% of patients^[7]. The clinical problem of cholelithiasis observed in chronic, non-cirrhotic PVCT patients is an under-mentioned topic and forms the basis of this compact review. Other clinical problems such as cirrhotic patients, biliary strictures or surgical management related to PD are beyond the scope of this paper.

PATHOGENESIS OF CHOLELITHIASIS IN PORTAL VEIN CAVERNOMATOUS TRANSFORMATION

Theoretically, cholelithiasis in the setting of PD and PVCT may be secondary to chronic cholestasis, changes in the constituents of bile or other factors such as reduced portal flow and associated liver atrophy.

Cholestasis and gallbladder functions

Cholestasis and related clinical findings such as jaundice, pruritus and cholangitis are the most common presenting symptoms in the setting of symptomatic PD and occurs in 5%-30% of all patients^[8,9]. Chronic biliary stasis (due to compression in the biliary outflow and loss of bile duct contractility due to either fibrosis or ischemia^[8]) may eventually result in stones formed *de novo* located in biliary ducts and/or gallbladder. The evidence for the proof of this hypothesis is obtained from clinical studies which generally observed pigment stones, located

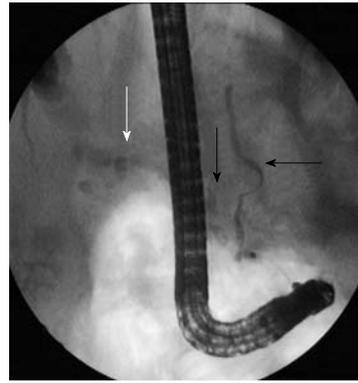


Figure 2 “Portal double ductopathy” sign with stones. Endoscopic retrograde cholangiopancreatography image of a patient with chronic portal vein cavernomatous transformation showing irregular pancreatic duct and biliary ducts (black arrows). Main bile duct shows local stricture and dilations with cholelithiasis (white arrow).

just proximal to the site of ductular stenosis without associated stones in the gallbladder^[10,11]. The reduced contractile function of the gallbladder may also contribute to development of stones due to the presence of varicose veins in the gallbladder wall, but in one study the contractile function of gallbladder has been found to be unchanged^[12]. Thus, cholestasis is believed to develop at later stages of PD secondary to fibrosis and compression of newly formed vessels, which are a major factors for development of stones. Once formed, stones further contribute to cholestasis and stricture formation by initiating recurrent cholangitis attacks.

Change in the constituents of bile

There is a small amount of evidence in the literature to indicate a change in the composition of bile resulting in a tendency for development of cholelithiasis. Previous studies investigating the contribution of reduced portal flow for development of cholelithiasis found that there is an increased tendency towards formation of stones after selective portal vein ligation in dogs^[13]. Also, temporary interruption of portal flow leads to decreased bile acid synthesis and biliary flow^[14]. There is no human study in the literature to indicate definitive changes in biliary composition take place during PVCT. Hypothetically speaking, presence of abnormal portal inflow might contribute to formation of lithogenic bile either by causing hypersplenism and increased pigment load in bile or abnormal enterohepatic circulation of bile acids due to portal hypertension.

FREQUENCY AND CLINICAL FINDINGS OF CHOLELITHIASIS IN PORTAL VEIN CAVERNOMATOUS TRANSFORMATION

The findings obtained from key studies concerning cholelithiasis observed in PVT-PVCT (in adult and pediatric populations) are summarized in Table 1. The frequency of cholelithiasis is highly variable (0%-84%). Probably, this variability is secondary to the problem of potential

Table 1 Cholelithiasis and important findings from selected studies

Author	Patient population	Frequency and location of cholelithiasis (%)	Notes
Bayraktar <i>et al</i> ^[1]	47 PVT patients evaluated by ERCP	8 (19) located in CBD	Unselected PVCT patients irrespective of symptoms, had a control group of 22 patients with other causes of portal hypertension
Condat <i>et al</i> ^[11]	25 consecutive patients in 2 yr evaluated by MRCP	4 (16)	Cholelithiasis associated with abrupt elevation of transaminase level, obstructive jaundice and cholangitis (2 patients)
Khare <i>et al</i> ^[27]	13 patients with obstructive jaundice evaluated by ultrasound	4 (31) located in GB, 4 (31) located in CBD	Retrospective selection of patients with obstructive jaundice
Sezgin <i>et al</i> ^[19]	10 consecutive patients in 6 yr presenting with jaundice and/or cholangitis evaluated by ERCP	0	Only patients with biliary symptoms are evaluated; Total number of patients or prevalence of cholelithiasis is not mentioned
Oo <i>et al</i> ^[16]	13 patients with symptoms related to PD in 13 yr	11 (84) located in GB, 9 (69) located both in GB and CBD	Symptomatic patients selected
Dumortier <i>et al</i> ^[28]	6 consecutive patients presented as case series	4 (66) located in GB, 2 also had stones in CBD	Upon follow up 3 patients suffered from cholecystitis and cholangitis requiring cholecystectomy
Chaudhary <i>et al</i> ^[20]	9 symptomatic PD patients managed surgically	2 (22) located in CBD	Selected patients with indications for surgery; Bile aspirates during surgery revealed <i>Escherichia coli</i> in patients with stones
Agarwal <i>et al</i> ^[29]	39 symptomatic PD patients managed surgically	12 (30) located in GB, 7 (18) located in CBD	Selected patients with indications for surgery
Dhiman <i>et al</i> ^[17]	53 symptomatic and asymptomatic patients	11 (20) (7 located in GB while 4 in CBD)	Stones were found more frequently in patients with symptoms than asymptomatic patients
Ozkavukcu <i>et al</i> ^[30]	16 patients diagnosed with PVT evaluated by MRCP	0 (there are 3 patients with history of cholecystectomy but the reasons are not given)	Patients with history of, chronic liver diseases, liver abscess and hydatid cyst were not eliminated with potential interference with findings of biliary system
Chiu <i>et al</i> ^[31]	29 children with PVT diagnosis evaluated for Rex shunt	5 (17) located in GB, 4 (14) had biliary sludge	Only children evaluated for possible surgery included
Yamada <i>et al</i> ^[32]	21 children with PVT	3 (14) located in GB	21 patients selected out of 35, selection criteria not mentioned

PVT: Portal vein transformation; ERCP: Endoscopic retrograde cholangiopancreatography; MRCP: Magnetic resonance cholangiopancreatography; PD: Portal ductopathy; CBD: Common bile duct; GB: Gallbladder; PVCT: Portal vein cavernomatous transformation.

bias in patient selection criteria. Most of the studies only included patients with symptoms in a retrospective design or included patients with chronic liver disease-cirrhosis or with mass-occupying lesions. In most of these studies, investigation methods [endoscopic retrograde cholangiopancreatography (ERCP), magnetic resonance cholangiopancreatography or ultrasound] were not uniform so it was not possible to make a definitive conclusion about incidence and prevalence of stones. The choice of investigation method has been found to have a clear impact on the results of anatomical location of cholelithiasis found in the biliary system. Therefore, current literature lacks prospective and well-designed studies to reveal the exact prevalence and incidence of cholelithiasis in a group of well selected PVCT patients irrespective of presence of symptoms.

The clinical findings related to cholelithiasis in PVCT patients are not different from patients who do not have PVCT, but it is evident that it results in a high frequency of PD symptoms. Cholelithiasis is found more commonly in patients with biliary findings such as jaundice, biliary colic, cholangitis and elevated liver enzymes compared with asymptomatic patients^[7,15].

TREATMENT

The management of stones in patients with PVCT merits special attention since concomitant changes in the biliary system may jeopardize standard therapeutic proce-

dures. The collaterals of Petren and Saint around major bile ducts enlarge in response to PVCT and they form the basis of collateral vessel compression to major bile ducts in PD^[6,7]. Also, secondary to ischemic injury and development of fibrous tissue in the major biliary ducts, biliary interventions should be performed with caution. Since these varicose collaterals and fibrous strictures may result in detrimental complications like hemobilia or recurrent cholangitis, clinicians should follow special management strategies being aware of these potential risks. Otherwise, therapeutic strategies for cholelithiasis in PD are not different from other patient groups.

In previous studies focusing on treatment of PD, the major concern and attention was given to the problem of biliary strictures. The treatment of strictures and associated cholestasis is important since resolving these factors also reduces the risk of formation or recurrence of cholelithiasis. Biliary stricture and associated jaundice can be managed by medical treatment with ursodeoxycholic acid^[11,16] or by interventions like biliary stenting^[16-18], balloon dilatation^[19] or portosystemic shunting^[20]. If present, cholelithiasis should first be managed by evaluation of varicose veins around the common bile duct, either by magnetic resonance angiography or endoscopic ultrasonography^[17]. After elimination of varicose veins, standard sphincterotomy and balloon trawl for extraction of common bile duct stones and/or sludge should be employed^[10]. The presence of any collateral varicose vein is very important since inadvertent interventions

like basket removal of stones or biliary stenting might result in bothersome hemobilia^[21-25] requiring massive transfusions, injection sclerotherapy^[26], terlipressin^[25] or urgent portosystemic shunting^[21]. In our personal experience, we propose that papillotomy should be performed at earlier stages of disease as a prophylactic measure to make future ERCP procedures safer and easier. If not performed earlier, varices and fibrosis in the distal main bile duct can preclude or complicate safe papillotomy.

In conclusion, cholelithiasis and choledocholithiasis in the setting of PVCT and PD is a specific problem that needs special attention. Stones can become very problematic and interventions may result in severe hemobilia. Stone extraction by balloon or basket trawl can be grueling due to strictures and fibrosis in biliary system. If present, biliary strictures and severe vascular compression should be treated by medical or interventional treatments in order to facilitate stone extraction and reduce recurrence. Further well designed prospective studies are required to clearly understand the exact natural history of cholelithiasis in PVCT patients.

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Acute pancreatitis in aging animals: Loss of pancreatitis-associated protein protection?

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Abstract

AIM: To investigate the effect of age on severity of acute pancreatitis (AP) using biochemical markers, histology and expression of the protective pancreatitis-associated proteins (PAPs).

METHODS: AP was induced *via* intraductal injection of 4% sodium taurocholate in young and old rats. Sera and pancreata were assayed at 24 h for the parameters listed above; we also employed a novel molecular technique to assess bacterial infiltration using polymerase chain reaction to measure bacterial genomic ribosomal RNA.

RESULTS: At 24 h after induction of AP, the pancreata of older animals had less edema (mean \pm SE histologic score of young *vs* old: 3.11 ± 0.16 *vs* 2.50 ± 0.11 , $P < 0.05$), decreased local inflammatory response (histologic score of stromal infiltrate: 3.11 ± 0.27 *vs* 2.00 ± 0.17 ,

$P < 0.05$) and increased bacterial infiltration ($174\% \pm 52\%$ increase from sham *vs* $377\% \pm 4\%$, $P < 0.05$). A decreased expression of PAP1 and PAP2 was demonstrated by Western blotting analysis and immunohistochemical staining. There were no differences in serum amylase and lipase activity, or tissue myeloperoxidase or monocyte chemoattractant protein-1 levels. However, in the most-aged group, serum C-reactive protein levels were higher (young *vs* old: 0.249 ± 0.04 mg/dL *vs* 2.45 ± 0.68 mg/dL, $P < 0.05$).

CONCLUSION: In older animals, there is depressed PAP expression related to a blunted inflammatory response in AP which is associated with worsened bacterial infiltration and higher C-reactive protein level; this may explain the more aggressive clinical course.

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Key words: Acute pancreatitis; Aging; Rats; Pancreatitis-associated protein; Molecular biology

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INTRODUCTION

Acute pancreatitis (AP) is a disease with significant impact in the United States. In 2003 there were about 226 000 admissions for this diagnosis, at a cost of over \$2.2 billion^[1]. The incidence of pancreatitis is higher^[2], and its severity is

worse^[3] in the older patient. While Frey *et al*^[3] showed that the increased comorbidities associated with age account, in part, for the worsened severity, he and others also showed that age is an independent prognostic indicator of survival, specifically in patients over the age of 70 years^[3-5].

The mechanism that puts the aged pancreas, and aged patient, at risk for severity in pancreatitis is unknown. Possibilities include: the immune response of the organism to the injury is impaired; the host organ response to the injury (e.g., lung or renal) is worsened; or protective mechanisms from the pancreas are depressed. The latter two concepts are supported by the observation that the aged patient with pancreatitis has a higher incidence of systemic complications, while there is a decreased local complication rate^[4].

We have recently defined that pancreatitis-associated proteins (PAPs) are proteins innate to the pancreas which are induced during AP. PAPs are members of the pancreatic regenerating (Reg) family of proteins, which are calcium-dependant lectins; all are secretory proteins^[6-8]. PAPs are homologues of pancreatic reg I, and in rats, mice and man, localize to the same chromosome^[7,9-12]. However, while reg I is constitutively expressed in normal pancreas, PAP mRNA expression is very low. PAPs are highly induced during AP^[7,13].

PAPs are endogenous protectors against pancreatic injury. We have shown that targeted inhibition of the three rat PAP isoforms, PAP1, 2 and 3, exacerbated AP^[14,15]. They likely protect by immunomodulation^[16-18]. In particular, PAP2 protects by inducing macrophages, and PAP1 has other anti-inflammatory and anti-apoptotic effects^[19].

Loss of protection by PAP with aging may put patients at risk for pancreatitis. The normal expression of the PAP homologue pancreatic regeneration gene I (*reg I*), decreases in aging^[20], resulting in impairment of its function in the aged animal. We postulate that PAP levels are also depressed in aging, resulting in less protection in AP and more severe disease. However, since baseline PAP levels are minimal in the normal pancreas, we needed to measure the extent of its induction after stimulation.

In this study, we used sodium taurocholate (NaT) to induce AP and determined: (1) the extent of pancreatitis in young and old rats; (2) the extent of PAP expression in young and old animals with pancreatitis; and (3) how PAP expression relates to severity of pancreatitis in the older animals. We employed a novel molecular method to measure bacterial infiltration.

MATERIALS AND METHODS

Animals

Sprague Dawley male rats weighing 260-1000 mg (Charles River, Wilmington, MA) were divided follows: animals in the young group had an average age of 91.9 ± 3.7 d (range 82-111 d, $n = 10$), and those in the older group averaged 339 ± 17.9 d (range 291-546 d, $n = 21$). Each group had 10 unoperated and 10 sham-operated animals as controls.

Animals were fed standard laboratory chow, given

water *ad libitum*, and were randomly assigned to control or experimental groups. All animal studies were approved by the Division of Animal and Laboratory Resources, SUNY Downstate Medical Center.

Pancreatitis

AP was induced with 4% NaT, by retrograde injection into the pancreatic duct as previously described^[14]. Briefly, under nembutal anesthesia (50 mg/kg intraperitoneally, Abbott Laboratories, North Chicago, IL), a midline incision was performed. The common bile duct was identified and cannulated in a retrograde direction with PE-10 tubing (Fisher Scientific, Pittsburgh, PA) through the ampulla of Vater *via* small needle puncture of the duodenum. The bile duct was ligated to prevent the flow of bile, and NaT was infused into the pancreatic duct at a rate of 1 mL/kg over 30 min. The abdomen was then closed with monofilament suture, and the animals allowed to recover.

Animals assigned to the sham group were anesthetized under the same protocol as the sodium taurocholate group. They were then subjected to a midline incision, pancreaticoduodenal manipulation, and then similarly closed and recovered.

Post-operative analysis

The animals were sacrificed 24 h after surgery. Swab cultures of the pancreata were taken, after which whole blood was taken *via* an 18-Gauge syringe in the inferior vena cava. The pancreas was then subdivided into 30 mg portions for pathology (10% formalin), RNA isolation (liquid nitrogen and rapid isolation), and protein isolation.

Qualitative cultures were performed in the clinical microbiology laboratory at SUNY Downstate, for which samples were plated on agar plates for 48-72 h.

To calculate edema, a pancreatic sample was immediately weighed on a coverslip and placed in a 37 °C dry oven. These samples were then re-weighed at 72 h on the same scale. The wet-to-dry ratio was then calculated.

Other pancreas samples were homogenized in 25 mmol/L Tris/0.5% Triton (Sigma, St. Louis, MO) in a glass homogenizer and centrifuged at 12 000 *g* for 10 min at 4 °C. After aspiration of the lipid layer, the supernatant was poured into a 1.5 mL tube and the pellet discarded. Proteins were assessed by the Bradford Assay (Bio-Rad Laboratories, Inc., Richmond, CA) and all samples were diluted to 2 mg/mL in phosphate buffered saline (PBS) (Lonza, Walkersville, MD), treated with protease inhibitor cocktail (Sigma) and stored at -20 °C until analyzed.

Ribonucleic acid isolation

Pancreas samples were flash frozen in liquid nitrogen and immediately processed using 600 μ L Trizol reagent (Fisher Scientific, Piscataway, NJ). The samples were homogenized and mixed with 75% ethanol. The tubes were centrifuged at 10 000 *g*, and the clear lysate was applied to an RNeasy column (Qiagen, Valencia, CA). They were

centrifuged at 10 000 *g* for 15 s and then rinsed with wash buffer, and subjected to DNase digestion.

Blood

Whole blood was collected in red-top tubes without anti-coagulant (Fisher, Piscataway, NJ) and allowed to clot for 30 min at room temperature. Tubes were centrifuged at 1800 rpm for 20 min. Sera were collected and stored at -80 °C until analyzed.

Serum amylase activity was measured using 4,6-ethylidene (G7)-p-nitrophenyl (G1)- α -D-maltoheptaoside as the substrate. Serum lipase was measured by the Clinical Laboratories, SUNY Downstate Medical Center. Similarly, C-reactive protein (CRP) levels were determined in the clinical laboratory using a conventional immunoassay system and Beckman nephelometer (Beckman Coulter, Inc., Galway, Ireland).

Myeloperoxidase, monocyte chemotactic protein-1 analysis

Pancreatic protein was assessed for rat monocyte chemo-tactic protein-1 (MCP-1) using the Rat MCP-1 ELISA Kit for tissue lysate (RayBiotech, Inc., Norcross, GA) and myeloperoxidase (MPO) by the rat MPO ELISA kit (Cell Sciences, Canton, MA) according to the manufacturer's protocol.

Polymerase chain reaction

One-step real-time quantitative reverse-transcriptase-polymerase chain reaction (PCR) for PAP 1, 2, 3, and *reg I* was performed as previously described^[13,14] using the LightCycler 480 (Roche, Indianapolis, IN) with β -actin as an endogenous control to standardize the amount of sample RNA added to a reaction. Primers and probes were designed as previously for PAP 1, 2, and 3^[13,14]; the primers for *reg I* were 5'-TACAGCTGCCAATGTCT-GGATT-3' (forward), 5'-CAGTGTCCAGGATTT-GTAGAGA-3' (reverse), and 5'-ATCCCAAAAATA-ATCGCCGCTGGC-3' (probe) (Applied Biosystems, Bedford MA). One hundred nanograms of total RNA was used to set up 20 μ L real-time quantitative PCRs that consisted of 1 \times Master Mix, 500 nmol/L forward and reverse primers, and 250 nmol/L TaqMan probe (Roche). Polymerase chain reaction amplification was performed with the temperature profile of: 3 min at 45 °C, 7 min at 95 °C, and 45 cycles of 5 s at 95 °C and 30 s at 60 °C. Assays were performed in triplicate. Data were analyzed with the relative standard curve method. Standard curves of the genes of interest and β -actin were prepared with six ten-fold dilutions of total RNA from the sample that was expected to have the highest amount of mRNA for the gene of interest. For each reaction tube, the amount of target or endogenous reference was determined from the standard curves. The mean amount of each sample was calculated from the triplicate data and was normalized by division by the mean quantity of β -actin RNA for the same sample. The mean and standard error of each

treated group were calculated from the normalized value for each rat in the group.

Bacterial 16s ribosomal DNA from pancreatic tissue was amplified by PCR as described^[21], using probes specific for gram-positive cocci (GPC) and gram-negative rods (GNR). The primers are specific for bacterial 16sRNA, a gene highly conserved in bacterial strains. Briefly, 0.2 μ g of total DNA extracted from each pancreas tissue isolated above was added to pre-decontaminated PCR cocktail for PCR amplification with the primers. The product is a 370 bp fragment.

Western blotting analysis

After SDS-polyacrylamide gel electrophoresis (15%), 15 μ g and 30 μ g protein/well was electrophoretically transferred to nitrocellulose (Nytan; Schleicher and Schuell, Keene, NH) and monoclonal antibody to *reg I*^[22] was used at 1:10 000 dilution, polyclonal antibodies to PAP 1, and PAP 2^[22] were used at 1:1000 dilution. Secondary antibody to IgG at 1:100 000 dilution was used for detection of primary antibody.

Densitometry was performed using the Bio-Rad Gel Documentation System with Quantity One software. Units are presented as Intensity (INT) \times area (mm²).

Histopathological analysis

The head of the pancreas from each rat was fixed in 10% buffered formaldehyde solution (Fisher Scientific, Pittsburgh, PA). Four to six micron section slides were generated for each pancreas, collected and stained with hematoxylin and eosin (H and E). Using previously described criteria, the histological severity of pancreatitis was examined in a blinded fashion by our pathologist. Using a scale ranging from zero (representing the least), to four (representing the greatest) severity, the degree of leukocytic infiltration (acinar and stromal) and tissue necrosis (acinar and stromal), edema and hemorrhage were determined for each specimen.

Immunohistochemical analysis was done as described in^[23] using PAP1 and 2 antibodies at 1:1500 dilution.

RESULTS

NaT experiments

There were three mortalities after surgery to induce pancreatitis; 2 in the young and one in the old group. Where appropriate, we show data from shams and unoperated controls for clarity and comparisons. Where noted, we performed subgroup analysis of the older animals, dividing them into: subgroup (a) aged, whose average age was 256.3 \pm 2.1 d (*n* = 10); subgroup (b) very aged, 372.5 \pm 0.4 d (*n* = 9); and subgroup (c) most aged, 544 \pm 2.8 d (*n* = 3). The latter group had the single mortality of the older group.

Twenty four hours after induction of NaT pancreatitis, older animals had slightly less edema, as measured by ratio of wet to dry weight, when compared to younger ones (1.35 \pm 0.04 *vs* 1.52 \pm 0.08, *P* = 0.07). By compari-

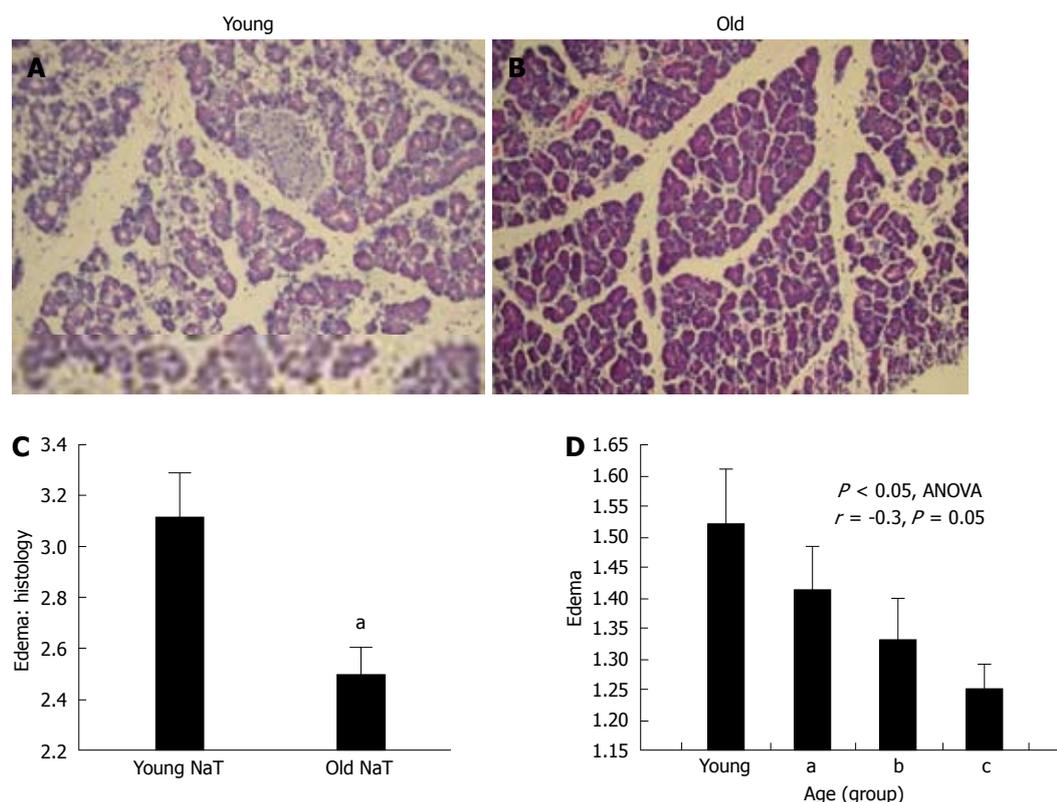


Figure 1 Effect of age on pancreatic edema 24 h after induction of sodium taurocholate pancreatitis. A: A young rat with severe, grade 4 edema [hematoxylin and eosin (H and E), 40×]; B: An old rat with moderate, grade 3 edema (H and E, 40×); C: Mean ± SE histologic scoring of edema for both groups; D: Edema data calculated by wet:dry ratio of pancreatic tissue. While young vs old ratios are discussed in the text, when animals were sorted into young, aged (group a), very aged (group b) and most aged (group c) groups, a progressive decrease in edema was seen with age. A significant negative correlation was also noted ($^{\#}P < 0.05$, Student's *t* test). NaT: Sodium taurocholate; ANOVA: Analysis of variance.

son, sham-operated animals had 1.12 ± 0.03 wet to dry ratios; unoperated controls had 1.01 ± 0.08 (all statistically significantly lower than the NaT group).

Histopathologic examination also showed that the extent of edema was significantly depressed in the older group (Figure 1A-C, $P < 0.05$) When subgroups of age (subgroups A-C), were subjected to analysis of variance (ANOVA), there was a statistically significant decrease (Figure 1D, $P = 0.02$), and significant negative correlation ($r = -0.3$, $P = 0.05$) with aging.

Histopathologic examination also showed that the extent of parenchymal and stromal inflammatory cell infiltrates was depressed in aging (Figures 2A and B, 3B and C). The extent of necrosis (acinar, peripheral acinar or fat necrosis), cellular vacuolization and hemorrhage was not different in the older animals (Figure 3A).

Markers of pancreatitis

When compared to sham-operated controls, animals with NaT pancreatitis showed significant increases in amylase ($628 \pm 63 \mu\text{g/L}$ vs $7919 \pm 2022 \mu\text{g/L}$, respectively, $P < 0.05$), and lipase ($37.7 \pm 16 \mu\text{g/L}$ vs $864 \pm 260 \mu\text{g/L}$, respectively, $P < 0.05$). Also, pancreatic levels of MPO were very high ($202 \pm 49 \text{ ng/mL}$ vs $2014 \pm 628 \text{ ng/mL}$, respectively, $P < 0.05$), as were levels of MCP-1 ($2172 \pm 463 \text{ pg/mL}$ vs $4508 \pm 251 \text{ pg/mL}$, respectively, $P < 0.05$). However, when values from old and young NaT animals

Table 1 Effect of age on biochemical markers of sodium taurocholate pancreatitis

	Young rats	Old rats
Amylase ($\mu\text{g/L}$)	$12\ 536 \pm 6141$	5941 ± 1162
Lipase ($\mu\text{g/L}$)	1288 ± 755	682 ± 194
MPO (ng/mL)	1962 ± 538	2066 ± 1219
MCP-1 (pg/mL)	4312 ± 350	4730 ± 376
CRP (mg/dL)	0.25 ± 0.04	0.61 ± 0.19

All serum levels were higher than sham and unoperated controls. No differences were seen between young and old animals. However, subgroup analysis of CRP levels revealed that the most-aged subgroup (c) had significantly higher levels (Figure 3). MPO: Myeloperoxidase; MCP-1: Monocyte chemoattractant protein-1; CRP: C-reactive protein.

were compared, no differences were noted (Table 1).

Serum CRP levels in control, unoperated animals were $0.17 \pm 0.07 \text{ mg/dL}$. A significant increase in CRP was seen after NaT ($0.25 \pm 0.04 \text{ mg/dL}$, respectively, $P = 0.05$ compared to unoperated controls); but when young and old NaT animals were directly compared, no difference was seen between younger and older groups (Table 1). However, subgroup analysis of age groups showed that serum CRP was statistically higher in the most-aged (subgroup c) (Figure 4) ($2.45 \pm 0.69 \text{ mg/dL}$, $P < 0.001$ ANOVA compared to all groups, $P < 0.001$ compared to young, Student's *t* test). This phenomenon was noted only for

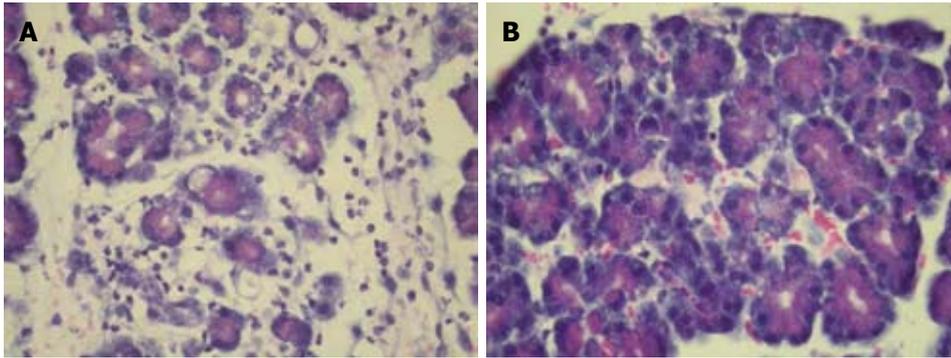


Figure 2 Histology (hematoxylin and eosin, 100 \times) of young (panel A) and old (panel B) inflammatory infiltrates 24 h after induction of sodium taurocholate pancreatitis. A: Severe, grade 4 parenchymal inflammation in the young animal; B: Mild, grade 2 inflammation in an older one.

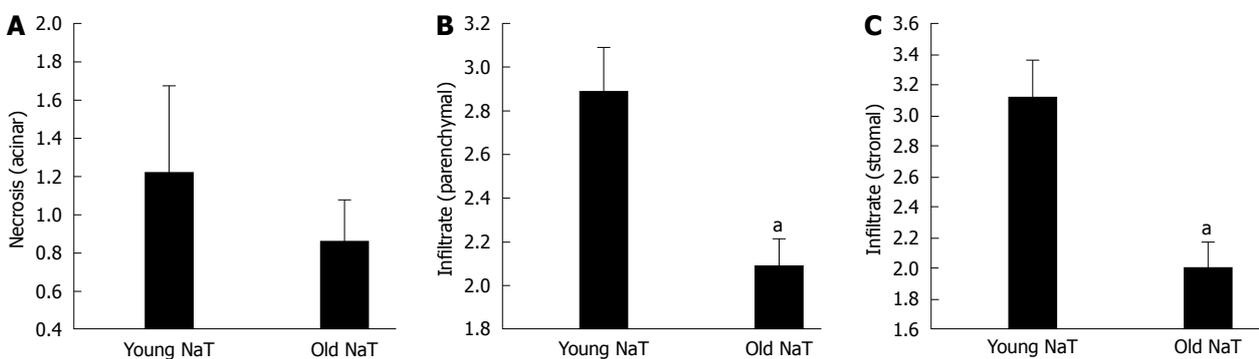


Figure 3 Histologic scores. There was no difference in necrosis scores between young and old (C), but significant decreases were observed in inflammatory infiltrates of the parenchymal (D) and stromal (E) tissue ($^aP < 0.05$, Student's *t* test). NaT: Sodium taurocholate.

CRP, not for any of the other markers measured above (amylase, lipase, MPO, MCP-1).

Real time PCR of mRNA isolated from rats with pancreatitis showed that *reg I*, PAP1, PAP2 and PAP3 all increased when compared to controls (not shown). The increase was similar to that which we have observed previously^[13,14] with *reg I* increasing twofold, and PAP1, 2, and 3 increasing 50-100 fold (not shown). Western analysis showed that protein levels increased but only minimally for *reg I* and by a factor of 3-8 fold for PAP1 and 2.

When young and old NaT animal mRNAs were compared, no differences were noted for pancreatic *reg I*, PAP1 and PAP2 mRNA (Table 2); and only PAP3 levels were significantly decreased. Conversely, when protein levels were compared, older animals had lower levels of PAP1 ($P < 0.05$); PAP2 proteins were depressed as well ($P = 0.07$) (Figure 5). PAP3 proteins were not measured since antibodies were not readily available.

Since we have previously shown *in vitro* that PAP2 directly activates leukocytes^[17], we postulated that its expression would correlate with MPO or MCP-1 activity. While there were no correlations between mRNAs or proteins and MCP-1 levels, a significant positive correlation of PAP2 mRNA levels with MPO protein was observed ($r = 0.81$, $P = 0.004$).

Immunohistochemistry

PAP1 and 2 immunohistochemistry showed no staining in normal pancreas, and positive staining pattern in the acinar cells (Figure 6A-C) after induction of NaT pancreatitis. Similar to our observation in the Western analysis, both PAP1 and 2 immunohistochemistry showed more intensity in the younger animals when compared to older (Figure 7); preparation of the slides was done side by side.

In young animals, PAP1 staining in AP was typically intracellular, uniform, and oriented in a base-to-apical intensity (Figures 6B, 7A). In contrast, PAP2 staining in AP was heterogeneous (Figures 6C, 7C): it too was intracellular, but was occasionally visible as dense, clumped proteins towards the base of the cells.

In older animals the staining patterns reversed: PAP1 staining became more heterogeneous (Figure 7B), and the pattern for PAP2 became more homogenous (Figure 7C).

Interestingly, in many sections, PAP2 staining was found in areas which corresponded to parenchymal and stromal inflammatory infiltrates (Figure 8).

Bacteriology

Qualitative cultures of the pancreata from almost all the animals showed colonization by GPC and GNR, with no difference between old and young animals. For example,

Table 2 Effect of age on pancreatic Reg1 and pancreatitis-associated protein mRNA levels

	Young rats	Old rats
Reg1	0.727 ± 0.19	1.27 ± 0.22
PAP1	119 ± 46	116 ± 18
PAP2	690 ± 267	475 ± 142
PAP3	57.8 ± 19	26.4 ± 4.3 ^a

Real time polymerase chain reaction analysis of RNA isolated from pancreata from young and old rats 24 h after induction of pancreatitis by sodium taurocholate. All were done against a β-actin control. No difference in RNAs were found except for pancreatitis-associated protein (PAP)3 (^a*P* < 0.05, Mann Whitney *U* test).

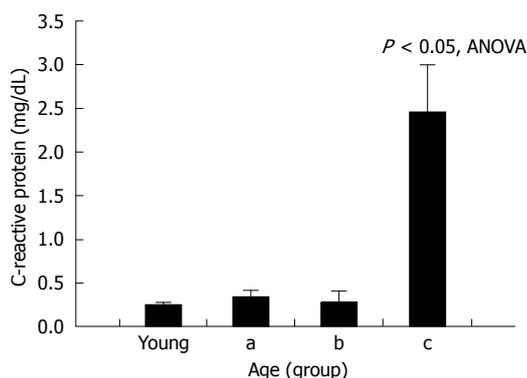


Figure 4 Effect of age on serum C-reactive protein level 24 h after induction of sodium taurocholate pancreatitis. Data for young vs old animals were not significantly different and are shown in the text and Table 1. However, when animals were sorted into young, and subgroups of aged (subgroup a), very aged (subgroup b) and most aged (subgroup c), a significant increase in C-reactive protein was seen in the most aged group (subgroup c). ANOVA: Analysis of variance.

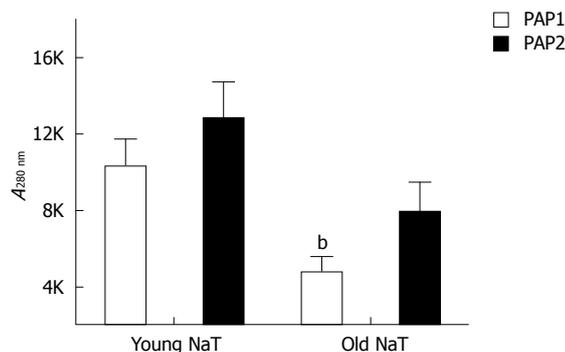


Figure 5 Western blotting analysis of pancreatitis-associated protein 1 and pancreatitis-associated protein 2 protein levels from pancreatic tissue 24 h after induction of sodium taurocholate pancreatitis. Both decreased with age, with statistical significance in the pancreatitis-associated protein (PAP)1 group (^b*P* = 0.003), and near significance in the PAP2 group (*P* = 0.06). Units are based on densitometry and expressed as intensity × area (mm²). NaT: Sodium taurocholate.

5 of 9 young animals with NaT pancreatitis grew GPC, while 8 of 9 grew GNR; only one animal had no culture positive. In the older group, 8 of 19 grew GPC, while 18 of 19 grew GNR; only one had no culture positive.

In order to accurately quantitate bacterial infiltration of the pancreas, we employed a novel molecular technique to discern the amount of bacterial genomic ribosomal RNA (rRNA) in the pancreata. As shown in Figure 9,

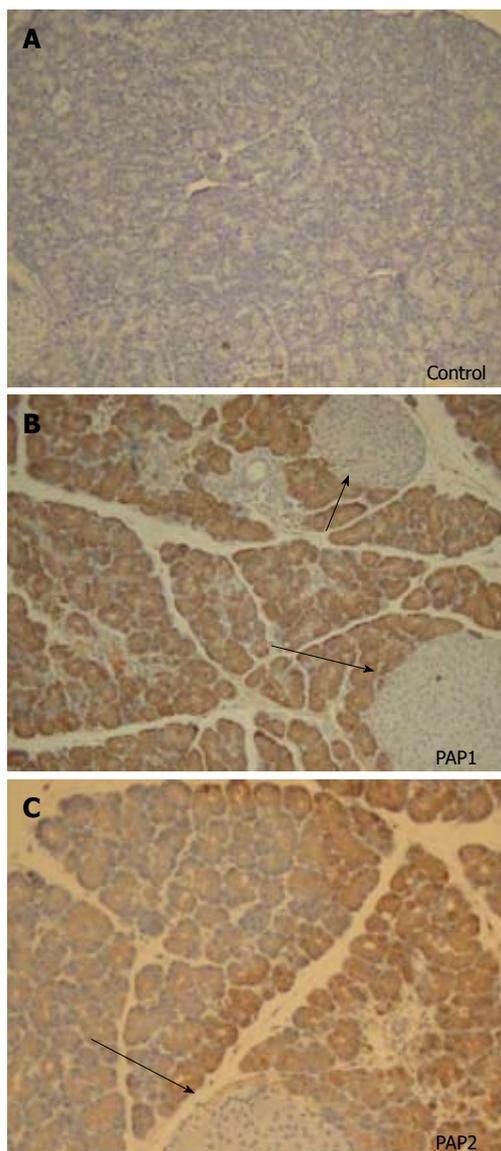


Figure 6 Immunohistochemistry for pancreatitis-associated proteins (40×) in a young control animal without pancreatitis (A) compared to pancreatitis-associated protein 1 (B) and pancreatitis-associated protein 2 (C) in young animals 24 h after sodium taurocholate pancreatitis. No staining is seen in controls, uniform brown colored staining is seen for pancreatitis-associated protein (PAP)1 (B). Preparation of the slides was done side by side. A different, heterogeneous pattern of staining is seen for PAP2 (C). Note that islets of Langerhans (arrows) do not stain at all for PAPs in normal animals or animals with acute pancreatitis.

compared to sham-operated animals, there was an increase in bacterial genomic rRNA in both the young and old NaT animals, and a more intense amount of bacterial rRNA in the older animals when compared to young.

DISCUSSION

In the present study, we found that 24 h after induction of AP, the pancreata of older animals have less edema, decreased inflammatory response and increased bacterial infiltration. This is associated with decreased expression of PAPs as measured by Western analysis and immunohistochemistry. While there were no differences between young

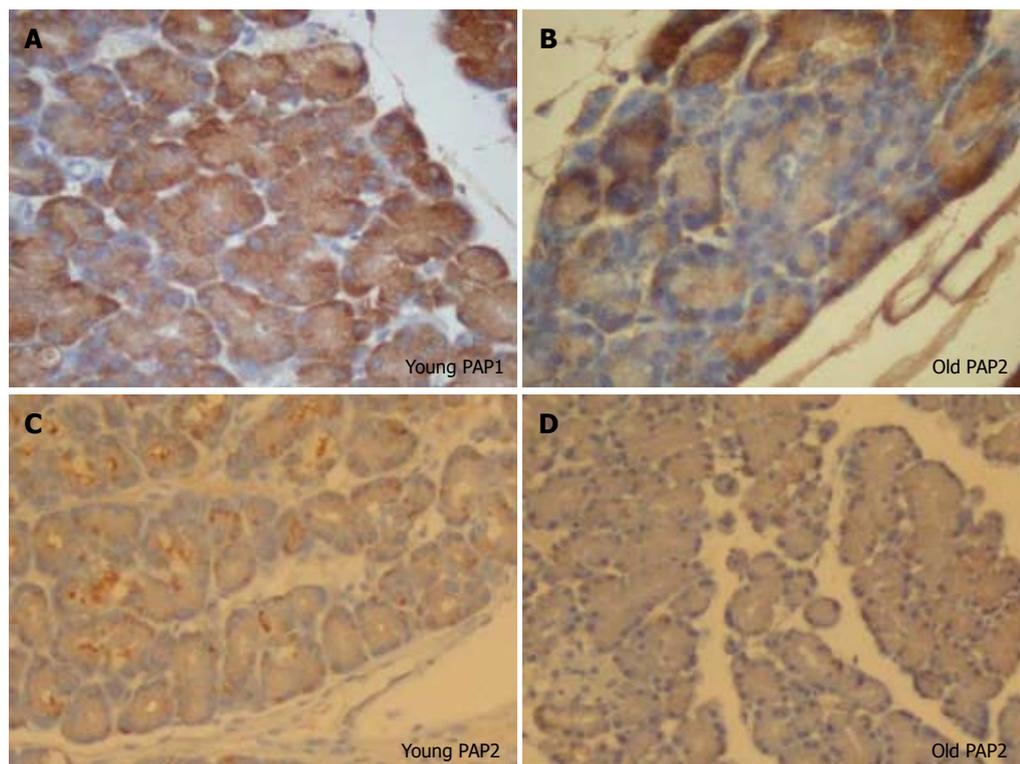


Figure 7 Immunohistochemical staining for pancreatitis-associated protein 1 and 2, high power (100×). Young animals (A, C) are compared to older (B, D). A decreased intensity of staining is observed in aging, and while pancreatitis-associated protein (PAP)1 staining in younger animals is uniform, in older animals it is heterogeneous. In contrast, while PAP2 staining patterns are heterogeneous in younger animals, they are more uniform in older ones. Note the clumping of PAP2 within the cells of the younger animals, which is absent in the older group.

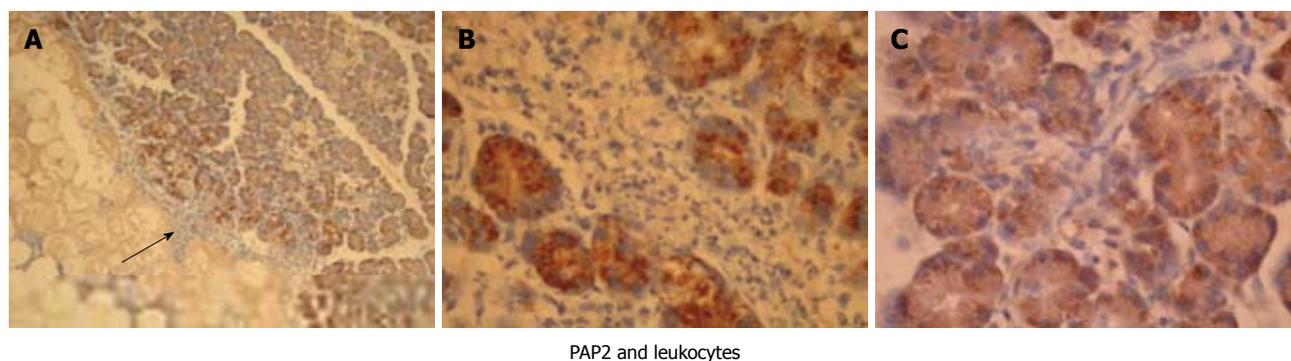


Figure 8 Association of pancreatitis-associated protein 2 staining with inflammatory infiltrates. A: Immunohistochemical staining in animals 24 h after sodium taurocholate. The arrow points to intense stromal infiltration near the periphery of intense pancreatitis-associated protein (PAP2) staining (40×); B: Association of PAP2 positive cells with stromal leukocyte infiltration depicted by arrow in A (100×); C: Association of PAP2 positive cells with parenchymal leukocyte infiltration (100×). This association was not seen for PAP1 (not shown).

and old animals in serum amylase and lipase activity, in the most-aged subgroup (c), there was an increase in CRPs.

These data suggest that the local response to AP is less severe in the older animals, and the systemic response may be worse. PAPs are endogenous protectors against pancreatic injury^[14-18], and it appears that the decrease in PAPs may not be related to the decreased inflammatory infiltrate. As will be described below, the effect may be a result of an altered balance of PAP 1 and 2. The decrease of PAPs may also result in increased bacterial infiltration into the pancreas, and ultimately a worsened outcome.

While the pancreas progressively atrophies with age,

its reserve capacity results in minimal clinical changes. Studies comparing baseline and stimulated levels of pancreatic enzymes have shown no significant differences^[24,25]. However, the regenerative capacity of the pancreas is diminished^[26,27], as evidenced by decreased recovery from pancreatitis. At the molecular level, this has been attributed to decreased phosphorylation of Akt^[27].

Very little is known about the aged pancreas in AP. As we have observed in animals, in humans there seems to be a blunted local response and an exaggerated systemic one. Fan *et al*^[4] noted decreased local complications but increased systemic complications, leading to death.

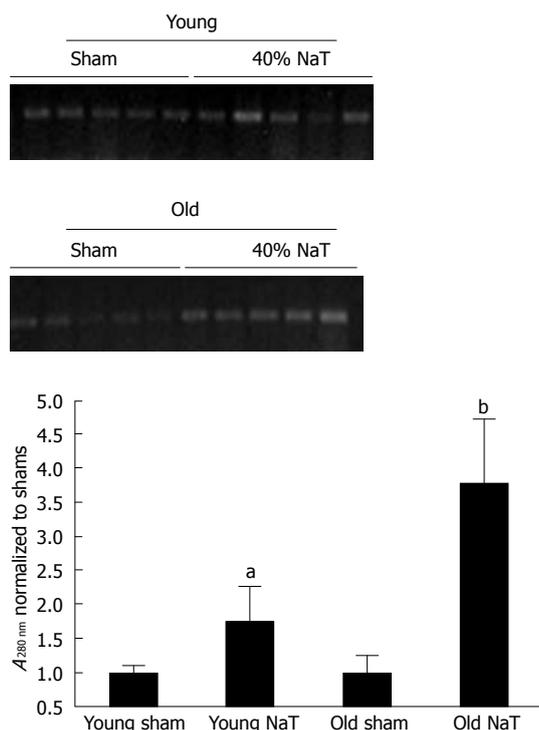


Figure 9 Molecular quantitation of bacterial levels in pancreatic tissue in unoperated controls, sham-operated animals and 24 h after sodium taurocholate pancreatitis. DNA from pancreas was subjected to semiquantitative polymerase chain reaction (PCR) using probes for the genomic sequences of bacterial ribosomal RNA. Top Panel shows representative polyacrylamide gels showing the 370 base pair PCR product; Bottom graph shows quantitated values from multiple experiments. No differences were seen between controls and sham-operated animals (not shown). There were significant differences in bacterial genomic levels between sham and sodium taurocholate (NaT) animals ($^aP < 0.05$), and between young and old ($^bP = 0.008$) NaT animals, when percent increases were compared (Mann-Whitney *U* test).

Kimura *et al.*^[28] described elderly patients with high mortality from AP, but autopsy evidence revealed only mild interstitial pancreatitis, where the inflammation was centered in the ductal and interstitial tissues regions.

We postulated that as animals age, there would be loss of protective mechanisms from the pancreas which would lead to worsened fate after pancreatitis. We focused on the PAP family since we and others have shown that they are endogenous proteins which are protective in AP^[14-16,29]. We know that expression of other genes of the PAP family decrease with aging^[20], and we formulated experiments to determine whether PAPs decreased too.

We showed that in older rats subjected to NaT, there is blunted PAP1 and 2 protein response in both Western analysis and immunohistologic staining patterns. How does this correlate with the histologic and biochemical data above?

PAP1's protective activity may be local or systemic. Locally, it has both mitogenic^[30] and anti-apoptotic^[31] effects, which affect regeneration after pancreatitis. PAP1 is a known anti-inflammatory agent^[16], and a potent modulator of lung injury in pancreatitis^[29,32]. Its loss with aging

may worsen the systemic response after acute pancreatitis.

However, our results differ from others who observed increased local inflammatory infiltrates in PAP1 knockout mice^[19]. Our model differs not only in that we employed a different rodent species, but in the fact that other proteins, such as PAP2, 3 and other inflammatory modulators, are likely also depressed.

PAP2's protective activity, on the other hand, is likely local. It can directly activate macrophages^[17], and macrophage activation has been shown to be protective in pancreatitis^[33-35]. PAP2 is a more potent immunomodulator than PAP1^[36]. In our present study we even noted that PAP2-positive pancreatic cells co-localize with leukocytes (Figure 8); this was not observed with PAP1. We also noted a positive correlation of PAP2 with pancreatic MPO activity. Activation of macrophages can induce MPO activity^[37,38], and it is possible that a decrease of PAP2 will be associated with decreased macrophages or macrophage activity, which is detrimental for the pancreas and host. The decrease in PAP2 may really be the reason for the decreased leukocyte infiltration seen in older animals.

Finally, PAP proteins directly interact with bacteria. They can bind gram-negative^[7] and gram-positive bacteria^[39], and we have discovered a bacteriocidal action of PAP1 and 2 (unpublished results). Our data suggest there is more bacterial infiltration in the older pancreata, as evidenced by increased quantity of bacterial rRNA. The blunted PAP responses may result in more acute pancreatic infection.

Increased bacterial colonization could lead to an increased systemic response to the injury, which is worsened by decreased PAP1 level. It is possible that PAPs are endogenous bacteriostatic or bacteriocidal agents that protect against infection during AP. Whether the loss of PAP with aging leads to increased clinical pancreatic infection remains to be seen. To study this, longer term experiments along with qualitative analysis of bacterial species will be necessary.

In conclusion, this is the first study to show a physiologic difference in AP between young and old animals, and the first to show that endogenous pancreatic molecules are related to the diathesis of the disease. In older animals, there is decreased edema and leukocyte infiltration, but increased bacterial infiltration and even serum CRP level. All are associated with decreased endogenous PAPs in the pancreas. Further studies are planned on the direct effect of all three PAPs in modulating the severity of pancreatitis, by focusing on their local immunomodulatory effect and effect on bacterial infiltrates, as well as the systemic response of the host.

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COMMENTS

Background

Acute pancreatitis (AP) is a disease with significant impact and expense worldwide. In the United States, the cost is significant, and only recently has the impact of this disease on the elderly begun to emerge.

Research frontiers

The mechanism that puts the aged pancreas, and aged patient, at risk for severity in pancreatitis is currently being explored. Impaired immune system, host organ response to the injury or protective mechanisms from the pancreas are possibilities. A molecular mechanism is the likely source.

Innovations and breakthroughs

The authors recently showed that pancreatitis-associated proteins (PAPs) are protective in pancreatitis. The authors postulated that their expression would be depressed in elderly animals with pancreatitis, resulting in worsened disease.

Applications

By understanding how PAPs affect the outcome of pancreatitis, the authors suggest that physicians can then employ strategies to enhance their expression in elderly patients with the disease and prevent complications. The authors also employed a new way of monitoring the infiltration of bacteria into the pancreas using polymerase chain reaction (PCR), which is much more sensitive than culture techniques.

Terminology

PAPs are members of the Reg family of pancreatic regenerative proteins. They have been shown to be important in pancreatic disease, including AP and gastrointestinal malignancies. Expression was monitored by standard techniques such as Western blot and immunohistochemical staining, as well as PCR. Myeloperoxidase and monocyte chemoattractant protein-1 analysis was used to monitor inflammation. Bacterial infiltration was monitored by PCR, using their 16S ribosomal RNA as the unique target.

Peer review

The overall goal of the paper is relevant. The data presented are solid and credible. The results are interesting and clinically important.

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Osteopontin increases hepatocellular carcinoma cell growth in a CD44 dependant manner

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Abstract

AIM: To investigate the role of osteopontin (OPN) and its splice variants in the proliferation of hepatocellular carcinoma (HCC).

METHODS: The expression of OPN variants in HCC cell lines as well as HCC tissue samples and non-tumour tissue was studied using polymerase chain reaction. OPN variant cDNAs were cloned into a mammalian expression vector allowing both transient expression and the production of stable OPN expressing cell lines. OPN expression was studied in these cells using Western blotting, immunofluorescence and enzyme linked immunosorbent assay. A CD44 blocking antibody and siRNA targeting of CD44 were used to examine the role of this receptor in the OPN stimulated cell growth observed in culture. Huh-7 cells stably expressing either OPN-A, -B or -C were injected subcutaneously into the flanks of nude mice to observe *in vivo* tumour

growth. Expression of OPN mRNA and protein in these tumours was examined using reverse transcription-polymerase chain reaction and immunohistochemistry.

RESULTS: OPN is expressed in HCC in 3 forms, the full length OPN-A and 2 splice variants OPN-B and -C. OPN variant expression was noted in HCC tissue as well as cognate surrounding cirrhotic liver tissue. Expression of these OPN variants in the HCC derived cell line Huh-7 resulted in secretion of OPN into the culture medium. Transfer of OPN conditioned media to naïve Huh-7 and HepG2 cells resulted in significant cell growth suggesting that all OPN variants can modulate cell proliferation in a paracrine manner. Furthermore the OPN mediated increase in cellular proliferation was dependent on CD44 as only CD44 positive cell lines responded to OPN conditioned media while siRNA knockdown of CD44 blocked the proliferative effect. OPN expression also increased the proliferation of Huh-7 cells in a subcutaneous nude mouse tumour model, with Huh-7 cells expressing OPN-A showing the greatest proliferative effect.

CONCLUSION: This study demonstrates that OPN plays a significant role in the proliferation of HCC through interaction with the cell surface receptor CD44. Modulation of this interaction could represent a novel strategy for the control of HCC.

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Key words: Osteopontin; Hepatocellular carcinoma; CD44 antigen; Nude mice; Xenograft

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a highly aggressive carcinoma of the liver, and is the fifth most common cancer worldwide, and the third leading cause of cancer related death^[1,2]. Risk factors for HCC include; infection with either hepatitis B virus (HBV) or hepatitis C virus (HCV), alcoholic cirrhosis and exposure to environmental toxins such as aflatoxin. HCC is a global disease most prevalent in Southeast Asia and sub-Saharan Africa^[3], however, in countries such as the United States and Japan, HCC incidence is on the increase, primarily as a result of infection with HCV^[4,5]. Despite intense investigation, therapeutic intervention for HCC is extremely limited, with a poor prognosis due to high rates of recurrence and intrahepatic metastasis following surgical resection. Furthermore, there is a significant gap in our knowledge of the molecular mechanisms responsible for HCC development and progression.

Osteopontin (OPN) is a secreted multifunctional matrix-glycoprotein that is emerging as a significant protein in the biology of HCC. It is involved in normal tissue remodelling processes and is secreted to high levels in numerous tumours including HCC^[6]. OPN is overexpressed in HBV-related metastatic HCC^[7], while OPN antibodies can suppress pulmonary metastasis of HCC cells in a nude mouse model, suggesting that it plays a significant role in the metastatic potential of HCC^[8]. However, its role in development and proliferation of HCC is relatively unexplored, although a recent study involving the silencing OPN mRNA expression in HCC cell lines, suggested it may also have proliferative effects^[9]. OPN binds to the family of $\alpha\beta$ integrins, and the cell-surface adhesion molecule CD44, to initiate cellular signals that enable tumour progression^[10-12]. However, the role, if any, of CD44 and OPN in modulating HCC cell growth, is unknown.

Osteopontin is expressed as a heterogenous protein, dependent on glycosylation patterns and the type of cell from which it is expressed. For example, OPN derived from osteosarcoma-derived cells is smaller than that from non-transformed bone cells, with expression of the smaller form correlating with anchorage independence, suggesting that different forms of OPN have different phenotypic effects^[13]. In addition, the existence of two OPN splice variants have been described, with deletions in exon 4 (termed OPN-C) and 5 (termed OPN-B)^[14]. These variants were originally described in glioma cells and more recently OPN-C has been implicated in the invasiveness of breast cancer cell lines^[15] while in HCC

derived cell lines OPN-C promotes the extracellular cleavage by matrix metalloproteinase (MMP)-9, releasing a distinct 5 kDa OPN fragment that is essential for HCC cellular invasion^[16]. However, the relative expression of the OPN variants in HCC has not been formally demonstrated, nor have their effects on HCC cell growth been studied. In this study we demonstrate that all splice variant forms of OPN are expressed in HCC at the mRNA level and that all have the ability to stimulate the growth of HCC derived cell lines *in vitro* and in an *in vivo* ectopic xenograft mouse model. Furthermore this growth promoting effect was mediated by interaction of OPN with CD44 and adds significantly to our understanding of the role of OPN in HCC.

MATERIALS AND METHODS

Cells and tissue samples

The human hepatoma-derived cell lines used in this study were Huh-7, Hep G2 and Hep3B, while Hepa 1-6 cells are of mouse hepatoma origin. All cells were maintained in Dulbecco's Modified Eagle Medium, containing 4.5 g/L D-Glucose, 25 mmol 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid and 2 mmol/L L-glutamine (Invitrogen, CA, United States). Media was supplemented with 10% fetal calf serum, 12 μ g/mL penicillin and 16 μ g/mL gentamycin. To monitor cell growth, cultured cells were seeded at a density of 7×10^4 cells per well in a 12-well plate and cell numbers monitored daily using trypan blue exclusion. All experiments were performed at least in triplicate. Human HCC tissue and cognate surrounding tissue were collected from patients undergoing HCC resection at the Royal Adelaide Hospital (collection was approved by the Hospital's ethics committee).

Construction of OPN expression plasmids and transfection

Full-length OPN cDNA and splice variants were amplified from Huh-7 cells by reverse transcription polymerase chain reaction (RT-PCR). Total RNA and cDNA synthesis were performed as described elsewhere^[17]. The coding sequence for OPN was amplified using the primers 5'-GTTGAAGCTTCTCACTACCATGAGATTGACAGTG-3' and 5-TAGTTCTAGACCTTTTAATTGACCTCAGAAGATG-3' and cloned into the mammalian expression vector pRC-CMV using *Xba* I and *Hind* III to generate pRC-CMV-OPN-A. During sequence verification, two smaller cDNAs were isolated that corresponded to OPN-B and OPN-C. These vectors were used in transient expression assays and to generate stable OPN expressing clones in Huh-7 cells using the transfection reagent Fugene (Roche, Australia) and G418 selection.

RNA extraction, cDNA synthesis and PCR analysis

Total cellular RNA was isolated from cells using Trizol (Invitrogen) and first strand cDNA was synthesized

and standard PCR performed as mentioned previously^[17]. Primers used for OPN cDNA detection were 5'-ATGAGA ATTGCAGTGATTTGCTTTTGCCT-3' and 5'-CATGGTCATCATCATCTTCATCATC-3'; primers utilised for the detection of CD44 were 5'-GACACATATTGCTTCAATGCTTCAGC-3' and 5'-GATGCCAAGATGATCAGCCATTCTGGAAT-3'; and glyceraldehyde-3-phosphate dehydrogenase primers were 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. Amplicons were electrophoresed through a 1% agarose gel before visualisation on a ultraviolet transilluminator.

Detection of OPN and CD44

Huh-7 cells, either stably selected or transiently expressing OPN, were lysed using RIPA buffer and total protein separated *via* sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose as previously described^[18]. Membranes were blocked with 5% skim milk in 0.1% phosphate buffered saline Tween-20 (PBS-T) and incubated overnight at 4 °C with either 400 ng/mL of goat anti-human OPN antibody (K-20: SCBT, SantaCruz, CA) or mouse anti-human CD44 antibody (Labvision, Fremont, CA, United States) at 200 ng/mL followed by either 33 ng/mL of anti-goat or anti-mouse horseradish peroxidase (HRP) antibody (Rockland, Gilbertsville, PA, United States). Washes between antibody binding were with 0.1% PBS-T. Protein bound to antibody was visualised *via* chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ, United States).

Cellular localisation of transiently expressed OPN was performed *via* indirect immunofluorescence as previously described^[19] with the exception that cells were incubated in 1 µg/mL of anti-OPN antibody followed by 10 mg/mL anti-goat Alexa 488-conjugated antibody (Molecular probes, Eugene, OR). CD44 expression was visualised using a mouse anti-human CD44 antibody at 4 µg/mL on cells that had been fixed in 5% formalin but not permeabilised for detection of surface CD44 only. Cells were visualised using a BioRad Radiance 2100 confocal microscope.

OPN concentration in cell culture supernatants was determined using an "in house" sandwich enzyme linked immunosorbent assay (ELISA) as described previously^[17]; where plates were coated with a monoclonal anti-OPN antibody (3 µg/mL R and D Systems, Minneapolis, MN, United States) and detection performed with a polyclonal anti-OPN antibody (200 ng/mL R and D Systems). CD44 blocking antibody (sc-7946; Santa Cruz, CA, United States) for 30 min at room temperature. The blocking antibody is a polyclonal antiserum raised against amino acids 21-320 of CD44^[20].

siRNA knockdown of CD44

StealthTM siRNA double stranded RNA oligonucleotides (Invitrogen) designed to "knock down" or minimise expression of the OPN receptor CD44 were transfected into Huh-7 and HeLa cells using the reverse transcription method of LipofectamineTM 2000 (Invitrogen) as

per the manufacturer's instructions. Cells were assayed for CD44 expression (and growth rate when required) 24-96 h post transfection *via* immunoblot assay. Three different siRNA oligonucleotides, designed to target the conserved exons present in all CD44 isoforms, were tested for their ability to minimise CD44 expression in a transient system using both RT-PCR and immunoblotting as described above. Of the three, two demonstrated consistent knockdown of CD44 at the RNA level by RT-PCR and immunoblot analysis and were pooled for further experiments.

In vivo tumour growth

Five week-old female Balb/c nude mice were implanted subcutaneously with 5×10^6 cells from Huh-7 OPN-A expressing cell lines and the Huh-7 parent cell line as a control. Cells were diluted in 100 µL of PBS and injected into both dorsal flanks ($n = 6$). Tumour growth was monitored by calculation of tumour volume [V (mL) = (width² × length²)/2] and measured daily for approximately 25 d. The mice were sacrificed and tumours removed for histological analysis. These experiments were approved by the University of Adelaide and Institute of Medical and Veterinary Science animal ethics committees.

Immunohistochemical staining of tissue sections

Paraffin embedded sections of liver and tumour tissue were processed as previously described^[17]. Five micron sections were re-hydrated and incubated in either anti-OPN (LabVision, Fremont, CA, United States), or normal rabbit IgG (Cell signalling, Boston, MA, United States) (diluted to 1 µg/mL in PBS + 3% (v/v) normal horse serum) for 30 min, before being incubated in ADVANCETM HRP-Link (Dako, Glostrup, Denmark), and then in ADVANCETM HRP Enzyme (Dako) for 30 min each. Slides were then incubated in diluted DAB + chromagen (Dako; 20 µL DAB + chromagen in 1 mL DAB + substrate buffer) for 5 min and then following a 5 min wash in H₂O, slides were counterstained with haematoxylin.

Statistical analysis

Student *t* tests were performed to analyse the distributions of 2 independent data sets and all statistical analysis were performed using SPSS 10.0.

RESULTS

Multiple OPN forms are expressed in HCC

The existence of multiple forms of OPN, termed OPN-A, OPN-B and OPN-C, has been previously described^[14], however, their expression and physiological roles in tumour biology, including HCC has not been well studied. We therefore investigated expression of these OPN variants in the HCC cell lines, Huh-7, HepG2, Hep3b and the mouse HCC line Hepa1-6, using PCR primers that spanned exons 2-6 (Figure 1A). As predicted, all HCC cell lines expressed the full-length OPN-A mRNA (Figure 1B). In addition, two smaller transcripts were identified

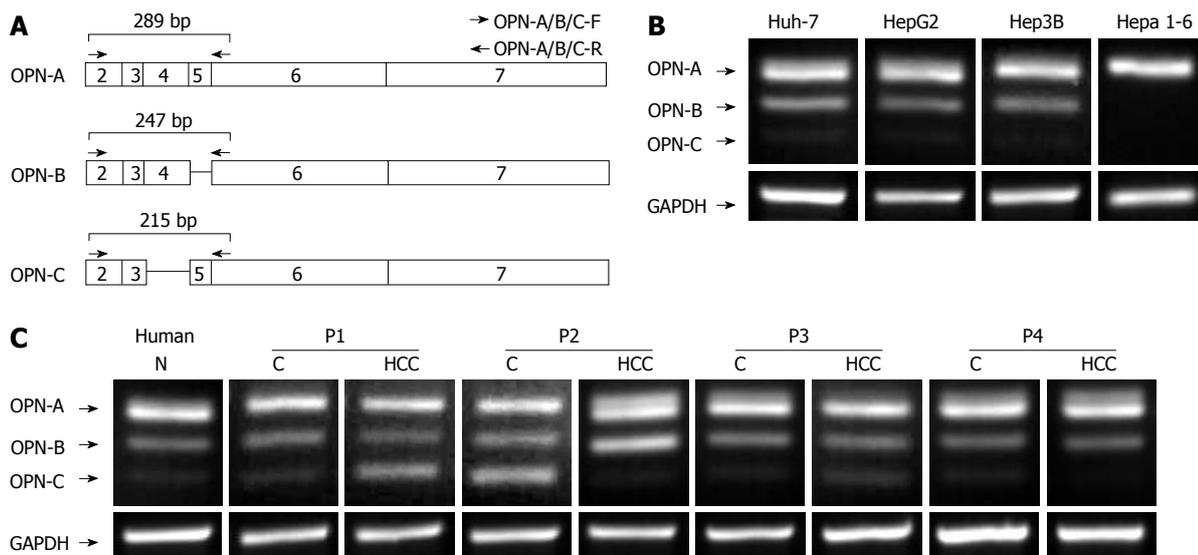


Figure 1 Osteopontin splice variants are expressed at different levels in the liver of hepatocellular carcinoma patients and in various cell lines. A: Schematic representation of the three osteopontin (OPN) splice variants; B: Polymerase chain reaction (PCR) performed on cDNA from different cell types shows varying distribution patterns of OPN mRNA; C: PCR performed on cDNA taken from patient hepatocellular carcinoma (HCC), and its surrounding tissue indicates altered OPN splice variant expression in HCC. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

in all cell lines (with the exception of Hepa 1-6 cells) with OPN-B consistently expressed to a higher level than OPN-C (OPN-C cDNA is readily visible on longer exposure). These smaller transcripts were identified by cloning and sequencing and shown to be specific for OPN-b and OPN-C that lack exons 5 and 4 respectively (Figure 1A). OPN-A is clearly the dominantly expressed OPN transcript followed by OPN-B and OPN-C respectively. Interestingly mouse tissue only expressed the full length OPN-A and this is consistent with a previous report indicating that OPN-A is exclusively expressed in mouse tissues^[15].

We next investigated OPN mRNA expression in HCC tissue samples and cognate non-tumour tissue using the primers described above. Attempts to generate a quantitative real-time assay for detection of all OPN variant simultaneously was unreliable. Nevertheless, it is clear that normal human liver tissue expressed the 3 OPN transcripts to variable degrees, while OPN variant expression was variable between HCC samples. For example, OPN-C expression was increased in HCC P1 compared to cognate liver tissue, while in P2, expression of OPN transcripts in non-tumour tissue was significantly different from that of the corresponding tumour (Figure 1C). This suggests that OPN transcript expression is variable and that individual OPN variants may have specific physiological roles. The detection of all OPN splice variants in normal liver tissue (OPN-C visible on long exposure) is in contrast to previous reports in which it was suggested that OPN-C expression is specific to tumour cells^[14,15].

Stable expression of OPN splice variants in Huh-7 cells

In order to study the physiological roles of the distinct OPN splice variants, cDNAs representing OPN-A,

OPN-B and OPN-C, were cloned into a mammalian expression vector to allow both transient expression and the production of stable OPN expressing cell lines. Following transfection of cDNA constructs representing the OPN variants, detection of OPN expression by confocal microscopy was unsuccessful, suggesting efficient secretion of OPN into the media. However, following treatment of transiently transfected cells with brefeldin A, which inhibits cellular secretion pathways and causes retention of OPN within the cell, we could detect intracellular expression of OPN variants (Figure 2A). OPN is predominantly thought to act at the extracellular level, however, OPN can be detected in tumourogenic hepatocytes by immunohistochemistry suggesting it may, at least in part, have a role at the intracellular level^[6]. Following brefeldin-A treatment, OPN variants exhibited different cytoplasmic distribution in Huh-7 cells, suggesting that each variant may have different effects on tumourogenic hepatocytes, however, this requires further investigation.

To investigate the role of the OPN variants on cell growth, stable clones were produced in a Huh-7 cell line that has low OPN mRNA expression and undetectable protein expression by ELISA (results not shown) and immunoblot. Multiple clones were isolated after G418 selection with no adverse effect on cell morphology or growth. Intracellular OPN was not detected in any clones (results not shown) consistent with efficient expression of OPN, however, OPN mRNA, specific for the OPN variant of interest, was detected by RT-PCR (Figure 2B), while OPN protein was detected by immunoblot (Figure 2C). Furthermore, OPN ELISA performed on media from stable cell lines revealed secretion of OPN in parent Huh 7 and vector transfected cells to be below the limit of detection (< 7.81 ng/mL).

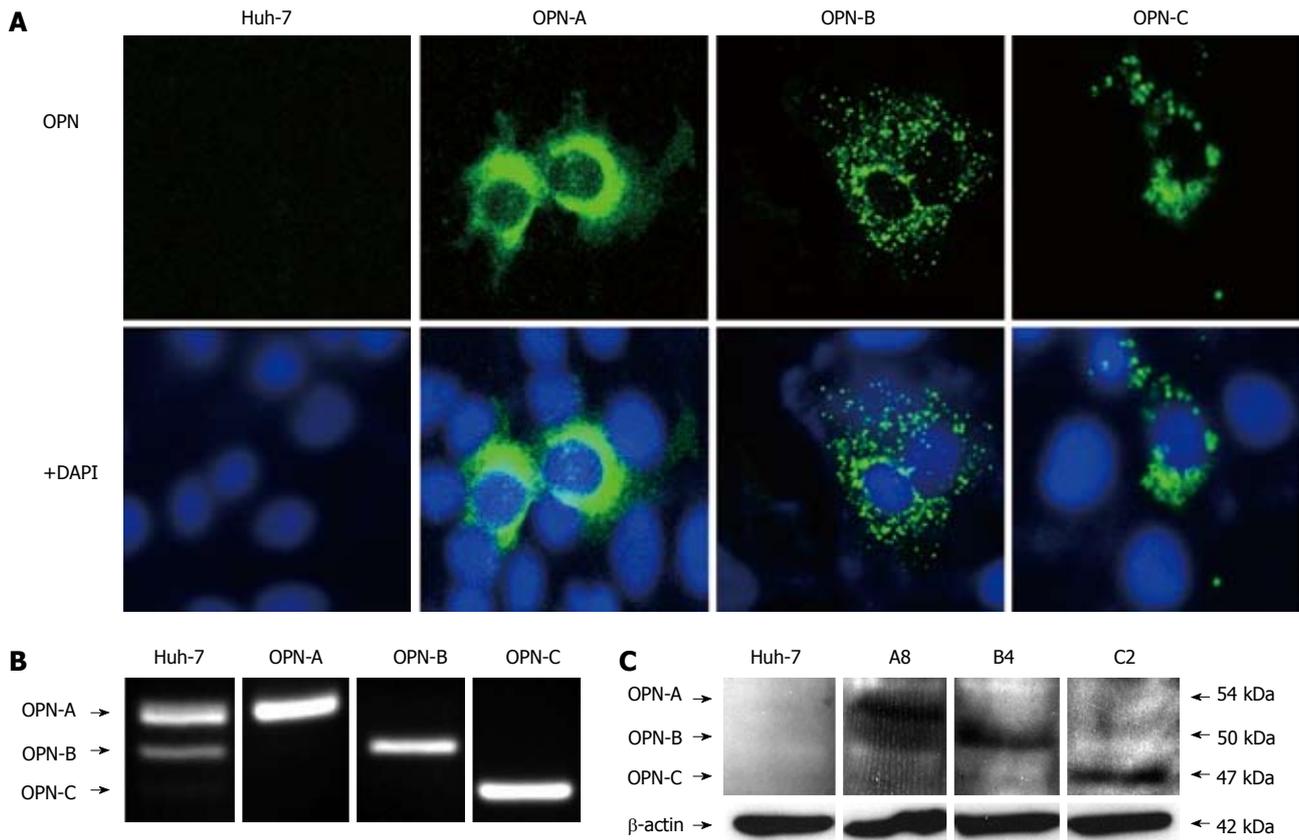


Figure 2 Stable expression of osteopontin splice variants demonstrates unique localisation patterns. Huh-7 cell stably overexpressing osteopontin (OPN)-A, -B, -C, or the control, were stained for OPN expression and visualised fluorescently to demonstrate localisation of the three splice variants (A); Stable expression was confirmed using polymerase chain reaction (B), and Western blotting (C). DAPI: 4',6-diamidino-2-phenylindole.

In contrast OPN-A was detected at 160.4 ng/mL and OPN-B at 127.1 ng/mL. We could not detect OPN-C by ELISA for reasons that are not entirely clear, however the OPN detection ELISA Ab was raised against OPN purified from human milk in which OPN-C is not present^[21]. However, OPN-C is clearly expressed in our system as detected by RT-PCR, immunofluorescence and immunoblotting (Figure 2).

OPN variants facilitate proliferation of HCC derived cell lines

Little work has been done regarding the role of OPN and associated splice variants on cell growth. However recent work from Sun *et al.*^[9] suggests that OPN can enhance cell growth of liver-derived cell lines, although the role of OPN splice variants was not investigated. Therefore, using our stably transduced OPN Huh-7 cells, we determined the impact of the individual variants on Huh-7 cell growth. Huh-7 cells expressing either OPN-A, -B or -C grew significantly faster after 4 d of culture compared to cells transfected with the vector only and the parent cell line (Figure 3A). This increase in growth rate was seen in multiple selected OPN transformants, removing the possibility of clonal selection bias. OPN is readily secreted from these Huh-7 cells and to determine if OPN was exerting its effect by either an autocrine or paracrine mechanism, we incubated Huh-7

cells with conditioned media from OPN-A, -B or -C stable transformants. As can be seen in Figure 3B, conditioned media from all stable transformants increased Huh-7 cell growth, suggesting OPN was exerting its effect in a paracrine manner. This increase in Huh-7 cell growth was directly attributable to the action of OPN because the addition of an anti-OPN antibody, but not a control immunoglobulin, to the cultures, suppressed the increase in cell growth when OPN-A conditioned media was added to Huh-7 cells (Figure 3C). Similar results were obtained for OPN-B and -C conditioned media (results not shown). These results suggest that all variants of OPN can enhance Huh-7 cell proliferation *in vitro*.

OPN exerts its growth proliferative effect through CD44

The C-terminal region of OPN binds the CD44 variant v6/7 to induce cellular signals responsible for tumour progression^[22]. To investigate if OPN was exerting its growth effect in a CD44-dependent manner, we stimulated a number of cell lines possessing different levels of CD44 with OPN conditioned media. As can be seen in Figure 4A, Huh-7, HepG2 and HeLa cells all express cell surface CD44 as determined by immunofluorescence microscopy of unpermeabilised cells, while Hep3b cells do not express CD44. Consistent with this observation, Huh-7, HepG2 and HeLa cells, when stimulated with OPN-A conditioned media, all showed a significant in-

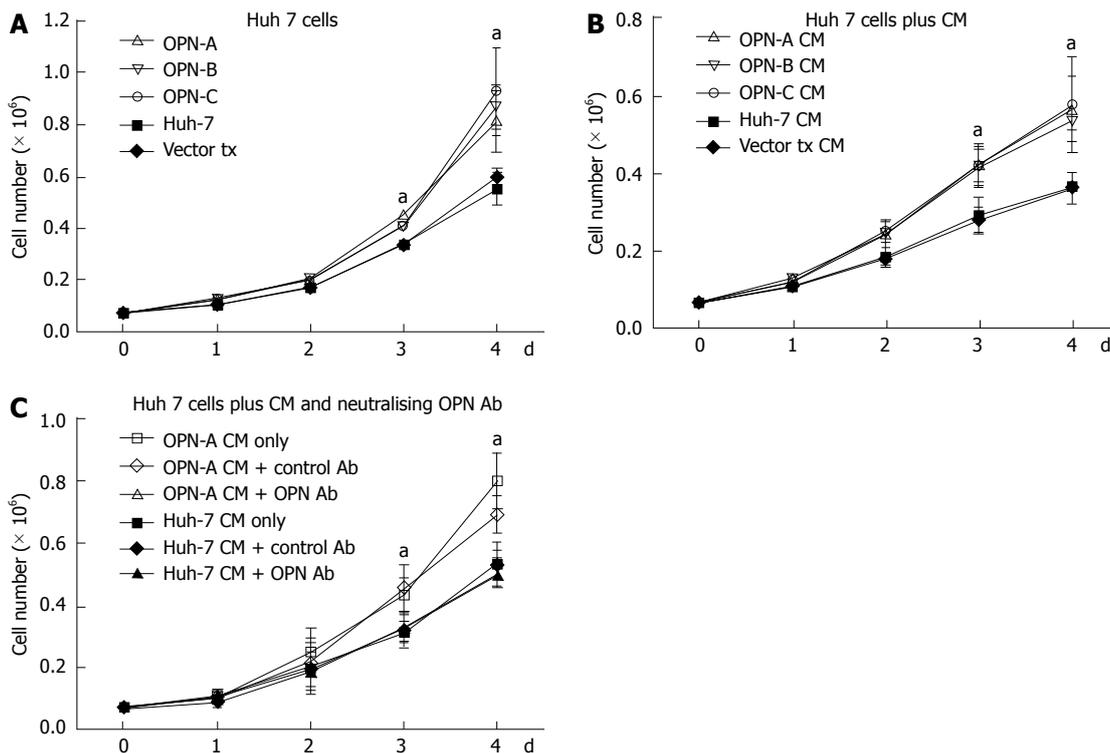


Figure 3 Osteopontin splice variants increase Huh-7 cell growth. A: Osteopontin (OPN)-A, -B, -C and control-expressing Huh-7 stable cell lines in conjunction with Huh-7 cells, were seeded at a density of 7×10^4 cells per well in a 12-well plate and cell numbers monitored daily using trypan blue exclusion; B: Conditioned media (CM) was removed from OPN-A, -B, -C and control-expressing Huh-7 stable cell lines in conjunction with Huh-7 cells and placed onto Huh-7 cells. Cells were monitored as mentioned above; C: Huh-7 cells were seeded at a density of 7×10^4 cells per well in a 12-well plate and conditioned media from OPN-A, -B, -C and control expressing-Huh-7 stable cell lines placed onto the cells in conjunction with either anti-OPN (OPN Ab) or an control immunoglobulin. ^a $P < 0.05$.

crease in cell growth (Figures 3B, 4B and C). However no cell growth was noted in Hep3Bs consistent with a lack of CD44 expression (Figure 4D). These observations were confirmed following the addition of CD44 blocking antibody to the conditioned media that completely suppressed the OPN growth effect (Figure 5A and B). Addition of control immunoglobulin to the cultures had no effect on OPN induced cell growth. To further confirm the role of CD44, we suppressed CD44 expression using an siRNA approach. Transfection of an siRNA targeting CD44 resulted in a significant decrease in CD44 mRNA and CD44 protein expression as determined by PCR and immunoblot in Huh-7 and HeLa cells (Figure 6A and B). This knockdown, in turn, correlated with a decrease in OPN-mediated dependent cell growth of both cell lines (Figure 6C and D). Collectively, these experiments indicate that the increase in OPN induced cell growth results from an interaction between OPN and CD44.

OPN-A induced tumour growth in an ectopic xenograft mouse model

Our *in vitro* data prompted us to examine whether the different OPN splice variants regulate tumour growth in an ectopic xenograft nude mouse model. Accordingly, Huh-7 cells stably expressing either OPN-A, -B or -C were injected subcutaneously into the flanks of nude mice. Mice injected with OPN-A expressing Huh-7 cells were sacrificed approximately 18 d post injection

as the tumour size was causing mice discomfort, while all other mice were sacrificed at approximately 22 d post injection. The typical gross appearance of the tumours is shown in Figure 7A, while the mean tumour volume throughout the experiment is shown in Figure 7B. Compared to parent control Huh-7 cells, mice injected with OPN expressing cell lines showed an increase in tumour growth. This increase in cell growth was most noticeable in mice injected with Huh-7 cells expressing full-length OPN-A (Figure 7B). These mice developed tumours at day 10, after which the growth rate was faster than that of the control tumours. To confirm OPN expression in isolated tumours, RNA was isolated and mRNA expression of the different OPN splice variants was confirmed by RT-PCR (Figure 7C). Detection of OPN *in situ* was performed by immunohistochemistry in which OPN-A expressing tumours expressed significantly more OPN-A than control Huh-7 cells (Figure 7D). OPN-B and -C were similarly detected by immunohistochemistry (results not shown). No differences were noted histologically between the tumours, with the exception that OPN-A tumours contained a greater number of cells undergoing various stages of the mitotic pathway. These data clearly suggest a role for OPN in promoting the growth of Huh-7 cells in an ectopic xenograft mouse model.

DISCUSSION

OPN is a secreted phosphoglycoprotein that binds to

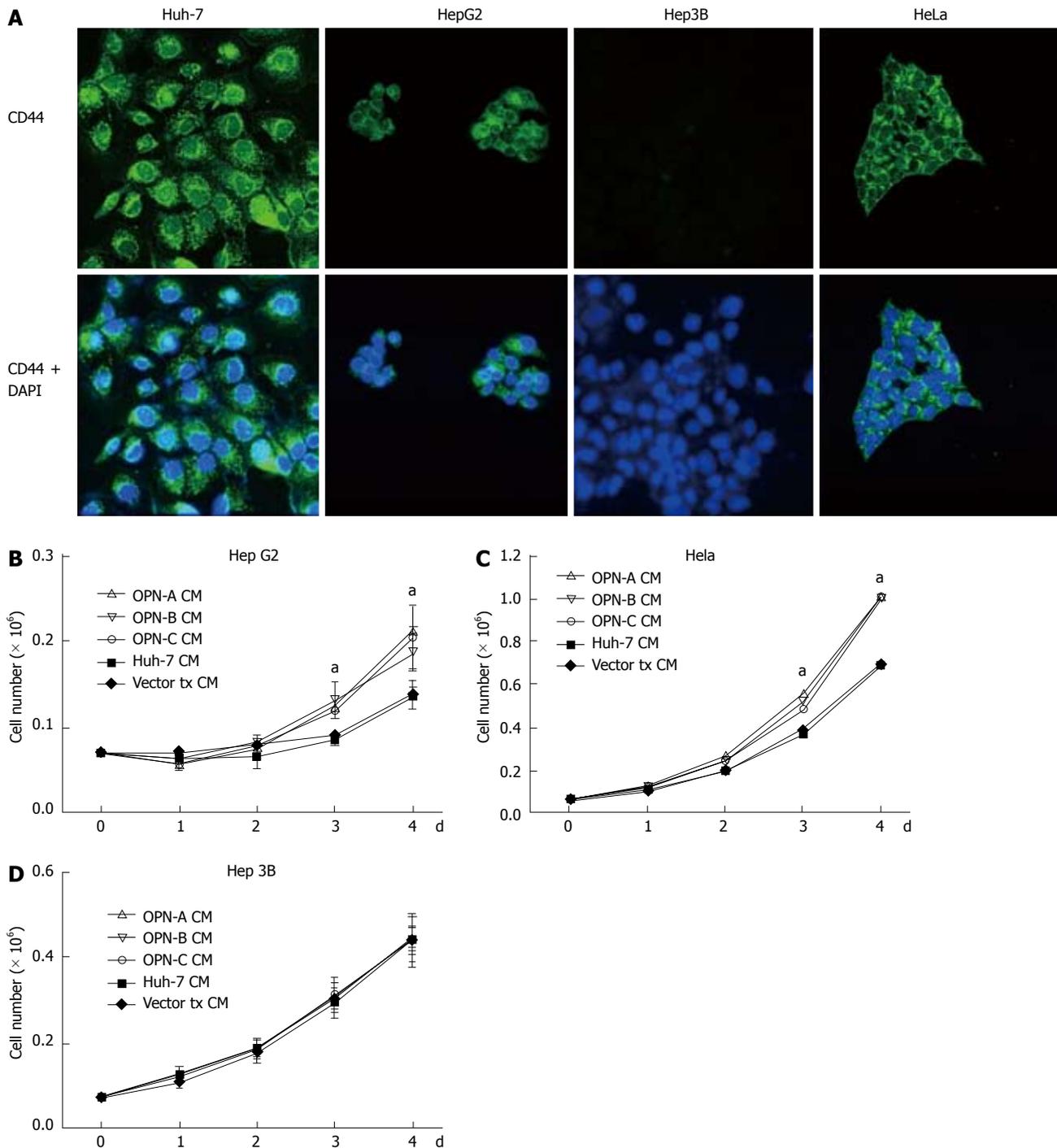


Figure 4 CD44 expression is necessary for increased cellular growth due to osteopontin expression. Huh-7, HepG2, Hep3B and HeLa cells were immunostained for 4',6-diamidino-2-phenylindole (DAPI) and CD44 expression (A); Conditioned media (CM) was removed from osteopontin (OPN)-A, -B, -C and control-expressing Huh-7 stable cell lines in conjunction with Huh-7 cells and placed onto Hep G2 cells (B); HeLa cells (C) or Hep 3B cells (D); cell numbers were monitored daily using a trypan blue exclusion. * $P < 0.05$.

the family of cellular receptors integrin $\alpha v \beta$ and CD44 to exert its biological effects, and of late has gained attention as its expression is associated with invasion and metastasis of a number of malignant tumours, including HCC^[8,23-26]. In many of these tumours, increased plasma levels of OPN have shown promise as potential prognostic biomarkers as OPN expression correlates with metastatic potential, early recurrence and decreased

patient survival. While our understanding of the role of OPN in HCC biology is expanding, there is still a paucity of information regarding the role of OPN (and its splice variants) in proliferation of HCC and the molecular mechanisms responsible.

OPN undergoes post-translational modifications *via* phosphorylation, glycosylation and cleavage by thrombin and MMPs to produce differentially active forms^[10].

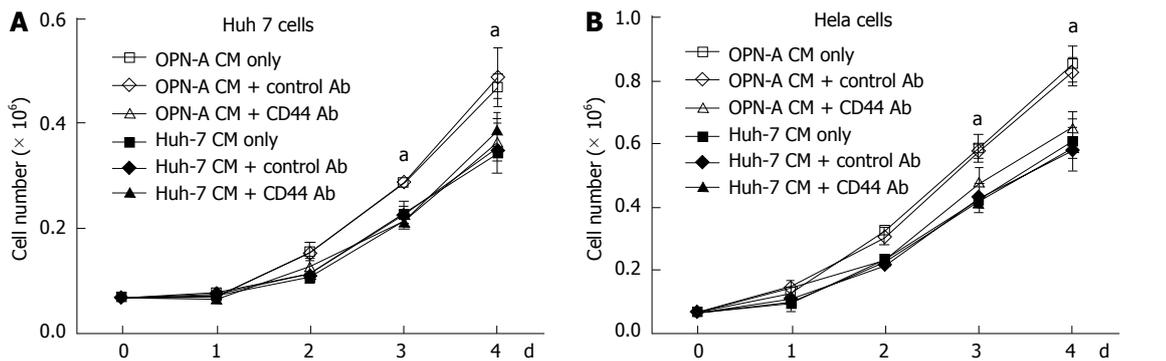


Figure 5 CD44 engagement is necessary for osteopontin-a induced cell growth in Huh-7 and HeLa cells. (A) Huh-7 or (B) HeLa cells were seeded at a density of 7×10^4 cells per well in a 12-well plate and conditioned media from osteopontin (OPN)-A Huh-7 stable cell lines or Huh-7 cells alone placed onto the cells in conjunction with either anti-CD44 or a control immunoglobulin. Cell numbers were monitored daily using trypan blue exclusion. CM: Conditioned media. ^a*P* < 0.05.

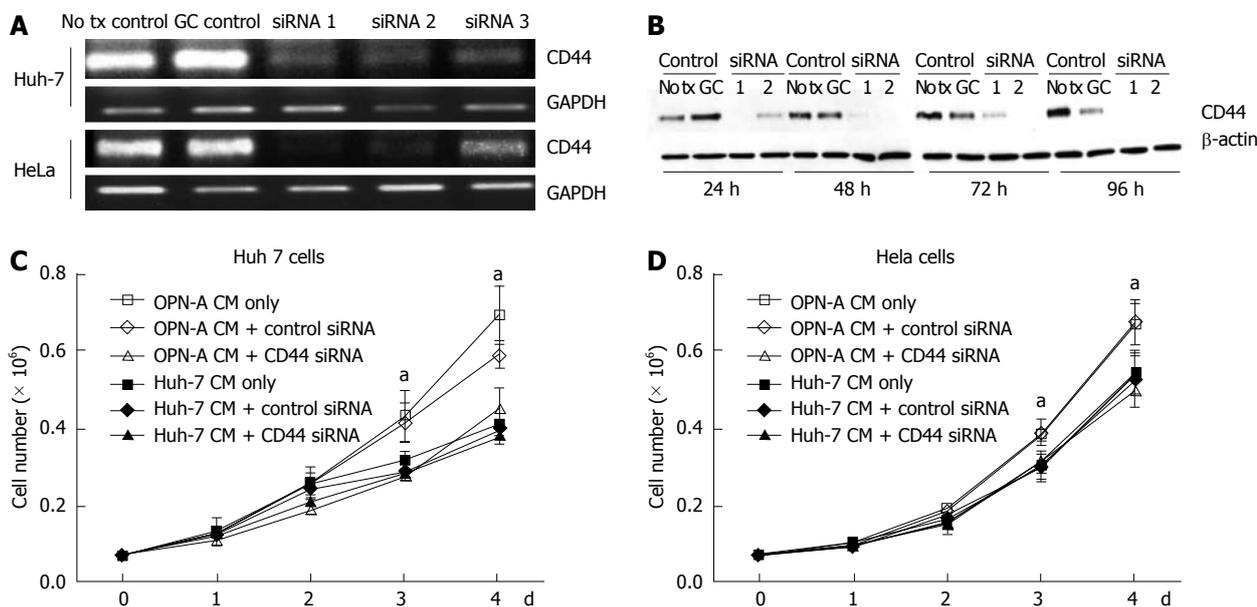


Figure 6 CD44 knockdown inhibits osteopontin-a-induced cell growth in Huh-7 and HeLa cells. A panel of 3 Stealth siRNA's were investigated for their ability to limit CD44 mRNA expression using polymerase chain reaction (A), further validation was achieved by examining protein reduction of CD44 using Western blotting (B). (C) Huh-7 or (D) HeLa cells were seeded at a density of 7×10^4 cells per well in a 12-well plate and a CD44 siRNA pool containing Stealth siRNA1 and 2 was transfected into the cultures 24 h prior to placing conditioned media from osteopontin (OPN)-A Huh-7 stable cell lines or Huh-7 cells alone onto the cells. Cell numbers were monitored daily using trypan blue exclusion. CM: Conditioned media; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. ^a*P* < 0.05.

For example, the MMP-9 cleaves OPN into specific fragments of which a 5 kDa fragment induces HCC cellular invasion *via* the CD44 receptor^[16]. Furthermore, stromelysin (MMP-3) and matrilysin (MMP-7) have been reported to cleave OPN at residues 166 and 210, resulting in tumour cell adhesion, while thrombin cleavage of OPN mediates increased adhesion and migration of tumour cells, *via* binding of the OPN RGD motif to cell surface integrins^[27,28]. Clearly the role of OPN and its derivatives in tumour biology is complex, which is further complicated by the expression of OPN splice variants. In humans, the existence of two OPN splice variants with deletion of exon 4 (OPN-C) or exon 5 (OPN-B) were first described in glioma cells^[29] and more recently in breast cancer cell lines^[15], however, in HCC, the roles of the OPN splice variants is relatively unde-

finied, although a recent report suggests that expression of OPN-A and -B in HCC-derived cell lines confers a robust migratory phenotype through activation of the p42/44 Map kinase pathway^[30].

There has been little investigation of the expression of OPN splice variants and the mechanisms by which OPN regulates cellular proliferation. Our initial investigations focused on OPN splice variant expression in various HCC cell lines, and in HCC and its cognate surrounding normal liver. At the mRNA level, we observed expression of all three OPN splice variants in human HCC cell lines, although OPN-A was clearly the dominant species followed by OPN-B and to a lesser extent-c. This observation is consistent with the work of Takafuji *et al*^[16] who showed that greater levels OPN-C were expressed in HCC cell lines with high metastatic potential

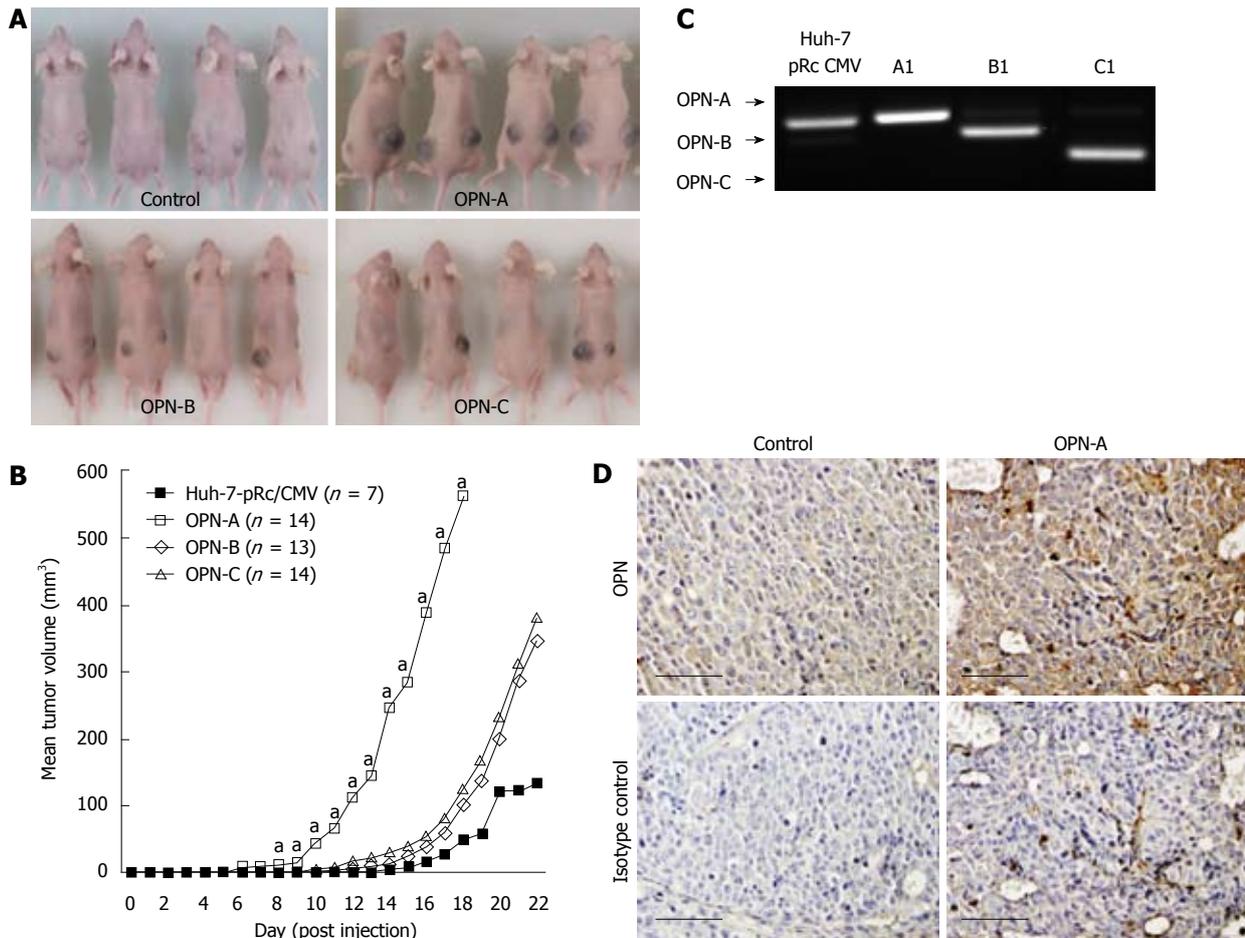


Figure 7 Over expression of the three osteopontin splice variants promotes Huh-7 tumour growth in Balb/c nude mice. A: Growth of tumours in mice injected in the hind flanks with osteopontin (OPN)-A, -B, -C or control-expressing stable cell lines, with mean tumour volumes at the time of euthanasia expressed graphically in (B) $^aP < 0.05$ OPN-A/Huh-7 vector only; C: Polymerase chain reaction analysis of a representative tumour from each of the 4 groups showing OPN mRNA expression in all tumours; D: OPN-a expressing tumours display increased levels of OPN protein. Control and OPN-A tumour sections were immunostained for OPN in conjunction with a rabbit isotype control. Bar represents 100 μm .

(the cell lines studied here are not of high metastatic potential). However, the expression of OPN-B and other splice variants was not investigated in primary HCC. Thus this study represents a comprehensive description of complete OPN variant expression *in vivo* and *in vitro*. Transcripts for all OPN variants were expressed in both primary HCC and cognate surrounding tissue, however, levels of expression varied between paired samples and between different patient HCCs. While our RT-PCR is only semi-quantitative (development of a quantitative real time assay for all OPN variants was not reliable) these results in-part confirm the work of He *et al*^[15] and show that expression of OPN splice variants can be detected in surrounding non-cancerous tissue. It has been reported that the OPN splice variants are specific to tumour cells and are rarely, if ever, expressed in normal tissue^[15]. However, we noted expression of all OPN variants in non-diseased liver tissue, suggesting that OPN splice variant expression is not restricted to tumour cells, and different forms of OPN may play different roles depending on the cell type that expresses OPN and the context in which it is expressed.

A significant finding of this study was the role of OPN and splice variants in *in vitro* proliferation and *in vivo* tumour growth of HCC. It is becoming increasingly clear that in addition to the role of OPN in the metastatic process it also has a role in cellular proliferation of a number of tumours. However, its role in HCC is not well studied, although, recently, an siRNA OPN knock-down approach suggested that OPN is important for HCC proliferation. Nevertheless, the relative roles of OPN variants or the cellular receptor responsible were not investigated^[9]. In contrast to the above OPN RNA interference study, we exogenously expressed OPN variants in the HCC Huh-7 cell line, and investigated cellular proliferation *in vitro* and in an ectopic xenograft nude mouse model. Stable ectopic expression of OPN-A, -B and -C resulted in secretion of all OPN forms into the culture media, and significant proliferation of Huh-7 cells, either directly or through the addition of conditioned media to naive Huh-7 cells. This suggests that at least *in vitro* OPN exerts its proliferative effect in either an autocrine or paracrine manner, which is consistent with the secreted nature of this protein, and was con-

firmed through the use of a neutralizing OPN antibody that abolished the proliferative effect of conditioned media. This proliferation effect was also seen in an *in vivo* HCC proliferation model in which Huh-7 cell lines, expressing either OPN-A, -B or -C, all proliferated faster than the control Huh-7 line. Interestingly compared to the *in vitro* proliferation assays, OPN-A showed the most significant proliferation advantage over OPN-B and -C. The reason for this discrepancy is unclear as all cell lines expressed similar amounts of OPN but may be related to stability or processing of OPN in an *in vitro* environment. To our knowledge, this is the first report that OPN and its splice variants play a significant role in cellular proliferation and growth of HCC, in addition to its role in invasiveness. Our collective studies strongly suggest that OPN plays a role in cellular proliferation however we cannot exclude the possibility that OPN expression also plays a role in increased cell survival given the recent observations from Zhao *et al.*^[31].

The CD44 family are cell surface adhesion molecules that mediate cell-matrix interactions, and in addition to the standard form, multiple isoforms arise from differential splicing of ten variant exons, and are designated CD44v6-15^[32]. CD44 variants, especially CD44v6, have been identified as protein markers for metastatic behaviour in a number of cancers, including HCC^[33-36]. A C-terminal region of OPN can specifically interact with CD44v6 and or v7 to mediate cellular chemotaxis^[37] and a thrombin-cleaved C-terminal OPN fragment induces macrophage migration *via* CD44^[38]. While OPN CD44v6-7 binding has been associated with metastasis and invasion, little work has been done in regard to the CD44 OPN interaction and tumour growth, although one study revealed that knockdown of CD44 expression reduces tumour growth in colon carcinoma cells^[39]. In this study, we show that the increased growth rate of Huh-7 cells in response to OPN conditioned media was as a result of CD44 expression because blocking CD44 with a neutralising antibody completely abolished this growth effect. Furthermore, HepG2 and HeLa cells, which both express CD44v6/7, also showed this growth effect while the Hep3B cell line, which is negative for CD44v6/7, was unresponsive to OPN in the conditioned media. To conclusively demonstrate a role for CD44, a siRNA approach was employed that successfully reduced CD44 expression in both Huh-7 and HeLa cells. Consistent with the above, this reduction in CD44 in both cell lines resulted in a decrease in cellular proliferation in response to OPN conditioned media. To our knowledge this is the first report that the interaction between OPN and CD44 results in signals that drive cellular proliferation in HCC. This is not surprising considering the recent work describing a role for OPN in regulating MKK3/6 and p38 dependent nuclear factor kappa B activation in cervical cancer leading to furin expression, a proprotein convertase that plays crucial roles in regulation of tumour progression metastasis and angiogenesis^[40]. Cervical cancer cells over expressing OPN

showed enhanced cell growth, while shRNA-mediated silencing of OPN suppressed cell growth in a CD44-mediated MKK3/6 dependent p38 activation mechanism^[40]. While similar mechanisms may be at play in HCC, the signalling pathways driven by OPN and CD44 activation in HCC require further investigation. Thus, understanding the molecular mechanisms that underpin increased HCC cell proliferation through the OPN/CD44 interaction and activation of downstream signalling pathways could provide potential targets for therapeutic strategies for the treatment of HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is a highly aggressive carcinoma of the liver, and is the fifth most common cancer worldwide. It is the third leading cause of cancer related death. Osteopontin (OPN) is a secreted multifunctional matrix-glycoprotein involved in normal tissue remodelling processes but is secreted at high levels in numerous tumours, including HCC. OPN is expressed as 3 isoforms and their role in HCC biology is not entirely known.

Research frontiers

Human OPN is known to be expressed in three different isoforms with potentially differing functions. Despite evidence of elevated OPN in several cancers and correlation of levels to malignant invasiveness, the expression of OPN isoforms in the context of HCC has not previously been investigated. Similarly the affect of OPN and its isoforms on HCC growth is unexplored.

Innovations and breakthroughs

This study demonstrates that all isoforms of OPN are expressed in HCC tissues and cell lines, at the mRNA level, and that each isoform can stimulate the growth of HCC derived cell lines both *in vitro* and *in vivo*, in an ectopic xenograft mouse model. Furthermore, this growth promoting effect has been shown to occur through a mechanism requiring functional CD44. These cellular proliferation signalling pathways, driven by OPN and CD44 activation, require further investigation.

Applications

Understanding the molecular mechanisms that underpin increased HCC cell proliferation could provide potential targets for therapeutic strategies for the treatment of HCC.

Peer review

Authors elegantly and convincingly demonstrated that OPN plays a relevant role in the expansion of HCC cells through interaction with the cell surface receptor CD44; this information is potentially interesting for novel anti HCC therapeutic approaches.

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Retrograde-viewing device improves adenoma detection rate in colonoscopies for surveillance and diagnostic workup

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Abstract

AIM: To determine which patients might benefit most from retrograde viewing during colonoscopy through subset analysis of randomized, controlled trial data.

METHODS: The Third Eye® Retroscope® Randomized Clinical Evaluation (TERRACE) was a randomized, controlled, multicenter trial designed to evaluate the efficacy of a retrograde-viewing auxiliary imaging device that is used during colonoscopy to provide a second video image which allows viewing of areas on the proximal aspect of haustral folds and flexures that are difficult to see with the colonoscope's forward view. We performed a post-hoc analysis of the TERRACE data to determine whether certain subsets of the patient population would gain more benefit than others from use of the device. Subjects were patients scheduled for colonoscopy for screening, surveillance or diagnostic workup, and each underwent same-day tandem examinations with standard colonoscopy (SC) and Third Eye colonoscopy (TEC), randomized to SC followed by TEC or *vice versa*.

RESULTS: Indication for colonoscopy was screening in 176/345 subjects (51.0%), surveillance after previous polypectomy in 87 (25.2%) and diagnostic workup in 82 (23.8%). In 4 subjects no indication was specified.

Previously reported overall results had shown a net additional adenoma detection rate (ADR) with TEC of 23.2% compared to SC. Relative risk (RR) of missing adenomas with SC *vs* TEC as the initial procedure was 1.92 ($P = 0.029$). Post-hoc subset analysis shows additional ADRs for TEC compared to SC were 4.4% for screening, 35.7% for surveillance, 55.4% for diagnostic and 40.7% for surveillance and diagnostic combined. The RR of missing adenomas with SC *vs* TEC was 1.11 ($P = 0.815$) for screening, 3.15 ($P = 0.014$) for surveillance, 8.64 ($P = 0.039$) for diagnostic and 3.34 ($P = 0.003$) for surveillance and diagnostic combined. Although a multivariate Poisson regression suggested gender as a possibly significant factor, subset analysis showed that the difference between genders was not statistically significant. Age, bowel prep quality and withdrawal time did not significantly affect the RR of missing adenomas with SC *vs* TEC. Mean sizes of adenomas detected with TEC and SC were similar at 0.59 cm and 0.56 cm, respectively ($P = \text{NS}$).

CONCLUSION: TEC allows detection of significantly more adenomas compared to SC in patients undergoing surveillance or diagnostic workup, but not in screening patients (ClinicalTrials.gov Identifier: NCT01044732).

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Key words: Colonoscopy; Colorectal cancer; Adenomas; Miss rates; Retrograde-viewing

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INTRODUCTION

Colonoscopy is generally regarded to be the “gold standard” for the detection of colorectal neoplasia, and is the only method that allows detection and removal during a single procedure^[1,2]. If the colon were a straight pipe, the standard colonoscope would be the perfect instrument for examining its lining, but lesions can be missed during colonoscopy, especially when located on the proximal aspect of haustral folds or on the inner curve of flexures^[3-8].

The Third Eye Retroscope (TER) (Avantis Medical Systems, Sunnyvale, California) provides an additional, retrograde view that helps to visualize areas behind folds and flexures^[9-11]. We recently showed in the Third

Eye Retroscope Randomized Clinical Evaluation (TERRACE) study that Third Eye colonoscopy (TEC) detected 23.2% additional adenomas compared to standard colonoscopy (SC)^[12].

As concerns about medical costs increase, there is growing interest in finding ways to target interventions and new technologies to specific patient sub-populations that will experience the greatest benefit^[13,14]. In this follow-up of our initial report of TERRACE results, we performed a post-hoc subset analysis of the data to determine whether specific indications for colonoscopy (screening, surveillance or diagnostic workup) or other patient characteristics were associated with higher additional adenoma detection rates (ADR) with TEC. Such information may be useful for targeting those segments of the colonoscopy population that would benefit most from use of this new technology. We also wished to evaluate the clinical significance of the additional adenomas detected through TEC and determine how finding those additional adenomas would affect recommendations for follow-up intervals.

MATERIALS AND METHODS

The TERRACE study was a prospective, randomized, controlled trial that used a “tandem” design involving two same-day, back-to-back procedures to provide a head-to-head comparison between TEC and SC.

Between March 2009 and February 2010, 15 experienced endoscopists at 4 European and 5 United States sites enrolled 448 patients who were scheduled to undergo colonoscopy for routine screening, surveillance after previous polypectomy, or diagnostic workup for symptoms possibly related to the lower gastrointestinal tract. Subjects were excluded for history of inflammatory bowel disease, colonic resection, polyposis syndrome or radiation therapy to abdomen or pelvis, or for active diverticulitis, suspicion of colonic stricture or concurrent enrollment in another study.

Upon arrival in the endoscopy suite, subjects were randomized to one of two groups by a research assistant utilizing a Web-based randomization module stratified both by center and by individual endoscopist. Each subject then underwent two complete examinations by the same endoscopist, who was informed of the order of exams immediately before beginning intubation.

Group A underwent SC first, followed by TEC, using the same colonoscope for both procedures. Group B underwent TEC first, then SC.

For TEC, following intubation of the cecum with a standard colonoscope, the TER was inserted through the biopsy channel of the colonoscope. When the TER emerges, its distal tip automatically turns 180 degrees so its miniature video camera and light are oriented backwards. As the colonoscope and TER are withdrawn together, the device provides a continuous retrograde view that complements the forward view of the colonoscope. The two video images are displayed side-by-side on a

monitor.

Tandem miss rate studies have demonstrated a “second-pass effect” - i.e., looking a second time generally yields additional lesions^[3-5]. In Group B the detection rate for SC as the second procedure served as a proxy for second-pass effect. Subtracting that from the additional detection rate with TEC in Group A yielded the net additional detection rate attributed to TEC.

Polyps were removed when detected and sent for histological evaluation. Advanced adenomas were defined as adenomas that measured at least 1 cm in diameter or those with villous or tubulovillous components, high-grade dysplasia or adenocarcinoma. An assistant recorded withdrawal time (from cecum to anal verge, subtracting pauses for polypectomy or extensive irrigation/suctioning) and total procedure time (from initial insertion of the colonoscope to withdrawal through the anal verge, not subtracting pauses). Bowel preparation was graded with the Ottawa Bowel Preparation Quality Scale Score^[15]. Telephone interviews were performed 24-72 h after procedures to assess each subject for adverse events.

Subjects were withdrawn from the study if they withdrew consent, had inadequate bowel preparation or developed complications, if the endoscopist was unable to intubate the cecum or identified a condition in which back-to-back examinations might create risk for the subject, or if there were device malfunctions, technical errors or deviations from the protocol.

The primary outcome measure was per-polyp detection rates for adenomas and for all polyps with SC and TEC. Secondary outcome measures were polyp size, polyp histology, withdrawal time and total procedure time.

Patients presenting for routine screening were regarded as having a low-to-average risk of developing colorectal cancer (CRC), while the non-screening patients - those presenting for surveillance colonoscopy as a follow-up after previous removal of adenomas or for diagnostic workup of symptoms - were considered to have an above-average risk for CRC.

The protocol was approved by the Institutional Review Board of each institution, and all patients signed an informed consent. The study was registered with ClinicalTrials.gov (Identifier Number NCT01044732) and was reported in compliance with the Consolidated Standards of Reporting Trials (CONSORT) 2010 Statement.

Statistical analysis

Statistical analyses were performed on the per-protocol population using the SAS statistical package, version 9.1 (SAS Institute, Cary, NC). Because non-adenomatous polyps are generally regarded as having limited clinical significance, subset analysis was applied only to adenomas. Sample size determination was discussed in our earlier report of TERRACE results^[12].

Because it is not possible to directly determine *P* values for the net additional ADR, we evaluated the statistical significance of the detection rates indirectly by calculating the relative risk (RR) of missing an adenoma

during first exams with SC *vs* first exams with TEC. RR for missing adenomas during the first exam was estimated by Poisson regression including only study group (TEC first *vs* SC first) as an explanatory variable. *P* values < 0.05 were considered statistically significant.

To further assess the effect of the order of procedures on adenoma miss rates during the first exam, an exploratory analysis was performed to examine the influence of the independent variables including study group, age, gender, bowel prep quality (Ottawa Score), withdrawal time and indication for procedure (screening, surveillance, or diagnostic workup). Interactions between study groups (i.e., order of exams) and each of the explanatory variables were also examined. A multivariate Poisson regression with backward selection was performed to determine whether the above variables predicted differences in the number of adenomas missed during first exams with SC *vs* TEC. Independent variables with *P* values < 0.10 were included in the model for the multivariate analysis. Independent covariates included in the final model from the multivariate Poisson regression were further evaluated through subset analysis, where *P* values < 0.05 were considered statistically significant.

For the subsets of subjects by indication for procedure, Fisher's exact test was performed to compare results for second exams in terms of additional subjects found to have at least one adenoma and additional subjects found to meet criteria for shortened surveillance intervals as a result of findings during the second exam.

RESULTS

Patient demographics and a study flow diagram describing withdrawal of subjects to yield a per-protocol population of 349 subjects are shown in Figure 1.

Indication for colonoscopy was screening in 176/345 subjects (51.0%), surveillance after previous polypectomy in 87 (25.2%) and diagnostic workup in 82 (23.8%). In 4 subjects no indication was specified.

Previously reported overall TERRACE results had shown a net additional ADR with TEC of 23.2% compared to SC (Table 1). The RR of missing adenomas with SC *vs* TEC was 1.92 (*P* = 0.029) (Table 2).

Using a multivariate Poisson regression, we determined the risk of missing adenomas during the first exam. Study group (TEC first *vs* SC first) and gender were selected as possibly significant factors, and the interaction between indication for procedures and study group was found to be predictive. Age, bowel prep quality and withdrawal time did not significantly affect the risk.

Subset analysis segmenting the population by indication showed that additional ADRs for TEC compared to SC were 4.4% in the screening group, 35.7% in the surveillance group, 55.4% in the diagnostic group and 40.7% in the non-screening patients (surveillance and diagnostic groups combined) (Table 1). The RR of missing adenomas with SC *vs* TEC was 1.11 (*P* = 0.815) for screening

Table 1 Additional adenoma detection rates for second exams by indication for procedure

Indication	Group A (SC, then TEC)			Group B (TEC, then SC)			Net additional detection with TEC (%)
	SC 1st	TEC 2nd	Additional in 2nd exam (%)	TEC 1st	SC 2nd	Additional in 2nd exam (%)	
All indications	107	49	45.8	115	26	22.6	23.2
Screening	54	19	35.2	52	16	30.8	4.4
Surveillance	37	20	54.1	49	9	18.4	35.7
Diagnostic	16	10	62.5	14	1	7.1	55.4
Surveillance + diagnostic	53	30	56.6	63	10	15.9	40.7

TEC: Third Eye colonoscopy; SC: Standard colonoscopy.

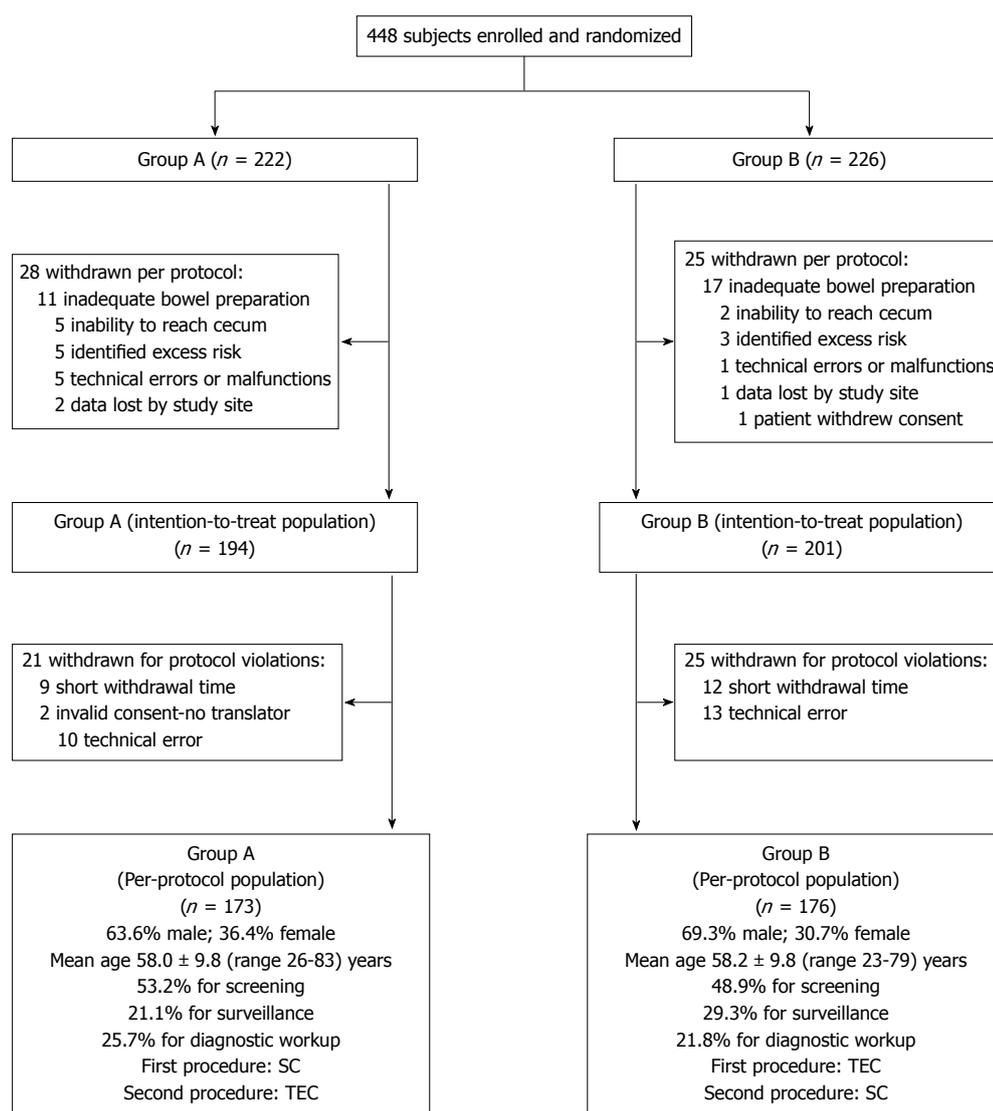


Figure 1 Study flow diagram and patient demographics. SC: Standard colonoscopy; TEC: Third Eye colonoscopy.

subjects, 3.15 ($P = 0.014$) for surveillance subjects, 8.64 ($P = 0.039$) for diagnostic subjects and 3.34 ($P = 0.003$) for the surveillance and diagnostic subjects combined (Table 2).

In the diagnostic group, due to detection of lesions with TEC that had been missed with SC (after correction for second-pass effect), 75.0% additional subjects were found to have at least 1 adenoma. In the surveillance group, 42.9% additional subjects were found to have at

least 1 advanced adenoma or at least 3 small adenomas criteria for 3-year follow-up according to joint guidelines published by the United States Multi-Society Task Force on CRC, American College of Radiology and American Cancer Society^[16]. However, the numbers of subjects with these findings were small, and the differences did not reach statistical significance.

Segmenting the population by gender, additional

Table 2 Adenoma miss rates¹ by indication for procedure

Indication	Group A standard colonoscopy first	Group B Third Eye colonoscopy first	Relative risk ² (95% CI)	P
All Indications ³	49/173 = 0.283	26/176 = 0.148	1.92 (1.25-2.94)	0.029
Screening	19/91 = 0.209	16/85 = 0.188	1.11 (0.62-2.04)	0.815
Surveillance	20/36 = 0.556	9/51 = 0.176	3.15 (1.63-6.10)	0.014
Diagnostic	10/44 = 0.227	1/38 = 0.026	8.64 (1.16-64.41)	0.039
Surveillance + diagnostic	30/80 = 0.375	10/89 = 0.112	3.34 (1.74-6.39)	0.003

¹Expressed in adenomas per patient; ²Relative risk of missing an adenoma during first exams with standard colonoscopy *vs* first exams with Third Eye colonoscopy, 95% CI calculated using 2 × 2 frequency table; ³In 4 subjects (2 in group A and 2 in group B), no indication was specified.

Table 3 Per-polyp miss rates for standard colonoscopy and Third Eye colonoscopy by size of adenoma and indication for procedure

	Adenomas missed with standard colonoscopy during 1st exams in group A (%)			Adenomas missed with Third Eye colonoscopy during 1st exams in group B (%)		
	< 10 mm	≥ 10 mm	All sizes	< 10 mm	≥ 10 mm	All sizes
All indications	33.8	11.8	31.4	20.8	0	18.4
Screening	27.7	12.5	26.0	28.1	0	23.5
Surveillance	37.7	0	35.1	16.7	0	15.5
Diagnostic	42.9	20.0	38.5	7.1	0	6.7
Surveillance + diagnostic	39.2	11.1	36.1	14.7	0	13.7

Table 4 Mean procedural times (min)

Indication	All exams with SC		All exams with TEC	
	Withdrawal time	Total procedure time	Withdrawal time	Total procedure time
All indications	7.6	17.0	9.5	20.9
Screening	7.5	16.0	9.6	19.4
Surveillance	7.9	16.4	9.5	21.8
Diagnostic	7.5	19.8	9.6	23.2
Surveillance + diagnostic	7.7	18.1	9.5	22.5

Total procedure time: Total time from initial insertion of the colonoscope to withdrawal through the anal verge; Withdrawal time: Time from cecum to anal verge, excluding pauses for polypectomy or extensive suctioning; TEC: Third Eye colonoscopy; SC: Standard colonoscopy.

ADRs for TEC compared to SC were 25.9% for males and 8.5% for females ($P = \text{NS}$). Segmenting by age, additional ADRs for TEC compared to SC were 22.0% for subjects less than 65 years of age and 25.8% for those at least 65 years ($P = \text{NS}$).

Per-polyp miss rates in the surveillance and diagnostic groups were lower for TEC than for SC, and no large adenoma (at least 10 mm) was missed with TEC in any subgroup (Table 3).

Mean sizes of adenomas detected with TEC and SC were similar at 0.59 cm and 0.56 cm, respectively ($P = \text{NS}$). Mean procedure times are shown in Table 4.

No adverse events were reported.

DISCUSSION

We report a subset analysis of results from the TER-RACE study, which was the first “head-to-head” comparison of TEC with SC and was designed to provide correc-

tion for the “second-pass effect” that invariably occurs in tandem studies^[12]. Reversing the order of procedures in the control group (TEC first, followed by SC) provided a proxy for the second-pass effect, which was then subtracted from the additional detection rate in the study group (SC first, followed by TEC) to yield the net additional ADR for TEC compared to SC.

This subset analysis provides additional insight regarding which segments of the population are likely to benefit most from TEC and the clinical significance of the adenomas that were detected with TEC after having been missed during SC.

As shown in Table 1, overall results demonstrated a 23.2% net additional ADR for TEC compared to SC. This was consistent with the outcome of a previous study by DeMarco *et al*^[11], in which investigators using a different methodology found a 25% additional ADR with the TER after each endoscopist had gained experience through performing 15 procedures.

Subset analysis segmenting the population by indication for procedure showed that in subjects undergoing colonoscopy for surveillance or diagnostic workup, TEC demonstrated additional ADRs of 35.7% and 55.4%, respectively, compared to SC. Pooling the results for those two groups of patients, who can be considered to have above-average risk for CRC, TEC showed an additional ADR of 40.7% compared to SC. In subjects undergoing screening colonoscopy, who can be regarded as having a low-to-average risk for CRC, TEC showed an additional ADR of 4.4% after correction for second-pass effect (Table 1).

In order to determine the statistical significance of these differences, we calculated the RR of missing an adenoma during first exams with SC *vs* first exams with TEC. The advantage with TEC proved to be statistically

significant for the surveillance and diagnostic groups, both separately and combined. However, the difference for the screening population was not significant (Table 2).

It is not surprising to find proportionally more lesions behind folds in surveillance patients because, by definition, they have had at least one previous colonoscopy during which polyps that were seen with the colonoscope were removed, while lesions that were hidden behind folds may have been left behind. In contrast, screening patients have not had adenomas removed, so whatever polyps they have are likely to be more evenly distributed. However, the reason for the difference in results between diagnostic patients and screening patients is not clear.

A multivariate analysis indicated that more adenomas were found during second exams with TEC than with SC, and more were found during second exams in males than in females. However, subset analysis showed that while the net additional ADR for TEC compared to SC trended lower for females than for males, the total number of adenomas detected in females during all exams was relatively small, and the difference between genders was not statistically significant.

The multivariate Poisson regression showed no significant influence of age, bowel preparation quality or withdrawal time on adenoma miss rates. These findings were confirmed by subset analysis in which patient age and bowel prep quality had no significant impact.

Mean withdrawal times for TEC were essentially identical for the groups segmented by indication for procedure, suggesting that the amount of time spent examining the mucosa could not explain the differences among those groups. However, total procedure times for TEC were somewhat longer for the surveillance and diagnostic populations, most likely because many more polypectomies were performed in those subjects compared to the screening group.

In this study, overall adenoma miss rates for SC were 31.4% for all-size and 11.8% for large adenomas (at least 10 mm). These miss rates are higher than suggested by previous studies involving two same-day colonoscopies. One such study found miss rates of 24% for all-size and 6% for large adenomas^[3]. Another found miss rates of 21% for all-size and 11% for advanced adenomas (but did not specify how many of those advanced adenomas were at least 10 mm)^[5]. These discrepancies are not surprising, since many authors have acknowledged the limitation inherent in comparing two examinations with identical technology: lesions that are hidden from the colonoscope during one exam are likely to be hidden during the second exam as well, causing them to be missed on the second exam and thus underestimating the true miss rate^[3-5,17].

Our 11.8% miss rate for large adenomas with SC is similar to the finding of a 12% miss rate for large adenomas in one study comparing colonoscopy with CT colonography (CTC)^[8]. Another comparison with CTC showed a 17% miss rate with SC for all polyps at least 10 mm, but did not indicate a miss rate specifically for large

adenomas^[17]. Due to the limited sensitivity of CTC for smaller lesions, neither study could reliably estimate the all-size adenoma miss rate for SC.

In Tables 1 and 2, we show that, except in the screening group, TEC provided a significant improvement in ADR on a per-polyp basis (i.e., number of adenomas per patient). As we currently think that each adenoma has the potential to develop into malignancy, it is desirable to detect and remove as many adenomas as possible except in the relatively uncommon circumstance in which the patient has a high risk for complications from a polypectomy of small lesions.

One might argue that it is important to determine ADR on a per-patient basis (i.e., the number of patients in whom at least one adenoma is found), because the detection of even one adenoma identifies the patient as a “polyp-former” who might be followed more carefully than a “non-polyp-former.” However, for example, if a patient had one diminutive tubular adenoma (5 mm or smaller) and two large advanced adenomas, this measure would attribute the same quality to an exam whether the endoscopist detected all 3 lesions or found only the small adenoma and missed the 2 more significant lesions.

Furthermore, the finding of a single small adenoma does not necessarily dictate close follow-up according to joint guidelines published by the United States Multi-Society Task Force on CRC, American College of Radiology and American Cancer Society^[16], which recommend follow-up at 3 years only for those patients who are at a high risk, defined as those with at least 1 advanced adenoma or at least 3 small adenomas. For patients with only 1-2 small tubular adenomas, who are at a low risk, the guidelines recommend repeat colonoscopy in 5-10 years.

Therefore, if we wished to consider a quality metric other than the per-polyp ADR, it might be more useful to calculate the number of subjects who are determined to be at high risk, i.e., those with at least 1 advanced adenoma or at least 3 small adenomas. In the surveillance group, due to detection of lesions with TEC that had been missed with SC, 42.9% additional subjects would be advised to return for 3-year follow-up per guidelines. However, the numbers were small and were not statistically significant.

Our results suggest that TEC offers an improvement in adenoma detection that can currently not be matched by other techniques or technologies. There is growing interest in improving quality in colonoscopy, and substantial evidence indicates that optimizing bowel preparation^[18-20], spending adequate time examining the mucosa^[21-24] and practicing high-quality withdrawal technique^[21,25,26] are associated with improved detection rates. However, none of these measures fully addresses the difficulty of detecting neoplasia located behind folds and flexures. According to one study comparing SC with CTC, two-thirds of adenomas missed with the colonoscope were located behind folds^[8], where they would likely be missed again during repeated exams.

CTC can reveal lesions on both sides of folds, but lacks sensitivity for small and flat lesions, and requires

referral for colonoscopy when abnormalities are identified^[27,28]. Cap-assisted colonoscopy does not require a second procedure, but studies have shown mixed results^[29-32] and even the most encouraging study demonstrated an advantage only for the detection of diminutive adenomas^[32]. Retroflexion of a colonoscope in the proximal colon has been found to be feasible by highly-experienced endoscopists, but results of studies so far have been mixed^[33-35]. Moreover, the safety, practicality and time requirement for routine retroflexion proximal to the rectum still need to be established.

Reducing the prevalence of CRC in a cost-effective manner requires use of the proper examination for each patient, so it is important to identify which segments of the population are most likely to benefit from various technologies. The findings in this subset analysis may have implications for evidence-based patient selection for TEC. In our study, TEC demonstrated a clinically and statistically significant benefit for the subsets of patients who presented for surveillance in follow-up of previous polypectomy and for diagnostic workup of symptoms. However, our results did not provide evidence for efficacy in patients who presented for routine screening.

Cost-effectiveness also requires appropriate timing of examinations. There is evidence that many endoscopists perform surveillance colonoscopy at shorter intervals than those recommended by guidelines^[16,36,37]. Their decisions regarding surveillance intervals may be influenced by the growing recognition that limitations in technology result in substantial miss rates during SC and by associated medico-legal concerns^[36,37]. Improving the quality of colonoscopy through use of a retrograde-viewing device could instill confidence in physicians and patients that the likelihood of missing significant neoplasia is very low and thus foster greater compliance with guidelines for surveillance intervals.

Limitations

The TERRACE study was not powered for analysis of subsets, and some numerical trends revealed by this post-hoc analysis, such as difference in additional detection rates between genders, did not reach statistical significance.

Only 33.5% of the subjects were female. Some of this discrepancy was due to one of the sites being a Veteran's Administration Medical Center, where 55 of the 57 subjects were male. Another, probably more significant, factor was that the TERRACE study was performed with the first-generation Retroscope, which was too large to fit through the working channel of "pediatric" size colonoscopes. With the growing trend toward using these smaller colonoscopes when examining female patients, it is likely that some investigators may have chosen not to enroll some of their female patients in the study on the basis that use of an "adult" colonoscope might have made the examination more difficult and/or uncomfortable (The second-generation device that is currently in use differs in that it has a higher-resolution camera and a smaller diameter that allows its use with all colonoscopes).

In conclusion, the results of this subset analysis demonstrated that adenoma miss rates for SC are even higher than those previously estimated through performance of back-to-back colonoscopy exams.

Use of a retrograde-viewing device significantly improves the quality of colonoscopy by increasing the detection of adenomas of all sizes. Use of the retrograde-viewing device along with a colonoscope appears to have the greatest value for patients undergoing colonoscopy for surveillance or for diagnostic workup of symptoms, and the study results do not provide evidence for efficacy of the device in routine screening colonoscopy. These findings could provide endoscopists with an evidence-driven basis for selection of patients most likely to benefit from this new technology.

COMMENTS

Background

This article expands on a previously-published report of the Third Eye Retroscope Randomized Clinical Evaluation (TERRACE), which was a large randomized, controlled trial. TERRACE evaluated the effectiveness of a device that provides an additional, retrograde (backward) view to allow examination of areas located behind folds in the colon wall that hide them from the forward-viewing camera during standard colonoscopy (SC). The previously-published overall TERRACE results showed that Third Eye colonoscopy (TEC) detected 23.2% additional pre-cancerous adenomas not found with SC.

Research frontiers

This new report focuses on post-hoc analyses of the TERRACE data to determine whether some subsets of patients might benefit more than others from TEC, to evaluate the clinical significance of the additional adenomas detected through TEC and to determine how finding those additional adenomas would affect recommendations for follow-up intervals.

Innovations and breakthroughs

These new analyses of the TERRACE data showed that, of all the variables that might influence the effectiveness of TEC, only the indication - i.e., the reason for performing the colonoscopy procedure - was significant. Patients who were having "surveillance" colonoscopy because they were found to have adenomas during previous exams, or "diagnostic" colonoscopy to look for a cause for symptoms, are considered to have above-average risk for colorectal cancer (CRC), and pooled results for those groups showed a 40.7% net additional adenoma detection rate (ADR) for TEC compared to SC. In contrast, patients who were having routine "screening" colonoscopy - who are considered to have low-to-average risk - had a 4.4% net additional ADR with TEC. In all of the groups combined, SC missed 11.8% of large adenomas, while none were missed during TEC.

Applications

These results confirm that use of a retrograde-viewing device allows detection of substantial numbers of clinically-significant adenomas that would be missed with the colonoscope alone. The finding that use of the device appears to offer more benefit for patients undergoing surveillance or diagnostic colonoscopy than for screening patients may provide physicians with a basis for selecting patients in whom they will use the device.

Terminology

Adenomas are polyps that have the potential to transform into CRC, and most cases of CRC arise from adenomas. The term "post-hoc" refers to additional analyses that were not anticipated in the original study protocol.

Peer review

The results of this post-hoc analysis will be helpful in supporting use of the Third Eye Retroscope (TER) in high-risk patients, which will allow for a more cost-effective approach as endoscopists continue to improve quality in colonoscopy. The authors are congratulated on demonstrating how TEC can detect additional adenomas during colonoscopy. Furthermore, the post-hoc analysis answers the question of who should have a TER used during colonoscopy. Based on the data from this manuscript, it would be those undergoing surveillance and diagnostic exams.

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Importance of early diagnosis of pancreaticobiliary maljunction without biliary dilatation

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Abstract

AIM: To clarify the strategy for early diagnosis of pancreaticobiliary maljunction (PBM) without biliary dilatation and to pathologically examine gallbladder before cancer develops.

METHODS: The anatomy of the union of the pancreatic and bile ducts was assessed by using endoscopic retrograde cholangiopancreatography (ERCP). Patients with a long common channel in which communication between the pancreatic and bile ducts was maintained even during sphincter contraction were diagnosed as having PBM. Of these, patients in which the maximal diameter of the bile duct was less than 10 mm were

diagnosed with PBM without biliary dilatation. The process of diagnosing 54 patients with PBM without biliary dilatation was retrospectively investigated. Histopathological analysis of resected gallbladder specimens from 8 patients with PBM without biliary dilatation or cancer was conducted.

RESULTS: Thirty-six PBM patients without biliary dilatation were diagnosed with gallbladder cancer after showing clinical symptoms such as abdominal or back pain ($n = 16$) or jaundice ($n = 12$). Radical surgery for gallbladder cancer was only possible in 11 patients (31%) and only 4 patients (11%) survived for 5 years. Eight patients were suspected as having PBM without biliary dilatation from the finding of gallbladder wall thickening on ultrasound and the diagnosis was confirmed by ERCP and/or magnetic resonance cholangiopancreatography (MRCP). The median age of these 8 patients was younger by a decade than PBM patients with gallbladder cancer. All 8 patients underwent prophylactic cholecystectomy and bile duct cancer has not occurred. Wall thickness and mucosal height of the 8 resected gallbladders were significantly greater than controls, and hyperplastic changes, hypertrophic muscular layer, sub-serosal fibrosis, and adenomyomatosis were detected in 7 (88%), 5 (63%), 7 (88%) and 5 (63%) patients, respectively. Ki-67 labeling index was high and K-ras mutation was detected in 3 of 6 patients.

CONCLUSION: To detect PBM without biliary dilatation before onset of gallbladder cancer, we should perform MRCP for individuals showing increased gallbladder wall thickness on ultrasound.

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Key words: Pancreaticobiliary maljunction; Pancreatobiliary reflux; Ultrasound; Gallbladder cancer; Endoscopic ultrasonography

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INTRODUCTION

Pancreaticobiliary maljunction (PBM) is a congenital anomaly defined as a junction of the pancreatic and bile ducts located outside the duodenal wall, and usually forming a markedly long common channel. As the action of the sphincter of oddi does not have a functional impact on the junction of the pancreatic and bile ducts in PBM cases, PBM causes a continuous reciprocal reflux of pancreatic juice and bile^[1,2]. This results in various pathological conditions of the biliary tract and pancreas. Given that the hydropressure in the pancreatic duct is usually greater than that in the bile duct, pancreatic juice frequently flows back into the biliary duct (pancreatobiliary reflux), and this becomes a high risk factor for biliary cancer^[3-5]. In a Japanese analysis^[6], biliary cancer was associated with 131 (10.6%) of 1239 cases with PBM with biliary dilatation (congenital choledochal cyst); 44 (33.6%) of the 131 cases had cancer of the extrahepatic bile duct and 85 cases (64.9%) had gallbladder cancer. On the other hand, PBM without biliary dilatation was associated with biliary cancer in 147 (37.9%) of 388 cases, and 137 (93.2%) of these cancers were gallbladder cancer.

Most PBM cases detected in childhood are associated with bile duct dilatation, but one third of PBM detected in adults do not show dilatation of the bile duct. Many patients with PBM with biliary dilatation have clinical symptoms due to cholangitis or pancreatitis in childhood, whereas in PBM without biliary dilatation, few patients have symptoms in childhood and they are usually not diagnosed until adulthood^[7]. Furthermore, many patients with PBM without biliary dilatation are diagnosed in association with advanced-stage gallbladder cancer, which carries a poor prognosis^[2,6,7]. It is necessary to clarify a strategy to diagnose PBM without biliary dilatation early, before cancer occurs. In the present study, we investigated the process for diagnosing patients with PBM without biliary dilatation and examined histopathological findings from the gallbladder of patients with PBM without biliary dilatation before cancer developed.

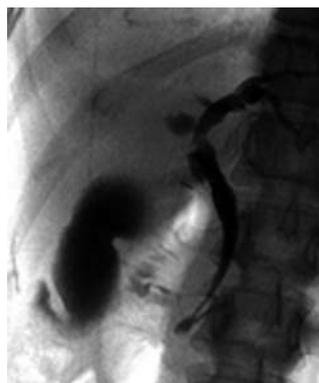


Figure 1 Endoscopic retrograde cholangiopancreatography of a patient with pancreaticobiliary maljunction without biliary dilatation showing a long common channel and deformity with fuzzy irregularity of the gallbladder.

MATERIALS AND METHODS

Study patients

We studied the anatomy of the union of the pancreatic and bile ducts using endoscopic retrograde cholangiopancreatography (ERCP). Patients with a long common channel in which communication between the pancreatic and bile ducts was maintained in both relaxation and contraction of the sphincter under serial observation during ERCP were diagnosed as having PBM^[1,2]. Of these, patients in whom the maximal diameter of the bile duct was less than 10 mm were diagnosed with PBM without biliary dilatation (Figure 1). When the common bile duct was involved with associated gallbladder cancer, diameter of the intact distal portion of the bile duct was measured.

Between January 1975 and December 2010, 104 patients were diagnosed with PBM with ERCP. Endoscopic ultrasonography (EUS) and magnetic resonance cholangiopancreatography (MRCP) were performed from 1990 and 2001, respectively. Of these, 54 patients (12 men and 42 women; median age at initial diagnosis of PBM 56.6 years, range 30-77 years) were diagnosed with PBM without biliary dilatation. The process leading to diagnosis of these patients was retrospectively investigated. This study was approved by the institutional review board.

Histopathological and immunohistochemical examinations

We conducted histopathological analysis of resected gallbladder specimens from 8 patients with PBM without biliary dilatation or cancer. Tissues were fixed in 10% buffered formalin and embedded in paraffin. Serial 3- μ m sections were stained with hematoxylin and eosin for evaluation. Thickness of the gallbladder wall and mucosal height of each specimen were measured using a semiautomatic image analyzer. Hyperplastic changes were defined as an increased number of mucosal folds that were longer than normal, irregular, and frequently branched. Immunohistochemistry was performed us-

Table 1 Clinical features of 8 pancreaticobiliary maljunction patients without biliary dilatation or cancer

Case	Age (yr)	Sex	Process leading to ultrasound	Amylase level in bile (IU/L)	Follow-up
1	30	F	Screening for hepatitis A	528 000	Follow-up after 236 mo
2	55	F	Screening for chronic hepatitis C	69 850	Follow-up after 47 mo
3	65	F	Cholangitis with mild acute pancreatitis	17 272	Follow-up after 40 mo
4	41	F	Right hypochondralgia	117 240	Follow-up after 12 mo
5	70	F	Screening for diabetes mellitus	254 900	Follow-up after 3 mo
6	42	M	Diarrhea	185 000	Unknown after 75 mo
7	47	M	Abdominal pain	300 000	Unknown after 18 mo
8	36	M	Abdominal discomfort	672 100	Unknown after 1 mo

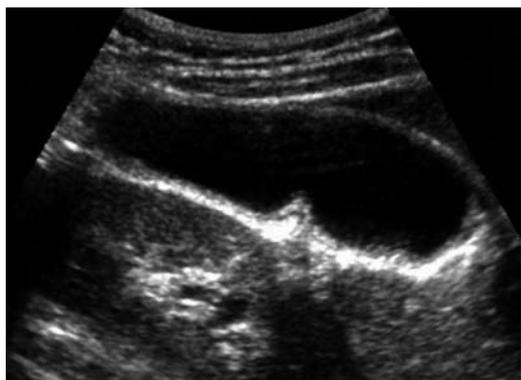


Figure 2 Abdominal ultrasound of a patient with pancreaticobiliary maljunction without biliary dilatation showing uniform smooth thickness of the gallbladder wall.

ing antisera for Ki-67 (clone MIB-1: Immunotech SA, Marseille, France) by the avidin-biotin horseradish peroxidase method (Vectastain Elite ABC kit; Vector, Burlingame, CA, United States). Ki-67-positive cells were defined as a cell with brown staining of the nucleus, and Ki-67 labeling index was determined by counting a minimum of 500 cells in the area representing the most homogenous region of positive cells.

Gallbladder epithelium was microdissected from 20- μ m formalin-fixed paraffin-embedded sections. Genomic DNA was extracted from the tissues by using Takara Dexpattm (Takara Shuzo, Otsu, Japan). A point mutation of K-ras codon 12 was analyzed by polymerase chain reaction enzyme-linked minisequence assay (Sumitomo Metal Industry, Tokyo, Japan). The precise methodology of this assay is described elsewhere^[8].

Ten cases of normal gallbladder that were resected at pancreatoduodenectomy for pancreatic diseases were selected as controls.

Statistical analysis

Statistical analysis was performed using chi-squared analysis or Mann-Whitney's *U* test. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Process of diagnosing PBM without biliary dilatation

Clues associated with a diagnosis of PBM without biliary dilatation were the presence of gallbladder cancer (*n* =

36), other malignancies (*n* = 4), chronic pancreatitis (*n* = 1), intrahepatic stones (*n* = 1), gallstones (*n* = 1), gastritis (*n* = 1), previous cholecystectomy (*n* = 2), and gallbladder wall thickening (more than 3 mm) on ultrasound (US; *n* = 8) (Figure 2). Thirty-one patients with gallbladder cancer had clinical symptoms such as abdominal or back pain (*n* = 16), jaundice (*n* = 12), and abdominal discomfort (*n* = 3), and the other 5 gallbladder cancers were detected using screening tests such as US (*n* = 4) and fluorode-oxyglucose positron emission tomography (*n* = 1). Radical surgery was possible in only 11 (31%) of 36 cases of gallbladder cancer and only 4 patients (11%) survived for 5 years. Three PBM patients without biliary dilatation associated with benign diseases (chronic pancreatitis, gallstones and gastritis) were diagnosed before 1985, and they were lost to follow up without surgery.

PBM patients without biliary dilatation diagnosed from gallbladder wall thickening on US

PBM without biliary dilatation was suspected in 8 patients based on finding of gallbladder wall thickening on US. Abdominal US was performed for symptoms such as abdominal pain in 5 patients to screen for associated diseases. Following US, computed tomography (*n* = 8), EUS (*n* = 5), MRCP (*n* = 4), and ERCP (*n* = 8) were done. The median age of patients was 48.2 years (range 30-70 years; 3 men and 5 women), which was significantly younger than that of PBM patients with gallbladder cancer (median age, 58.5 years; *P* < 0.01). Based on the diagnosis of PBM without biliary dilatation, all patients underwent prophylactic cholecystectomy. Amylase levels in the bile were markedly elevated in all patients. Five patients have been regularly followed, and 3 patients were lost to follow up 1-75 mo after surgery. Bile duct cancer has not occurred in any patient (Table 1). On EUS, thickening of the inner hypochoic layer and outer hyperechoic layer was detected in 2 patients (Figure 3), and thickening of the inner hypochoic layer, a middle, more hypochoic layer, and the outer hyperechoic layer was observed in 3 patients.

Histopathological findings of the gallbladder of PBM patients without biliary dilatation diagnosed from gallbladder wall thickening on US

On histological findings of the resected gallbladder, the gallbladder wall was more thickened in PBM (7.7 ± 7.0 mm, mean \pm SD) than in control cases (2.0 ± 0.5 mm, *P*

Table 2 Histopathological findings of gallbladders from patients with pancreaticobiliary maljunction without biliary dilatation diagnosed based on gallbladder wall thickening on ultrasound

Case	Thickness of the wall (mm)	Mucosal height (mm)	Hyperplastic change	Hypertrophic muscular layer	Subserosal fibrosis	Adenomyomatosis	Gallstone	Ki-67 labeling index (%)	K-ras mutation
1	7	2.4	+ ^a	-	+	-	-	8	GAT (3+)
2	6	1.8	+	-	+	+	-	5	NA
3	3	0.6	-	-	-	-	+	6	-
4	5	2.2	+	+	+	+	-	4	-
5	5	1.7	+	+	+	+	-	5	NA
6	8	1.9	+	+	+	+	-	8	AGT (1+)
7	6	1.4	+	+	+	-	+	9	AGT (2+)
8	25	0.9	+	+	+	+	-	3	-
PBM, %, mean ± SD	7.7 ± 7.0 ^b	1.06 ± 0.7 ^b	88 (7/8) ^b	63 (5/8) ^b	88 (7/8) ^b	63 (5/8) ^b	25 (2/8)	6.0 ± 2.1 ^b	50 (3/6) ^a
Controls, %, (n = 10), mean ± SD	2.0 ± 0.5	0.58 ± 0.24	0 (0/10)	0 (0/10)	0 (0/10)	0 (0/10)	10 (1/10)	1.2 ± 0.3	0 (0/10)

PBM: Pancreaticobiliary maljunction; NA: Not amplified. ^a*P* < 0.05, ^b*P* < 0.01 *vs* controls.



Figure 3 Endoscopic ultrasonography of a patient with pancreaticobiliary maljunction without biliary dilatation showing thickening of the inner hypoechoic layer and the outer hyperechoic layer.

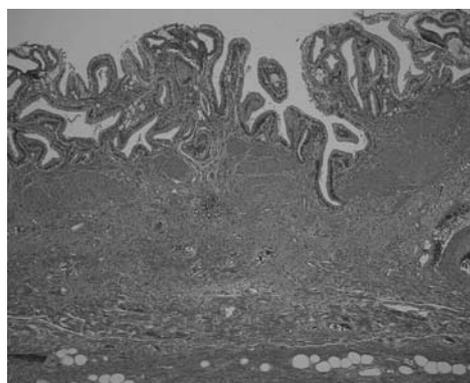


Figure 4 Histological findings of the gallbladder of a patient with pancreaticobiliary maljunction without biliary dilatation showing wall thickness composed of epithelial hyperplasia, hypertrophic muscular layer, and subserosal fibrosis.

< 0.01). Mucosal height was significantly higher in PBM (1.0 ± 0.7 mm) than in control cases (0.5 ± 0.2 mm, *P* < 0.01). Hyperplastic changes, hypertrophic muscular layer, subserosal fibrosis, and adenomyomatosis were detected in 7 (88%), 5 (63%), 7 (88%) and 5 (63%) of PBM patients, respectively, compared with no control cases (Figure 4). No dysplastic changes were observed. Ki-67 labeling index was higher in PBM ($6.0\% \pm 2.1\%$) than control cases ($1.2\% \pm 0.3\%$, *P* < 0.01). K-ras mutation was detected in 3 (50%) of 6 PBM patients (Table 2). Adenomatous changes were not observed in any patient.

DISCUSSION

Because PBM patients have a high incidence of biliary cancer due to pancreatobiliary reflux, once PBM is diagnosed, prophylactic biliary surgery is recommended before the onset of malignant change^[2]. In particular, PBM without biliary dilatation is frequently associated with gallbladder cancer. However, PBM cases without biliary dilatation rarely evoke symptoms, and most patients are not diagnosed until the onset of advanced-stage gallbladder cancer^[2,6,7].

In this series, 36 (67%) of 54 PBM patients without biliary dilatation were diagnosed based on associated gallbladder cancer. Most gallbladder cancers were in an advanced stage, and resection was possible in only 11 patients (31%). Since a paper^[9] published in 1991 reported that gallbladder wall thickening was sometimes observed in PBM cases without biliary dilatation, we have prospectively checked for PBM after finding gallbladder wall thickening on US. This additional investigation using MRCP and ERCP allowed us to identify 8 patients with PBM without biliary dilatation before the onset of gallbladder cancer. All 8 patients underwent prophylactic cholecystectomy, and bile duct cancer has not occurred in any patient. The median age of the 8 patients was about 10 years younger than PBM patients with associated gallbladder cancer. The age of PBM patients with gallbladder cancer at the time of diagnosis peaked in the 50s, and was younger by a decade than that of gallbladder cancer patients without PBM^[10]. Chronic injury to the epithelium of bile duct by pancreatic juice induces severe inflammation and malignant changes in PBM patients; therefore, age can be a major determinant of

risk. To eliminate the risk of gallbladder cancer, we must detect PBM without biliary dilatation early in adults.

Epithelial hyperplasia of the gallbladder induced by longstanding continuous stasis of the bile intermingled with refluxed pancreatic juice has been reported to be one of the characteristic pathological changes in PBM patients^[11-13]. The incidence of epithelial hyperplasia of the gallbladder of PBM patients without biliary dilatation was reported to be 72%^[11] to 91%^[12]. Tanno *et al*^[12] reported that the Ki-67 labeling index of epithelial hyperplasia of PBM patients was elevated to $6.1\% \pm 1.5\%$ and K-ras mutation was detected in 2 (13%) of 15 patients. Histopathological findings of our 8 cases were similar to the above findings. Considering that increased cell proliferation is linked to the development of cancer by means of tumor promotion and an increased rate of random mutations, gallbladder epithelium of PBM patients can be considered to represent a premalignant lesion.

Interestingly, hypertrophic muscular layer, subserosal fibrosis, and adenomyomatosis were detected in 63%, 88% and 63%, respectively. Sugai *et al*^[14] reported that subserosal fibrosis with chronic inflammation was detected in 33% of gallbladders of 27 PBM patients, and Tsuchida *et al*^[11] found K-ras mutations in 5 (50%) of 10 gallbladders of PBM without biliary dilatation. Long-standing continuous stimulation with bile containing pancreatic juice may induce these three changes as well as epithelial hyperplasia.

EUS shows the normal gallbladder wall to be a two-layered structure consisting of an inner hypoechoic layer composed of the mucosa and the muscular layer, and an outer hyperechoic layer composed of the subserosal layer and the serosa^[15]. On EUS, the gallbladder wall of patients with PBM appeared as two thickened layers showing epithelial hyperplasia and subserosal fibrosis or three thickened layers containing a middle, more hypoechoic, layer showing a hypertrophic muscular layer.

Although ERCP is the gold standard for diagnosis of PBM, MRCP has recently become a common non-invasive method for obtaining quality images of the pancreaticobiliary tract. MRCP can diagnose many PBM cases based on findings of an anomalous union between the common bile duct and the pancreatic duct, although some atypical PBM cases with relatively short common channel cannot be diagnosed only by MRCP, and should be confirmed by ERCP^[16]. To detect patients with PBM without biliary dilatation early requires that certain patients undergo MRCP before gallbladder cancer occurs. Gallbladder wall thickness on US during medical checkups may serve as an indication for MRCP and EUS before ERCP for suspected PBM without biliary dilatation. Once PBM is diagnosed in patients with increased gallbladder wall thickness, prophylactic biliary surgery is recommended before the onset of malignant change.

In conclusion, hyperplastic gallbladder mucosa of PBM patients represents a premalignant lesion. To detect PBM without biliary dilatation before onset of gallbladder cancer, we should perform MRCP for individuals showing gallbladder wall thickness on US.

COMMENTS

Background

Pancreaticobiliary maljunction (PBM) causes a continuous reciprocal reflux of pancreatic juice and bile. PBM cases without biliary dilatation rarely evoke symptoms, and most patients are not diagnosed until the onset of advanced-stage gallbladder cancer. It is necessary to clarify a strategy to diagnose PBM without biliary dilatation early, before cancer occurs.

Research frontiers

The authors investigated the process for diagnosing patients with PBM without biliary dilatation and examined histopathological findings from the gallbladder of patients with PBM without biliary dilatation before cancer developed.

Innovations and breakthroughs

Thirty-six (66%) out of 54 PBM patients without biliary dilatation were diagnosed with gallbladder cancer. Radical surgery for gallbladder cancer was only possible in 11 patients and only 4 patients survived for 5 years. Eight patients were suspected as having PBM without biliary dilatation from the finding of gallbladder wall thickening on ultrasound and the diagnosis was confirmed by endoscopic retrograde cholangiopancreatography and/or magnetic resonance cholangiopancreatography (MRCP). The median age of these 8 patients was younger by a decade than PBM patients with gallbladder cancer. All 8 patients underwent prophylactic cholecystectomy and bile duct cancer has not occurred. Wall thickness and mucosal height of the 8 resected gallbladders were significantly greater than controls, and hyperplastic changes were detected in 7 (88%) patients. Ki-67 labeling index was high and K-ras mutation was detected in 3 of 6 patients.

Applications

To detect PBM without biliary dilatation before onset of gallbladder cancer, people should perform MRCP for individuals showing increased gallbladder wall thickness on ultrasound.

Peer review

The study has a good-sized patient cohort and involves appropriate methods of analysis. The interpretation and presentation of the results are both appropriate. The findings of this study should contribute in the effective management of patients with PBM.

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Effect of sumatriptan on gastric emptying: A crossover study using the BreathID system

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Abstract

AIM: To determine the effect of oral sumatriptan on gastric emptying using a continuous ¹³C breath test (BreathID system).

METHODS: Ten healthy male volunteers participated

in this randomized, 2-way crossover study. The subjects fasted overnight and were randomly assigned to receive a test meal (200 kcal/200 mL) 30 min after pre-medication with sumatriptan 50 mg (sumatriptan condition), or the test meal alone (control condition). Gastric emptying was monitored for 4 h after administration of the test meal by the ¹³C-acetic acid breath test performed continually using the BreathID system. Then, using Oridion Research Software (β version), the time taken for emptying of 50% of the labeled meal ($T_{1/2}$) similar to the scintigraphy lag time for 10% emptying of the labeled meal (T_{lag}), the gastric emptying coefficient (GEC), and the regression-estimated constants (β and κ) were calculated. The statistical significance of any differences in the parameters were analyzed using Wilcoxon's signed-rank test.

RESULTS: In the sumatriptan condition, significant differences compared with the control condition were found in $T_{1/2}$ [median 131.84 min (range, 103.13-168.70) vs 120.27 min (89.61-138.25); $P = 0.0016$], T_{lag} [median 80.085 min (59.23-125.89) vs 61.11 min (39.86-87.05); $P = 0.0125$], and β [median 2.3374 (1.6407-3.8209) vs 2.0847 (1.4755-2.9269); $P = 0.0284$]. There were no significant differences in the GEC or κ between the 2 conditions.

CONCLUSION: This study showed that oral sumatriptan significantly delayed gastric emptying of a liquid meal.

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Key words: Sumatriptan; Gastric emptying; Breath test; Liquid meal; BreathID system

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INTRODUCTION

Sumatriptan, a selective 5-HT₁ receptor agonist, was launched in the market in 1991, representing the single most remarkable advance to date in the treatment of migraine^[1]. It was suspected that the drug might delay gastric emptying of liquids in healthy subjects by prolonging the lag phase of gastric emptying^[2,3]. Several studies have reported that sumatriptan restores gastric accommodation in patients with functional dyspepsia^[4-9].

A recent study showed that the accelerated gastric emptying of nutrient liquids can be a surrogate marker of impaired distension-induced accommodation^[10]. After aggressive filling of the stomach with a nutrient liquid, the resultant increase in intragastric pressure determines the rate of gastric emptying; higher luminal pressure caused by defective accommodation accelerates gastric emptying^[10,11]. Conversely, it would be logical to consider that the gastric emptying of a nutrient liquid would be delayed when distension-induced accommodation is enhanced. Compared with second-generation 5HT_{1B/1D} receptor agonist antimigraine drugs, sumatriptan shows poor oral bioavailability and a relatively short elimination half-life^[6]. According to a previous report, the T_{max} of sumatriptan is 2-3 h, the T_{1/2} is 2 h, and the oral bioavailability is 14%^[6]. The aim of this preliminary study was to test our hypothesis that oral sumatriptan intake would cause a delay in the gastric emptying of nutrient liquids. If the hypothesis were proven to be true, it would lend indirect support to the suggestion that sumatriptan enhances distension-induced gastric accommodation^[11].

In this study, we investigated the physiologic effect of sumatriptan on the rate of gastric emptying using a continuous real-time ¹³C breath test (BreathID system: Exalenz Bioscience Ltd., Israel).

MATERIALS AND METHODS

Ethics

The study was conducted in accordance with the principles laid down in the Declaration of Helsinki. The study protocol using the BreathID system was approved by the Ethics Committee of Yokohama City University School of Medicine (No. A110929010).

Subjects

The subjects were 10 asymptomatic male volunteers, all of whom were students of Yokohama City University,

non-smokers, none were habitual drinkers, and none had a history of gastrointestinal disease or abdominal surgery. None of the subjects were on any routine medications at the time of the study.

¹³C-acetic acid breath test

Ten subjects participated in this randomized, 2-way crossover study. The 2 tests were conducted as follows: (1) The subjects were assigned to receive a test meal 30 min after intake of a 50 mg tablet of sumatriptan; and (2) The subjects only received the test meal. The two test protocols were administered in crossover fashion separated by a washout period of at least 7 d. In both experiments, the protocols were started after the patients had fasted overnight (for at least 8 h), and the breath test was performed for 4 h while the subjects were seated.

In experiment A, subjects were asked to take a 50 mg tablet of sumatriptan prior to the administration of a test meal. The test meal consisted of a 200 kcal/200 mL liquid meal (Racol with milk flavor, Otsuka Pharmaceutical, Tokyo, Japan) containing 100 mg of ¹³C-acetic acid (Cambridge Isotope Laboratories, Boston, MA, United States)^[12-23], which the patients were requested to consume within 5 min. In experiment B, subjects underwent the breath test after ingestion of the test meal alone. Breath samples were continuously collected *via* a nasal tube using the BreathID system (Exalenz Bioscience Ltd., Israel) from baseline, before administration of the test meal, until 4 h after completion of the test meal ingestion (time 0)^[14-23].

Analysis of the ¹³C-acetic acid breath test data

The data from the ¹³C acetic acid breath test were analyzed using the Oridion Research Software, version β (Oridion Medical Ltd., Israel)^[14-23]. The time *vs* ¹³CO₂ excretion rate curve was fitted to the conventional formula of $z(t) = m(1 - e^{-\kappa t})^\beta$, and the regression-estimated constants κ and β were determined^[24]. After the mathematical analysis, the time required for emptying of 50% of the labeled meal (T_{1/2}) similar to the scintigraphy lag time for 10% emptying of the labeled meal (T_{lag}), the gastric emptying coefficient (GEC), and the regression-estimated constants (β and κ) were also calculated. A larger (smaller) value of β indicates slower (faster) emptying in the early phase, and a larger (smaller) value of κ indicates faster (slower) emptying in the later phase.

Statistical analysis

Statistical evaluation was carried out using the Wilcoxon's signed-rank test. The level of significance was set at $P < 0.05$. The statistical analysis were performed with the StatView software (SAS Institute, Cary, NC, United States).

RESULTS

The study was completed in 10 male subjects (mean age, 30.7 years; median age, 31 years; range, 24-38). No adverse events occurred during the study. The subjects'

Table 1 Comparison of breath test parameters

	Sumatriptan	Control	P value
T _{1/2}	131.84 (103.13-168.70)	120.27 (89.61-138.25)	0.0166
T _{lag}	80.085 (59.23-125.89)	61.11 (39.86-87.05)	0.0125
GEC	3.545 (3.20-3.97)	3.55 (3.19-4.03)	0.6460
β	2.3374 (1.6407-3.8209)	2.0847 (1.4755-2.9269)	0.0284
κ	0.5833 (0.3689-0.7836)	0.6180 (0.4661-0.8872)	0.3329

T_{1/2}: Time to emptying of 50% of the labeled meal (min); T_{lag}: Very similar to the percentage dose recovery peak time (min); GEC: Gastric emptying coefficient; β and κ: Regression-estimated constants. A larger (smaller) value of β indicates slower (faster) emptying in the early phase, and a larger (smaller) value of κ indicates faster (slower) emptying in the later phase.

mean height was 171.5 cm, their median height was 171 cm (range, 165-180 cm), their mean weight was 72.6 kg, and their median weight was 69.5 kg (range, 62-92 kg).

Table 1 summarizes the sumatriptan-induced changes in the breath test parameters. In the sumatriptan condition, significant differences compared with the control condition were found in T_{1/2}, [median, 131.84 min (range, 103.13-168.70) *vs* 120.27 min (89.61-138.25); *P* = 0.0016], T_{lag}, [median, 80.085 min (59.23-125.89) *vs* 61.11 min (39.86-87.05); *P* = 0.0125], and value of β [median 2.3374: 1.6407-3.8209) *vs* 2.0847 (1.4755-2.9269); *P* = 0.0284]. On the other hand, there were no significant differences in GEC [median, 3.545 (3.20-3.97) *vs* 3.55 (3.19-4.03); *P* = 0.6460] or κ [median, 0.5833 (0.3689-0.7836) *vs* 0.6180 (0.4661-0.8872); *P* = 0.3329] between sumatriptan and control conditions, respectively.

DISCUSSION

In this study, we examined the changes in the rate of gastric emptying during the first 4 h after oral administration of a 50 mg tablet of sumatriptan half an hour prior to a test meal in healthy subjects. The rate of gastric emptying was measured by the ¹³C-acetic acid breath test.

The ¹³C-acetic acid breath test is a non-invasive and well-established test for measuring the rate of gastric emptying of liquid meals, and the results of this test have been shown to be significantly correlated with those of scintigraphy^[12-24]. The subject ingests ¹³C-labeled acetic acid, which passes through the stomach and is absorbed in the duodenum and upper small bowel. The ¹³C-labeled acetic acid is then metabolized in the liver and excreted from the lung as ¹³CO₂. This test thus enables gastric emptying to be measured in a non-invasive manner^[12-24]. The BreathID system allows continuous evaluation of gastric emptying. In patients, it allows real-time breath analysis with a shortened examination time and minimal patient discomfort. Continuous analysis also provides quick, immediate results^[14-23].

In general, administration of sumatriptan, a 5HT₁ receptor agonist, has been reported to delay gastric emptying of calorie-containing liquids, and to restore gastric accommodation. In other words, sumatriptan allows both increased gastric relaxation after ingestion of

a meal and an increased caloric intake at maximum satiety^[9,25,26]. However, several studies have reported these effects of sumatriptan after subcutaneous sumatriptan injection^[3,9,26-28]. As previously mentioned, sumatriptan shows poor oral bioavailability and a relatively short elimination half-life profile. To the best of our knowledge, no researchers have reported the aforementioned effects following administration of sumatriptan *via* the oral route. Therefore, we studied the effect of oral sumatriptan on the rate of gastric emptying.

Sumatriptan has been reported to be effective in relieving nausea associated with a migraine attack^[7], although specific antiemetic agents are often required to control the symptoms and to restore normal gastrointestinal motor patterns^[7,9].

In the present study, the amount of sumatriptan administered was not adjusted to the body weight of each individual, with the drug being administered *via* the oral route. T_{lag} of sumatriptan has been reported to be 2 or 3 h. In a recent study, the BreathID examination was started half an hour after the subjects took sumatriptan orally, and was continued for 4 h. Therefore, the T_{lag} of sumatriptan would have been reached while the BreathID examination was still ongoing.

In the present study, an increase in T_{lag} and T_{1/2}, and also in the value of β, was observed in the subjects administered sumatriptan prior to the test meal. Thus, sumatriptan delayed gastric emptying. In addition, the increase in the value of β indicated that sumatriptan in particular delayed the early phase of gastric emptying. We think that these findings were attributable to restoration of distention-induced gastric accommodation by sumatriptan. Several studies have shown that in patients with functional dyspepsia, who showed impaired accommodation of the proximal stomach, subcutaneously administered sumatriptan restored gastric accommodation, thereby significantly improving meal-induced satiety^[9,26,27]. In healthy volunteers, accommodation of the proximal stomach may be thought to increase after administration of sumatriptan. A previous study using real-time ultrasonography and computed tomography demonstrated that subcutaneous administration of sumatriptan, after distention of the stomach with liquids, produced a reduction in the proximal and distal transverse area and an increase in the sagittal axis of the proximal stomach^[28]. Another study using duplex sonography showed that subcutaneous administration of sumatriptan 10 min postprandially caused a significant widening of both the gastric antrum and the proximal stomach. Therefore, the authors concluded that the time to commencement of peristalsis-related emptying is delayed following administration of sumatriptan^[26].

The effect of 5-HT on gastric fundus tone has not been studied. However, Tack *et al.*^[29] recently showed that sumatriptan, which is a 5-HT₁ receptor agonist, is an agonist at 5-HT_{1P} receptors on nitrergic myenteric neurons in the stomach. We consider this action was induced through 5-HT_{1P} receptors as one of the side effects of

sumatriptan.

In the present study, we found that despite its low bioavailability, oral administration of even one tablet of sumatriptan consistently affected the rate of gastric emptying. Delayed gastric emptying may represent an increase in gastric accommodation.

In conclusion, this study suggests that sumatriptan, which can restore gastric accommodation, may be of benefit to many patients suffering from the distress of early satiety.

COMMENTS

Background

The incidence of functional gastrointestinal disorders is currently increasing worldwide though the cause of this disease is largely unknown. Rome III criteria included postprandial distress syndrome, which might come from impaired gastric accommodation and gastric emptying.

Research frontiers

It is accepted that both gastric emptying and gastric accommodation are important in functional gastrointestinal disorders. However, how gastric accommodation affects gastric emptying in a breath test has not been researched. In this study, the authors demonstrated that oral sumatriptan significantly delayed gastric emptying and suggest that delayed gastric emptying may represent an increase in gastric accommodation.

Innovations and breakthroughs

This is the first study to report that oral administration of sumatriptan affected the rate of gastric emptying. Furthermore, the studies would suggest that delayed gastric emptying may represent an increase in gastric accommodation.

Applications

By understanding both gastric emptying and gastric accommodation, this study may represent a future strategy for therapeutic intervention in the treatment of patients with functional dyspepsia.

Terminology

The BreathID system allows measurement of gastric emptying by a breath test. This allows continuous and real-time evaluation of gastric emptying. Continuous analysis also provides quick, immediate results.

Peer review

This paper demonstrated a new way to identify the gastric emptying in human subjects; this is the main merit of this study.

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Safety and effectiveness of propofol sedation during and after outpatient colonoscopy

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Abstract

AIM: To study the safety and effectiveness of propofol sedation for outpatient colonoscopy.

METHODS: Propofol was given by bolus injection with an age-adjusted standard protocol consisting of 60 mg for patients < 70 years old, 40 mg for patients age 70-89 years, and 20 mg for those \geq 90 years, and additional injections of 20 mg propofol were given up to a maximum of 200 mg. The principal parameters were the occurrence of adverse events within 24 h after colonoscopy and overall satisfaction for this procedure. Secondary parameters included successful procedure, respiratory depression, and other complications.

RESULTS: Consecutive patients were entered prospectively and all 2101 entered successfully completed

outpatient colonoscopy. The mean dose of propofol used was 96.4 mg (range 40-200 mg). Younger patients required higher doses of propofol than older patients (20-40 years *vs* \geq 61 years: 115.3 ± 32 mg *vs* 89.7 ± 21 mg, $P < 0.001$). Transient supplemental oxygen supply was needed by five patients (0.2%); no other complications occurred. The questionnaires were completed by 1820 (87%) of 2101 patients and most rated their overall satisfaction as excellent (80%) or good (17%). The majority (65%) of patients drove home or to their office after their colonoscopy. Most (99%) were willing to repeat the same procedure. No incidents occurred within 24 h after colonoscopy.

CONCLUSION: Propofol sedation using a dose < 200 mg proved both safe and practical for outpatient colonoscopy.

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Key words: Colonoscopy; Propofol; Colorectal cancer

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INTRODUCTION

Colorectal cancer is one of the main causes of death

from cancer in western countries and in Japan. There is no clear strategy for reducing the incidence of colorectal cancer, therefore, reduction in mortality relies on removal of premalignant lesions and detection of cancer at an early stage by colonoscopy^[1,2]. In the face of increasing demands for gastroenterological services, the success of colonoscopic screening in the outpatient setting depends on patient acceptability^[3]. CO₂ insufflation and the water method for unsedated colonoscopy have been shown to be more acceptable than unsedated conventional colonoscopy^[4,5]. We previously demonstrated that use of a small-caliber pediatric colonoscope resulted in completed colonoscopies in patients who had an unsuccessful procedure using a standard colonoscope^[6]. The use of variable stiffness colonoscopes significantly reduced procedure-related pain and the dose of propofol required for sedation during colonoscopy^[7].

Recently, the feasibility and safety of endoscopist-directed propofol administration was confirmed in a study of 646 080 cases from 28 centers^[8]. Endoscopist-directed propofol sedation refers to administration of propofol by a non-anesthesia specialist under the direct supervision of the endoscopist performing the endoscopic procedure. Propofol sedation for colonoscopy was shown to be superior to other sedation methods in that propofol was associated with a low incidence of cardiopulmonary complications and was superior to benzodiazepines with regard to rapidity of both induction of sedation and recovery^[9-11].

These results have been confirmed by meta-analysis^[12]. We have previously reported that propofol sedation was both safe and practical for diagnostic esophago-gastroduodenoscopy and endoscopic procedures in patients aged ≥ 90 years^[13-16]. In addition, we have allowed our patients to drive home after colonoscopy, based on the experience with diagnostic esophagogastroduodenoscopy in our endoscopy unit^[13,14]. Here, we report a prospective evaluation of the safety and effectiveness of propofol sedation with follow-up for 24 h after outpatient colonoscopy.

MATERIALS AND METHODS

Patients

The study was done at the Showa Inan General Hospital and included outpatients who underwent only colonoscopy. Emergency procedures were excluded. Patients were also excluded if they were < 20 years old, pregnant, assigned to American Society of Anesthesiologists (ASA) class III and IV, overweight (> 100 kg), or allergic to the drugs used or their components (soybeans or eggs). Routine standard monitoring at this unit included continuous assessment of peripheral oxygen saturation (SpO₂) and heart rate. Clinical assessment of the patients included measurement of respiratory effort by visual assessment and by palpation of the chest wall and abdominal excursion and/or palpation of exhaled breath. When oxygen desaturation (SpO₂ < 90%) continued for

> 20 s, supplemental oxygen was given. The endoscopic team consisted of the nurse who administered the drugs and was responsible for the patient, the endoscopist, and a second nurse to assist the endoscopist and the patient-monitoring nurse.

Propofol was administered for endoscopic sedation by nurses supervised by endoscopists. Both the nurses and endoscopists had advanced cardiac life support certification, advanced airway training, didactic training on propofol, observation of cases, and supervised administration of propofol by anesthesiologists before beginning propofol administration supervised by the endoscopist. The training period typically lasted about 2 wk.

The study was conducted in accordance with the Helsinki Declaration and was approved by the ethics committee at the hospital. Verbal and written informed consent was obtained from all patients.

All colonoscopies were performed by six skilled endoscopists (Horiuchi A, Nakayama Y, Kajiyama M, Kato N, Kamijima T, Ichise Y) who each perform > 300 colonoscopies/year. All the procedures were conducted under propofol sedation (AstraZeneca, Osaka, Japan)^[13,14]. The standard bowel preparation was a polyethylene glycol solution (Ajinomoto Pharmaceutical Co, Tokyo, Japan).

Study design

Colonoscopy was performed in the lateral decubitus position. A butterfly needle for the bolus injection of propofol was placed on the patient's forearm shortly before the start of colonoscopy and was removed after completion of the procedure. Propofol was given by bolus injection with an age-adjusted standard protocol of 60 mg for patients aged < 70 years, 40 mg for patients aged 70-89 years, and 20 mg for those aged ≥ 90 years. Adequate sedation was considered achieved when the patients passed through the following sequence: eyes closing, one or two yawns, and cessation of body movements. The target level of sedation was moderate conscious sedation with the patients still being able to respond purposefully to verbal commands. When the target level was not obtained or the patients were under-sedated, additional injections of 20 mg propofol were given up to a maximum of 200 mg.

A decline in SpO₂ to < 90% that continued for > 20 s was regarded as respiratory depression associated with sedation. Vital signs were frequently assessed but not on a periodic basis. In addition to monitoring of vital signs, the patient's condition was assessed more globally by visual inspection. Monitoring and complications were recorded by a registered nurse. SpO₂ was routinely captured by visual inspection of the monitor and the value was recorded on the vital sign sheet.

After the procedure, patients were moved to a waiting room after they could stand by themselves and they were discharged after they were fully awake. Full recovery, including consciousness and psychomotor function was assessed using three criteria: (1) Level of consciousness (fully awake and responding to questions from the recov-

Table 1 Demographic and clinical data in 2101 patients who underwent colonoscopy using propofol sedation

	<i>n</i> (%)
Sex: Male	1149 (55)
Mean age (range) (yr)	66 (20-94)
Mean body weight (range) (kg)	56.7 (32-98)
Indication	
Screening	785 (37)
Hemopositive stool	538 (26)
Abdominal symptoms	406 (19)
Hematochezia	140 (7)
Surveillance	133 (6)
Anemia	70 (3)
Others	29 (1)
Propofol dose (mg):	
About 40	110 (5)
60	239 (11)
80	623 (30)
100-120	973 (46)
140-160	131 (6)
180-200	25 (1)
Successful procedure	2101 (100)
Mean procedure time (range) (min)	14 (8-46)
Oxygen administered	5 (0.2)
Mask ventilation required	0 (0)
Heart rate < 50 beats/min	0 (0)
Complications	0 (0)
Full recovery 60 min after the procedure	2101 (100)

Values are numbers (percentages) of patients except for mean age, mean body weight, and mean procedure time.

ery room nurse); (2) Ability to stand on one-foot; and (3) Ability to walk in a straight line without instability for 5 m. These three criteria were assessed every 15 min starting 30 min after the procedure; full recovery was defined as meeting all three criteria. The nurses reconfirmed the absence of re-emerging sedative effects and finally permitted patients to leave the endoscopic unit.

In addition, we provided questionnaires. Within 2 wk after the procedure, patients were contacted by telephone and asked about overall satisfaction for this procedure, whether they drove home or to their office after colonoscopy, the occurrence of any accidents within 24 h after colonoscopy and their willingness to repeat the same procedure next time (yes/no).

Study parameters

The principal parameters were the occurrence of adverse events within 24 h after colonoscopy and overall satisfaction for the procedure. Secondary parameters included successful procedure, respiratory depression, and other complications. Respiratory depression was defined as the need of oxygen supply due to an oxygen desaturation ($SpO_2 < 90\%$) that continued more than 20 s.

Instruments

The Olympus PCF-Q260AI videoscope used has an insertion diameter of 11.3 mm, an accessory channel diameter of 2.8 mm, a total length of 1335 mm, and a working length of 1030 mm.

Table 2 Relationship between age or sex and dose of propofol used in 2101 patients who underwent colonoscopy

Age (yr)	No. (M/F)	Propofol dose (mg)			<i>P</i> value
		Total (range)	Male	Female	
20-40 ¹	150 (89/61)	115.3 ± 32 (40-200)	113.4 ± 27	118.2 ± 24	0.43
41-60	563 (316/247)	107.7 ± 27 (40-200)	106.9 ± 28	108.7 ± 25	0.19
≥ 61	1388 (744/644)	89.7 ± 21 (40-200)	87.7 ± 23	92.1 ± 19	0.32
Total	2101 (1149/952)	96.4 ± 27	95.0 ± 26	98.1 ± 25	0.18

Values are mean ± SD except for number of patients and range of dose used of propofol. *P* value shows the difference between male and female patients. ¹There were significant differences in the doses of propofol in each group between age 20-40 and ≥ 61 years ($P < 0.001$).

Statistical analysis

Data are presented as mean ± SD. The χ^2 test, with Yates' correction for continuity where appropriate, was used for comparison of categorical data. Fisher's exact test was used when the numbers were small. For parametric data, Student's *t* test was used when two means were compared. Analysis of variance was used when the three groups were compared and positive results were confirmed using Tukey's Honestly Significantly Different procedure. A value of $P < 0.05$ was regarded as significant. Statistical analysis was performed by using JMP[®] 9.0.2 version software (SAS Institute Inc., Cary, NC, United States).

RESULTS

Between January 2010 and December 2010, 2101 consecutive patients received outpatient colonoscopy based on a standard protocol of age-adjusted doses of propofol (Table 1). All procedures were completed successfully. The patients' ages ranged from 20 to 94 years. The most common indications for colonoscopy were: colorectal cancer screening in 785 (37%), hemopositive stools in 538 (26%), and abdominal symptoms in 406 (19%). Mean procedure time was 14 min (range, 8-46 min). A biopsy and/or polypectomy was performed in 775 patients (37%). Oxygen desaturation requiring supplemental oxygen (1-3 L/min) occurred in 0.2% (five patients); mask ventilation or endotracheal intubation was not required in any case. In no case did respiratory event or laryngospasm occur. No other complications occurred (Table 1). Full recovery within 60 min after the procedure was present in 100%.

The mean dose of propofol used was 96.4 mg (Table 2). There were no significant differences in the dose of propofol used between men and women (95.0 ± 26 mg *vs* 98.1 ± 25 mg, $P = 0.18$), however younger patients required higher doses of propofol than older patients (20-40 years *vs* ≥ 61 years, 115.3 ± 32 mg *vs* 89.7 ± 21 mg, $P < 0.001$). Of the 2101 patients, 495 (24%) had at least an adenoma detected. Fifty-two (3%) had invasive colorectal cancer. Colorectal adenomas with high-grade dysplasia were found in 32 (2%) patients. The detection rate of adenoma in this study was 53 adenomas/100

Table 3 Demographic data and results of questionnaires in 1820 patients who underwent colonoscopy using propofol sedation

Sex: Male	954 (52)
Mean age (range) (yr)	65 (33-80)
Mean body weight (range) (kg)	58.7 (45-98)
Did you find propofol sedation for your colonoscopy satisfactory?	
Excellent	1456 (80%)
Good	309 (17%)
No	11 (0.6%)
NA	8 (2.4%)
Did you drive home or to the office after your colonoscopy?	
Yes	1183 (65%)
No	637 (35%)
Did you experience any accidents within 24 h after your colonoscopy?	
Yes	0 (0%)
No	1820 (100%)
Do you want to repeat the same procedure next time?	
Yes	1805 (99%)
No	15 (1%)

Values are numbers (percentages) of patients except for mean age and mean body weight. NA: Not available.

colonoscopies. Adenomas were distributed evenly in the colon and rectum.

The questionnaires were completed by 1820 (87%) of 2101 patients (Table 3). Their mean age and mean body weight were 65 years and 58.7 kg. The majority rated their overall satisfaction for this procedure as excellent (80%) or good (17%). The majority (65%) of subjects drove home or to their office after their colonoscopy. No associated incidents within 24 h after colonoscopy occurred. Most (99%) were willing to repeat the same procedure.

DISCUSSION

This study describes the safety and outcomes of propofol sedation given to 2101 patients for outpatient colonoscopy. Previous studies have involved propofol dosages ranging from 60 to 300 mg using an indwelling catheter for endoscopic sedation^[9-12,17-21]. In the present study, the mean dose of propofol used was 96.4 mg, which corresponded to that of a previous study in the Japanese population^[17]. On the basis of our previous experiences^[13,14], the protocol adopted here focused on safety with the initial dose of 40 or 60 mg propofol designed to minimize hypoxemia during the procedure; only 0.2% required oxygen, which is less than previous studies^[9-12]. No subject required mechanical ventilation during the procedures via either endotracheal intubation or a mask (Table 1). The routine use of supplemental oxygen during colonoscopy may mask respiratory depression^[22], therefore, we chose to administer supplemental oxygen only when needed. No subjects experienced bradycardia (heart rate < 50 beats/min). Other studies with low-dose propofol have reported rates of bradycardia up to 10%^[23]; the differences may relate to difference in the sedation method because studies with bradycardia have typically used a combination of propofol, midazolam

and meperidine. All procedures were done with pediatric variable stiffness colonoscope, thus, it is impossible to assess whether the pediatric variable stiffness colonoscope, or the lower dose of propofol required, was responsible for the low incidence of cardiopulmonary depression.

Generally, patients are not permitted to drive themselves within the first day after endoscopic sedation. Riphaut *et al.*^[24] have reported that current recommendations that patients should be refrain from driving and unescorted use of public transport for 24 h after sedation may need to be reconsidered in patients who receive propofol sedation. Based on our previous study using a driving simulator showing that driving ability recovered to the basal level within 60 min of low-dose propofol sedation^[13], patients have been permitted to drive themselves after colonoscopy as well as esophagogastroduodenoscopy at our endoscopy unit. In this study, the majority (65%) of patients drove home or to their office after their colonoscopy. No sedation-associated incidents within 24 h after colonoscopy occurred in the 1820 subjects responding.

Our recent study used a number connection test and a driving simulator test to assess psychomotor recovery before and 1 h and 2 h after colonoscopy^[25]. Psychomotor recovery was evident as early as 1 h after propofol sedation. Additional studies are needed before it can be routinely recommended that patients be permitted to drive home after endoscopy using only propofol sedation. Effective endoscopic sedation depends on the type of procedure and the procedure time. In most patients, an appropriate level of sedation can be reached through the use of a benzodiazepine combined with a narcotic. In contrast, we gave propofol by bolus titration with an initial dose of 40-60 mg followed by dose of 20 mg beginning 30-60 s later. The appropriateness of additional bolus doses was determined by the level of sedation and the respiratory effect. When moderate conscious sedation was not obtained or the patients were undersedated, additional injections of 20 mg propofol were given up to a maximum of 200 mg; 180-200 mg were required in only 25 patients (1.2%). The advantages of propofol using a butterfly needle include immediate onset of action and fast recovery, which likely contributed to the high level of acceptability and cost-effectiveness for outpatient colonoscopy. In our facility, a butterfly needle has been used instead of an indwelling cannula for about 60 000 patients. Even if a patient in conscious sedation moves his/her arm, the butterfly needle is usually placed stably in the vein. This butterfly needle method is thought to be a safe and practical way to administer propofol over 15-30 min.

The average procedure time was 14 min and the detection rate of adenoma was 24% as the proportion of patients in whom at least one adenoma was identified in this study. The adenoma detection rate that was reported to be an independent predictor of the risk of interval colorectal cancer after screening colonoscopy was compatible to our

data^[26]. Therefore, this sedation method is likely an important variable in ensuring quality in colonoscopy.

Unsedated colonoscopy is still the main type of procedure in many countries. Even if sedation is safe, it requires an extra nurse and a team educated for monitoring, resulting in higher costs. The requirement for an escort and time burden of recovery from sedation are both barriers to the acceptance of screening colonoscopy. Improved acceptance of colonoscopy is important to allow full use of colonoscopy in cancer screening and prevention. Colonoscopy can be completed without sedation in the majority of patients and undoubtedly there is a place for unsedated colonoscopy, especially in areas where cost containment is a primary concern^[27-29].

This study had some limitations. The sedation level was assessed using minimal, moderate, and deep sedation on the basis of the ASA level^[30], but this was not recorded for each patient during the study. Although the patients may have had amnesia after propofol sedation, the questionnaire in this study did not include any questions related to amnesia. Blood pressure monitoring was not routinely performed in this study. Blood pressure monitoring is generally a standard practice whenever administering propofol in the United States but not in Japan. We were therefore unable to comment on episodes of hypotension in our series. The monitoring used (e.g., continuous assessment of SpO₂ and heart rate) is the standard in Japan.

In conclusion, propofol sedation was associated with good acceptance of colonoscopy and willingness to repeat the procedure and enabled patients to drive home safely by themselves after colonoscopy. Propofol sedation using a dose < 200 mg was safe and practical. Increased use of this sedation method may improve the acceptability of colonoscopy, which may enable population wide screening and decrease colorectal cancer mortality.

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COMMENTS

Background

Despite the increasing usage of short-acting sedatives, the recommendations that patients should refrain from driving and the unescorted use of public transport for 24 h after sedation remain unchanged. The authors previously reported that recovery after low-dose propofol sedation for diagnostic esophagogastroduodenoscopy was such that the patients were able to drive home. In addition, the recent study using a number connection test and a driving simulator test demonstrated that psychomotor recovery was evident as early as 1 h after propofol sedation for outpatient colonoscopy.

Research frontiers

The authors report a prospective evaluation of the safety and effectiveness of propofol sedation with follow-up for 24 h after outpatient colonoscopy.

Innovations and breakthroughs

Propofol sedation was associated with good acceptance of colonoscopy and willingness to repeat the procedure and enabled patients to drive home safely by themselves after colonoscopy. Propofol sedation using a dose < 200 mg was safe and practical. Increased use of this sedation method may improve the

acceptability of colonoscopy, which may enable population-wide screening and decrease colorectal cancer mortality.

Applications

The findings in this study indicate that patients sedated with propofol may be capable of safely driving 1 h after colonoscopy. The ability to drive home after sedation reduces the need for an accompanying individual and thus lowers the costs and burden associated with endoscopy.

Peer review

This is a well-written paper providing new data on safety of propofol sedation and new information about patients checking out and driving home after propofol sedation.

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Efficacy of hepatic arterial infusion chemotherapy in advanced hepatocellular carcinoma

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Abstract

AIM: To investigate the efficacy of hepatic arterial infusion chemotherapy (HAIC) using floxuridine (FUDR) in patients with advanced hepatocellular carcinoma (HCC) confined to the liver.

METHODS: Thirty-four patients who had advanced HCC with unresectability or unsuccessful previous therapy in the absence of extrahepatic metastasis were treated with intra-arterial FUDR chemotherapy at our

hospital between March 2005 and May 2008. Among the 34 patients, 9 patients were classified as Child class C, and 18 patients had portal vein tumor thrombus (PVTT). One course of chemotherapy consisted of continuous infusion of FUDR (0.3 mg/kg during day 1-14) and dexamethasone (10 mg on day 1, 4, 7 and 11), and this treatment was repeated every 28 d.

RESULTS: Two patients (5.9%) displayed a complete response, and 12 patients (35.3%) had a partial response. The tumor control rate was 61.8%. The median overall survival times were 15.3 mo, 12.4 mo and 4.3 mo for the patients who were classified as Child class A, Child class B and Child class C, respectively ($P = 0.0392$). The progression-free survival was 12.9 mo, 7.7 mo and 2.6 mo for the patients who were classified as Child class A, Child class B and Child class C, respectively ($P = 0.0443$). The cumulative survival differed significantly according to the Child-Pugh classification and the presence of PVTT. In addition to hepatic reserve capacity and PVTT, the extent of HCC was an independent factor in determining a poor prognosis. The most common adverse reactions to HAIC were mucositis, diarrhea and peptic ulcer disease, but most of these complications were improved by medical treatment and/or a delay of HAIC.

CONCLUSION: The present study demonstrates that intra-arterial FUDR chemotherapy is a safe and effective treatment for advanced HCC that is recalcitrant to other therapeutic modalities, even in patients with advanced cirrhosis.

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Key words: Hepatic arterial infusion chemotherapy; Floxuridine; Advanced hepatocellular carcinoma; Child-Pugh classification; Portal vein tumor thrombus

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INTRODUCTION

Hepatocellular carcinoma (HCC), which is one of the most common malignancies worldwide, causes over 600 000 deaths per year and is the third most common cancer in South Korea^[1,2]. Although surgical resection or liver transplantation can be curative for HCC, most patients are not candidates for surgery at the time of diagnosis because it is difficult to detect HCC at an early stage. In addition, some patients have advanced cirrhosis by the time of diagnosis^[3].

Although recent advances in therapeutic modalities, such as hepatic resection, percutaneous ethanol injection, radiofrequency ablation (RFA), transarterial chemoembolization (TACE), radiotherapy and liver transplantation, have improved the treatment of HCC, the prognosis for patients with advanced HCC remains poor. In addition, these current therapies have many limitations, and recurrence and metastasis are relatively common^[4-7]. Among the currently available therapies, TACE is the current standard of care for patients who are not candidates for curative therapy. The survival benefit conferred by TACE was reported in a randomized controlled trial^[8], which showed that the median survival of the patients in the TACE group was approximately 14 mo. However, TACE is contraindicated in advanced HCC with main portal vein tumor thrombus (PVTT), massive or diffuse infiltration, poor liver function with Child class C, and severe hepatic arterioportal shunt.

Regional hepatic arterial infusion chemotherapy (HAIC) has also been used in patients with advanced HCC because liver cancers receive most of their blood supply from the hepatic artery, whereas normal liver tissue has a dual vascular supply (i.e., 20% of the blood supply comes from the hepatic artery, and the remaining 80% comes from the portal vein). Thus, HAIC may be used, albeit with caution, in cases in which TACE is not indicated or is ineffective^[9]. HAIC may provide higher concentrations of chemotherapeutic agents directly to the HCC and produces minimal systemic concentrations of chemotherapeutic agents, which can minimize systemic toxicity^[10].

Floxuridine (FUDR) is an active metabolite of 5-fluorouracil (5-FU) that has the advantage of being rapidly metabolized, with a 94%-99% extraction rate within the liver *via* first-pass metabolism. FUDR is maintained at an intrahepatic concentration that is more than tenfold

greater than that of 5-FU, cisplatin, mitomycin or doxorubicin, which permits maximal tumor cell death while preventing systemic toxicity^[11,12].

Most previous studies have reported the efficacy of HAIC using 5-FU and cisplatin in advanced HCC or HAIC using FUDR in patients with hepatic metastasis from colorectal cancer^[10,13,14].

In the present study, we evaluated the efficacy and toxicity profile of HAIC using FUDR in patients with advanced HCC confined to the liver who failed to respond to previous therapy or who were unable to receive other therapeutic modalities.

MATERIALS AND METHODS

Patient eligibility

Thirty-four patients with advanced HCC that was unresectable or resistant to previous therapy in the absence of extrahepatic metastasis were treated with intra-arterial FUDR chemotherapy at our hospital between March 2005 and May 2008. The criteria for unresectability included bilobar disease with 4 or more lesions, large tumors occupying more than 50% of the liver, and invasion of the tumor into major vascular structures. Previous therapies included RFA, TACE and radiotherapy. All of the patients belonged to tumor node metastasis stage IIIA or IIIB. To assess the eligibility for inclusion, each patient received a computed tomography (CT) scan of the abdomen and pelvis. Each patient provided a full medical history and underwent a physical examination, chest X-ray, and laboratory tests, including a test for alpha-fetoprotein. Additional examinations, such as magnetic resonance imaging (MRI) scans, liver biopsy and angiography, were performed if the CT scan and tumor marker analyses were insufficient for diagnosis. Among the 34 patients, disease was histologically confirmed in 5 patients. When metastasis was suspected, positron emission tomography scans and bone scans and/or CT scans of suspicious areas were conducted. Patients with distant metastases were excluded. Informed consent was obtained from all of the patients.

A pump catheter was inserted at the proper hepatic artery from the superior mesenteric artery. The distal gastroduodenal artery, the right gastric artery, the small branches supplying the stomach or duodenum, and all of the accessory hepatic arteries received ligations to prevent gastrointestinal toxicity. The patients with hepatitis B received prophylactic or therapeutic antiviral agents before HAIC. Some of the patients with hepatitis C had previously been treated with pegylated-interferon/interferon and ribavirin. The remaining hepatitis C patients were carefully monitored because antiviral treatment was deferred because of underlying bone marrow and immune suppression complications.

Assessment of responses

The patient responses were classified according to the Response Evaluation Criteria In Solid Tumors guidelines.

Table 1 Baseline characteristics *n* (%)

Characteristics	IA-FUDR (<i>n</i> = 34)
Age (yr)	62.2 ± 7.4
Sex	
Male	27 (79.4)
Female	7 (20.6)
Cause of HCC	
Hepatitis B virus	27 (79.4)
Hepatitis C virus	2 (5.9)
Alcoholism	5 (14.7)
Child-Pugh classification	
Child class A	7 (20.6)
Child class B	18 (52.9)
Child class C	9 (26.5)
Portal vein thrombosis	
Yes	18 (52.9)
No	16 (47.1)
Tumor morphology	
Multinodular	16 (47.1)
Huge, massive > 50% of liver	18 (52.9)
Bilirubin (mg/dL)	2.6 ± 2.3
Albumin (g/dL)	2.8 ± 1.1
Prothrombin time (INR)	2.0 ± 0.7
AFP (ng/mL)	5136.03 (median)
Treatment prior to chemotherapy or supportive care	
Surgery	0 (0)
RFA	3 (8.8)
TACE	20 (58.8)
RFA and TACE	1 (2.9)
Radiation	1 (2.9)
No treatment	9 (26.5)

HCC: Hepatocellular carcinoma; AFP: Alpha-fetoprotein; RFA: Radio-frequency ablation; TACE: Transarterial chemoembolization; IA-FUDR: Intra-arterial floxuridine; INR: International normalized ratio.

Complete response (CR) was defined as the disappearance of all evidence of disease and the normalization of tumor markers for at least 4 wk. Partial response (PR) was defined as a $\geq 30\%$ reduction in unidimensional tumor measurements without the appearance of any new lesions or the progression of any existing lesion. Progressive disease (PD) was defined as any of the following: a 20% increase in the sum of the diameters of five measurable lesions, the appearance of any new lesions, or the reappearance of any lesion that had previously disappeared. Stable disease (SD) was defined as a tumor response that did not fulfill the criteria for CR, PR or PD. CT scans or MRIs of the measurable lesions were carried out within 4 wk before the start of treatment and repeated every 2 cycles (2 mo). Responses were confirmed by subsequent CT or MRI scans after the documentation of the initial response.

Toxicity assessment

Toxicity was assessed according to the National Cancer Institute Common Toxicity Criteria. The FUDR dose was modified when grade 3-4 toxicity was observed.

Hepatic toxicity was defined as a significant increase over baseline values (3- to 4-fold for aspartate transaminase or alanine transaminase and greater than 1.5-fold for bilirubin), and the increases in hepatic enzyme levels

caused by the disease varied across patients. If a patient complained of epigastric pain, an evaluation that included an upper gastrointestinal endoscopy was performed. If an ulcer or gastroduodenitis was identified, then chemotherapy was stopped until recovery. If a patient had severe diarrhea or abdominal pain, chemotherapy was stopped until recovery. In addition, angiography was performed to block collateral vessels when extrahepatic perfusion was suspected.

Chemotherapy regimen

Local chemotherapy was started between 3 and 5 d after pump insertion. The patients received FUDR (0.3 mg/kg per day for 14 d) and dexamethasone (10 mg on day 1, 4, 7 and 11) *via* an intra-arterial pump. FUDR was synthesized by APP Pharmaceuticals, LLC (Schaumburg, IL, United states). After the 14 d of treatment with FUDR, the pump was emptied and refilled with 30 000 units of heparin in 0.9% saline for 14 d. This treatment was repeated every 28 d. FUDR was given indefinitely until the disease progressed or the therapy was discontinued due to toxicity or patient death.

Statistical analysis

The objective of the present study was to estimate the efficacy and toxicity of continuous HAIC with FUDR *via* an implantable pump. All of the analysis were performed using SPSS version 18.0.

Survival times were calculated from the start of the study treatment until patient death or the final follow-up. Progression-free survival (PFS) was calculated from the first day of chemotherapy until the date of progression. PFS and overall survival (OS) curves were obtained using the Kaplan-Meier method, and comparisons were made using the log rank test. Multivariate analysis to evaluate the influence of prognostic factors on survival was performed using Cox proportional hazard methods. Statistical significance was established as $P < 0.05$.

RESULTS

Patient characteristics

A total of 34 patients, 27 men and 7 women, with a median age of 62.2 years received intra-arterial FUDR chemotherapy between March 2005 and May 2008. The patient baseline characteristics are shown in Table 1. The major etiology of the patients' liver disease was hepatitis B virus (27 of 34, 79.4%). Eighteen patients had PVTT, whereas 16 patients did not have PVTT. The majority of patients had received TACE as the previous therapeutic modality (20 of 34, 58.8%).

Response to treatment

Patients received 2-10 (the median was 3.5) cycles of chemotherapy. All of the patients received at least 2 cycles of intra-arterial FUDR chemotherapy, and 17 (50%) patients received more than 4 cycles.

The patients' responses to treatment are summarized

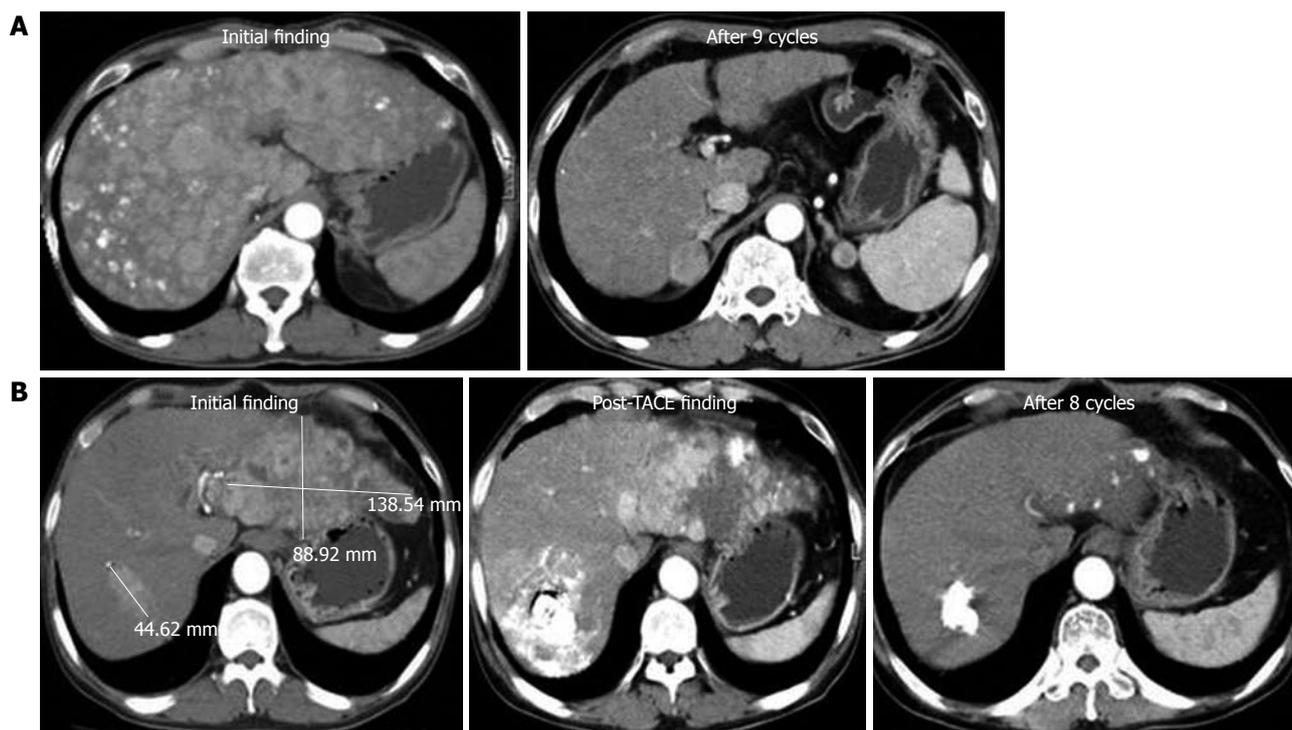


Figure 1 Two cases of nearly complete response. A: The first case was a 54-year-old patient with diffuse, multinodular hepatocellular carcinoma throughout the whole liver. After 9 cycles of hepatic arterial infusion chemotherapy (HAIC) with floxuridine, no enhancing nodular lesions were observed by dynamic contrast-enhanced computed tomography (CT); B: The second case was a 48-year-old patient with a large mass in the left lobe and intrahepatic metastasis in the right lobe. After transarterial chemoembolization treatment, viable masses were still observed in sequential CT images, and HAIC was started. After 8 cycles of chemotherapy, no viable masses were observed. TACE: Transarterial chemo-embolisation.

Table 2 Treatment response rate *n* (%)

Tumor response	Intra-arterial FUDR chemotherapy			
	Total	Child class A	Child class B	Child class C
CR	2 (5.9)	1 (14.3)	1 (5.6)	0 (0)
PR	12 (35.3)	2 (28.6)	9 (50)	1 (11.1)
SD	7 (20.6)	2 (28.6)	3 (16.7)	2 (22.2)
PD	13 (38.2)	2 (28.6)	5 (27.8)	6 (66.7)
Response rate	14 (41.2)	3 (42.9)	10 (55.6)	1 (11.1)
Disease control rate	21 (61.8)	5 (71.4)	13 (72.2)	3 (33.3)
Total	34 (100)	7 (100)	18 (100)	9 (100)

FUDR: Floxuridine; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

in Table 2. Two patients (5.9%) had CR, 12 (35.3%) had PR, seven (20.6%) had SD, and 13 (38.2%) had PD. The CT findings for the two patients with CR (Figure 1). Among the patients who were classified as Child class A or B, 2 (8%) had CR and 11 (44%) had PR. The patients who were classified as Child class A or B had a response rate of 52% and a disease control rate of 72%.

Survival

The cumulative survival of the 34 patients is presented in Figure 2A and B. The median OS was 8.9 mo, and the median PFS was 6.6 mo.

We assessed the cumulative survival according to the Child-Pugh classification and the presence of PVTT

(Figure 3). The median OS times were 15.3 mo, 12.4 mo and 4.3 mo in the patients who were classified as Child class A, Child class B and Child class C, respectively, and there were significant differences between the groups ($P = 0.0392$). Similarly, there were significant differences in the median PFS times, which were 12.9 mo, 7.7 mo and 2.6 mo in the patients who were classified as Child class A, Child class B and Child class C, respectively ($P = 0.0443$) (Figure 3A and B). These findings showed that the patients who were classified as Child class C had significantly shorter survival times compared with the patients who were classified as Child class A or Child class B.

Further differences were found in the OS and PFS according to the presence of PVTT, which indicated a negative impact of PVTT on survival time. The median OS and PFS of the patients without PVTT were 13.1 mo and 8.2 mo, respectively, compared with 8.2 mo and 3.4 mo, respectively, for the patients with PVTT (Figure 3C and D).

Prognostic factors

We conducted univariate and multivariate analyses of baseline characteristics, such as age, sex, Child-Pugh classification, PVTT, extent of HCC, bilirubin, albumin and PT international normalized ratio (INR), using a Cox proportional hazards model to evaluate the prognostic factors for survival (Table 3). The multivariate analysis showed that 4 variables (i.e., child classification, PVTT, HCC type and PT INR) were independent predictors of survival.

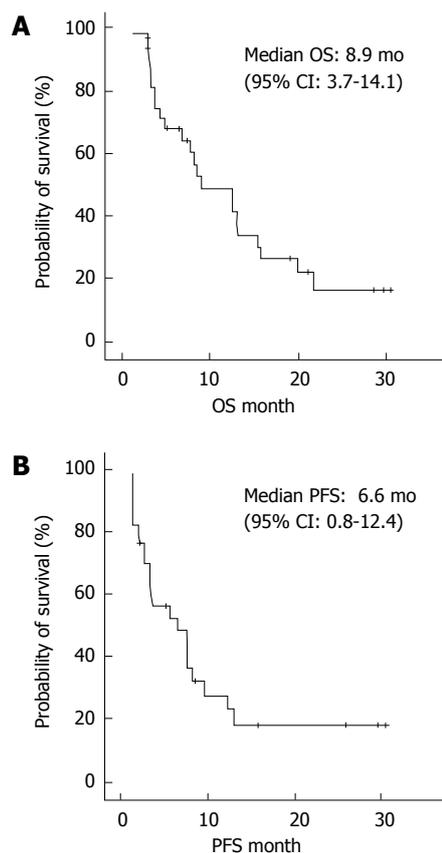


Figure 2 The overall survival and the progression-free survival determined by Kaplan-Meier analysis. A: The overall survival (OS) determined by Kaplan-Meier analysis; B: The progression-free survival (PFS) determined by Kaplan-Meier analysis.

Adverse reactions and complications

The treatment-related adverse reactions and complications that were observed in the 34 patients who were treated with HAIC (Table 4). Severe hematologic toxicity during HAIC was not noted in any of the patients. The most common grade 3-4 adverse reaction was gastric or duodenal ulcer (11.8%). Most of the adverse reactions were improved by medical treatment and/or delaying HAIC. One patient with hepatitis C experienced progressive hepatic failure during the third cycle of chemotherapy and eventually died. In addition, a major complication related to the indwelling catheter occurred in 1 patient. Indeed, an infection occurred around the catheter, but it was controlled by antibiotics and removal of the port. This patient continued HAIC after insertion of another catheter in the opposite site. Another major complication that has commonly been reported to be associated with FUDR treatment is biliary sclerosis; however, the patients in the present study received prophylactic dexamethasone on a regular schedule, and none of the present patients experienced biliary sclerosis.

Cause of death

During treatment and follow-up, 9 patients died from various causes, including tumor progression, hepatic failure, gastrointestinal bleeding and sepsis. Among the

Table 3 Prognostic significance of the clinical factors influencing survival

Multivariate	Hazard ratio	95% CI	P value
Age (≥ 60 yr, < 60 yr)	1.381	0.403-4.734	0.608
Sex (male, female)	1.462	0.433-4.937	0.541
Child-Pugh classification	3.710	1.490-9.238	0.005
Portal vein thrombosis (without, with)	0.086	0.019-0.387	0.001
Extent of HCC (multinodular, massive $> 50\%$ of liver)	0.185	0.051-0.679	0.011
Bilirubin (≥ 3 , < 3)	0.837	0.319-2.195	0.718
Albumin (< 3 , ≥ 3)	1.059	0.430-2.603	0.901
PT INR (< 2.3 , ≥ 2.3)	0.218	0.066-0.715	0.012

HCC: Hepatocellular carcinoma; PT INR: Prothrombin time international normalized ratio.

Table 4 Adverse reactions to floxuridine *n* (%)

Treatment group (toxicity)	NCI-CTC grade	
	1-2	3-4
Fever	2 (5.9)	0 (0)
Nausea/vomiting	0 (0)	0 (0)
Gastric or duodenal ulcer	3 (8.8)	4 (11.8)
Mucositis	4 (11.8)	3 (8.8)
Diarrhea	4 (11.8)	3 (8.8)
Leukopenia	2 (5.9)	1 (2.9)
Thrombocytopenia	2 (5.9)	1 (2.9)
Bilirubin elevation	4 (11.8)	1 (2.9)
AST/ALT elevation	5 (14.7)	1 (2.9)
BUN/Cr elevation	0 (0)	1 (2.9)
Catheter infection	1 (2.9)	
Total	19	9

NCI-CTC: National Cancer Institute Common Toxicity Criteria; AST: Aspartate transaminase; ALT: Alanine transaminase; BUN: Blood urea nitrogen; Cr: Creatinine.

patients who died, 4 (44.4%) died of hepatic failure related to an advanced cirrhotic condition. Only 1 of the 4 patients had hepatic failure in relation to therapy, and the remaining patients died after loss to follow-up or the incidence of another illness. Tumor progression and sepsis were the causes of death in 2 patients each (22.2%). One patient died of upper varix bleeding.

DISCUSSION

Many therapeutic modalities are available for the treatment of HCC, such as hepatic resection, percutaneous ethanol injection, RFA, TACE, radiotherapy and liver transplantation^[15]. Among these therapies, TACE has been the main treatment modality for the management of unresectable or recurrent HCC. A randomized controlled trial revealed that the median survival for patients undergoing TACE was approximately 14 mo^[8]. However, this treatment has not been useful in patients with PVTT or large infiltrative HCC because of the potential risk of hepatic failure resulting from ischemia^[16,17].

HAIC can be safely used in patients with impaired liver function due to advanced HCC or underlying liver

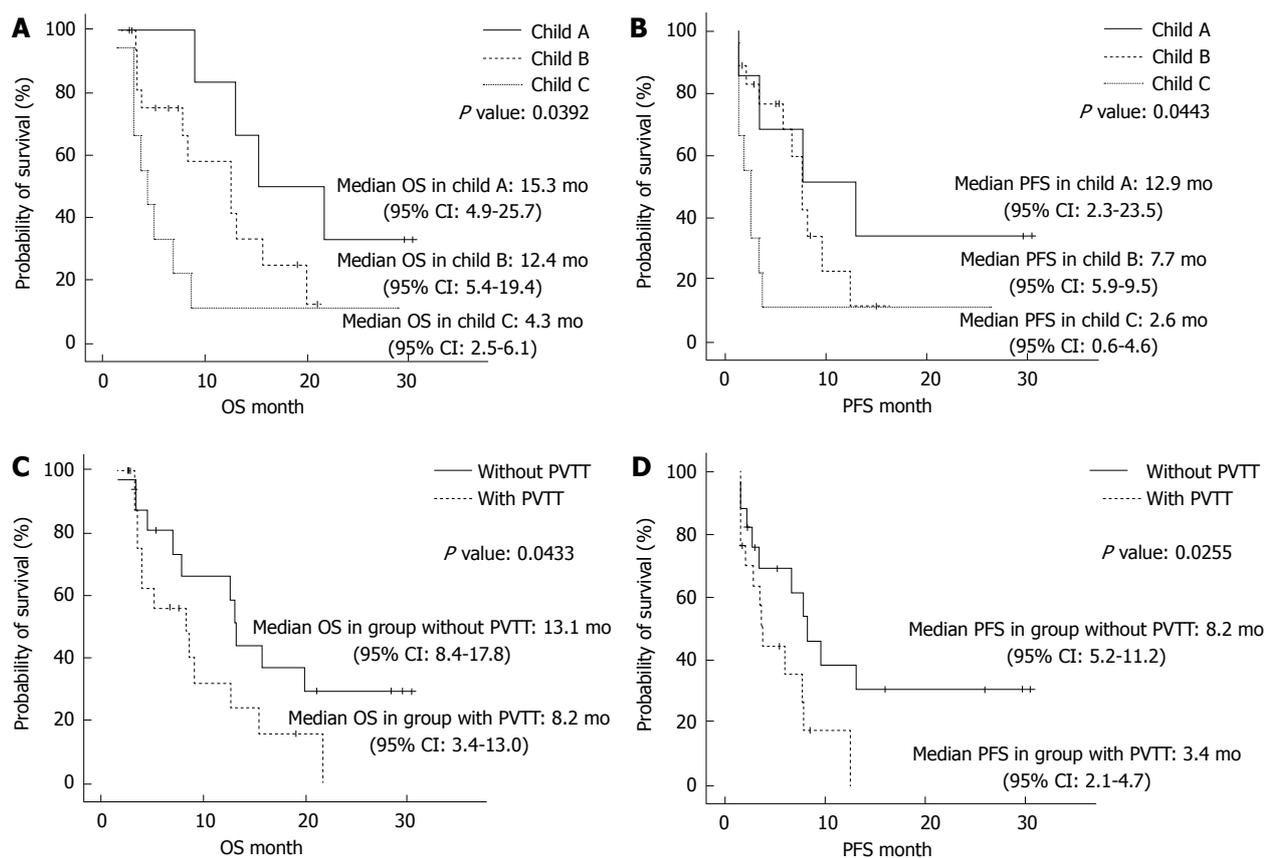


Figure 3 Kaplan Meier plot estimates of the overall survival and the progression-free survival according to the Child-Pugh classification and existence of portal vein tumor thrombus. A: Kaplan Meier plot estimates of the overall survival (OS) to the Child-Pugh classification; B: Kaplan Meier plot estimates of the progression-free survival (PFS) according to the Child-Pugh classification; C: Kaplan Meier plot estimates of the OS according to existence of portal vein tumor thrombus (PVTT); D: Kaplan Meier plot estimates of the PFS according to existence of PVTT.

cirrhosis because hepatic arterial blocking agents, such as lipiodol or gelatin sponges, are not used in HAIC^[18]. Thus, HAIC is not limited by tumor size, number, and/or proximity to major vasculature, all of which are common contraindications to resection and/or ablation. In addition, HAIC has several other advantages. For example, in most cases, there is no need for the administration of antiemetics or exogenous hydration, which can cause ascites or peripheral edema. Moreover, the higher first-pass hepatic extraction of infused drugs produces elevated local concentrations with lower systemic exposure, which results in fewer side effects than systemic chemotherapy^[10].

HAIC using 5-FU with cisplatin has been extensively studied as a treatment for advanced HCC^[19,19,20]. However, the pharmacokinetics of 5-FU are not linear over a hepatic extraction gradient of 19%-90%, and there are decreases in both systemic clearance and hepatic extraction at higher doses, which reduce the selective regional advantage^[21].

Intra-arterial FUDR, which is a metabolite of fluorouracil, is preferable because it is associated with an increased response rate due to its higher hepatic extraction (> 95%) and an intrahepatic concentration that is more than tenfold greater than that of 5-FU, cisplatin, mitomycin or doxorubicin^[11,12]. Therefore, FUDR is

associated with decreased toxicity and improved survival through maximal tumor cell death. Despite these advantages, regional chemotherapy using FUDR *via* an implantable pump has rarely been studied in HCC, although it has been studied extensively in patients with liver metastasis from colorectal cancers^[14,22-24]. Therefore, we hypothesized that the study of HAIC using FUDR in patients with advanced HCC could yield significant results.

In general, the prognosis is poor for patients with advanced HCC. Many studies have reported a median survival of 3-6 mo for unresectable and untreated HCC^[25]. Recently, a multinational phase III, randomized, double-blind, placebo controlled trial was conducted to assess the efficacy and safety of new therapeutic options in a group of Asian-Pacific patients with advanced HCC^[26]. The controlled trial reported a median OS and PFS of 4.2 mo and 1.4 mo, respectively, in the patients without treatment. Interestingly, most of the patients were classified as Child class A (Child class A: 220 and Child class B: 6). Many studies of HAIC have reported good results compared with those of untreated cases^[27,28]. In the present study, the response rate and the median survival time of the 34 patients who were treated with intra-arterial FUDR were 41.2% and 8.9 mo, respectively. Despite the inclusion of patients who were classified as Child class

C, the present outcomes are similar or better than those observed in other studies.

Studies have clearly shown that survival times are longer in patients with a good functional hepatic reserve. Many studies have shown that a patient's Child-Pugh status significantly influences survival, which is consistent with the present results^[29,30]. The OS times of the patients who were classified as Child class A, B and C were 15.3 mo, 12.4 mo and 4.3 mo, respectively. Similarly, the PFS times of the patients who were classified as Child class A, B and C were 12.9 mo, 7.7 mo and 2.6 mo, respectively. Most other studies excluded patients who were classified as Child class C because early reports demonstrated poor outcomes in these patients and no differences in survival between treated and untreated groups^[29]. The present study included patients who were classified as Child class C if they demonstrated a desire to receive therapy and had a relatively good performance status. The nine patients in the present study who were classified as Child class C had similar or slightly better outcomes compared with untreated patients. Interestingly, only 1 of the Child class C patients suffered from a serious adverse event (i.e., hepatic failure). Compared with other studies, the present study also showed relatively good results in the patients who were classified as Child class B.

Like the Child-Pugh classification, PVTT is a major independent factor in the determination of a poor prognosis in patients with advanced HCC^[30]. The median survival of untreated HCC with PVTT was reported to be 2.7 mo^[31]. One study reported a median survival time of 6 mo in patients with advanced HCC with PVTT (excluding Child class C patients) who received HAIC with 5-FU and cisplatin along with systemic chemotherapy^[32]. In the present study, the median OS in the groups with and without PVTT were 8.2 mo and 13.1 mo, respectively, and this difference was significant. In addition, the difference in the median OS times in the groups with and without PVTT also demonstrated the relatively good outcomes that were observed in the present study compared with those of other studies (despite the inclusion of Child class C patients with PVTT). The present study showed that the presence of PVTT, the extent of HCC, and hepatic function (as assessed by the Child classification) were major predictors of survival. Indeed, the present study demonstrated the significance of these factors using multivariate analysis.

Treatment tolerability and patient quality of life are also important when deciding therapies for advanced cancers. Only 1 of the 34 patients in the present study (2.9%) experienced progressive impaired hepatic function that justified the withdrawal of FUDR, and the majority of the patients demonstrated a relatively sustained quality of life during the HAIC.

Several studies have reported the outcomes of HAIC using FUDR in advanced HCC. One study investigated the efficacy of HAIC using FUDR for 5 patients with HCC and reported a response rate of 80% and a 1-year survival rate of 100%^[22]. In 2009, Jarnagin *et al.*^[33] report-

ed that HAIC with FUDR therapy could be effective and safe in patients with unresectable primary liver cancer. They reported a response rate of 25% and a tumor control rate of 62.5%. These two studies demonstrated positive results of HAIC with FUDR for HCC; however, they only included 5 and 8 patients, respectively. Compared with these two reports, the present study was significant in that it included more patients and demonstrated the efficacy and safety of HAIC with FUDR even in patients with advanced HCC.

Sorafenib was the first systemic agent to be approved for the treatment of advanced HCC. Both the Study of Heart and Renal Protection trial, which was conducted in Europe and North America, and an Asia-Pacific trial showed that sorafenib prolonged the time to progression by 1.4-2.7 mo and prolonged the OS by 2-3 mo^[26,34]. In the present study, the outcomes of HAIC with FUDR were superior to treatment with sorafenib. In addition, there are limitations to sorafenib therapy, namely its decreased efficacy over time (i.e., disease-stabilization for only a few months) and potential side effects. Moreover, the safety and efficacy of sorafenib in patients who are classified as having Child class B or C cirrhosis remain unclear. Many factors could eventually limit the potential advantages of anti-angiogenic sorafenib effects. Several mechanisms of resistance to vascular endothelial growth factor signaling-targeted therapy have been proposed, and other pre-existing or distinct oncogenic signaling pathways may begin to drive tumor growth during therapy^[35]. Recently, approaches to overcome resistance to anti-angiogenic sorafenib therapy in advanced HCC are being pursued. One of these approaches is the combination of anti-angiogenic therapy and metronomic chemotherapy to induce durable tumor shrinkage or disease stabilization in refractory cancer^[36,37]. Therefore, the combination of sorafenib and HAIC with FUDR may be a promising therapeutic approach for advanced HCC.

The present study has several limitations. For example, high doses of FUDR in HAIC can produce toxicity, which results in a fibrotic narrowing of the bile ducts that is similar to primary biliary sclerosis (up to 30% of patients)^[38]. The use of regional dexamethasone with FUDR, however, can reduce hepatic toxicity. In some studies, the biliary sclerosis rate was 3% or lower, and the patient response rate and survival also improved with the addition of dexamethasone^[39]. None of the patients in the present study had biliary sclerosis, which was likely due to the inclusion of dexamethasone in our treatment protocol.

Another limitation of the present study was that FUDR had to be administered for 14 d with continuous infusion, which might have been associated with poor patient compliance. Hepatic drug uptake and metabolic capacity can be saturated at high drug delivery rates^[40]. Therefore, continuous hepatic arterial infusion is regarded as the most effective means of delivery to maximize the regional advantage. In the present study, all but one patient continued therapy and showed relatively good compliance.

The final limitation of the present study was that we did not use a randomized design (i.e., HAIC was not compared with other therapeutic modalities). However, the present study was the first formal attempt to test HAIC using FUDR in patients with advanced HCC, including patients with advanced cirrhosis. Importantly, the present study demonstrated that survival was better in patients who received HAIC with FUDR compared with patients with unresectable tumors or in whom other therapeutic modalities had been used unsuccessfully.

In conclusion, the present study shows that intra-arterial FUDR chemotherapy is safe and effective for patients with severely advanced HCC confined to the liver for which other therapeutic modalities are ineffective, even in patients with advanced cirrhosis. Based on these results, additional large prospective randomized clinical trials should be performed to prove the efficacy and safety of HAIC using FUDR. Eventually, HAIC using FUDR could be widely applied for the treatment of advanced HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Despite recent advances in therapeutic modalities, the prognosis of advanced HCC remains poor. The current therapies have many limitations, and recurrence or metastasis is relatively frequent. Regional hepatic arterial infusion chemotherapy (HAIC) has been used in patients with advanced HCC. HAIC is believed to provide higher concentrations of chemotherapeutic agents directly to the HCC, which would minimize systemic concentrations of chemotherapeutic agents and potentially minimize systemic toxicity. However, previous studies of HAIC in HCC have shown various results.

Research frontiers

Floxuridine (FUDR) is an active metabolite of 5-fluorouracil (5-FU) that has the advantage of being rapidly metabolized (i.e., a 94%-99% extraction rate within the liver). Furthermore, FUDR is maintained at an exceptionally high intrahepatic concentration (i.e., more than tenfold greater than 5-FU, cisplatin, mitomycin or doxorubicin), which permits maximal tumor cell death while preventing systemic toxicity. In the present study, the authors demonstrated the efficacy and safety of HAIC using FUDR in patients with advanced HCC that was confined to the liver.

Innovations and breakthroughs

Most previous studies have reported the efficacy of HAIC using 5-FU and cisplatin in advanced HCC, whereas HAIC using FUDR in advanced HCC has rarely been reported. The present paper describes the results of HAIC using FUDR in 34 patients with advanced HCC.

Applications

HAIC with FUDR can be performed in patients with advanced HCC that is unresectable or has not responded to previous therapy.

Terminology

HAIC provides chemotherapeutic agents directly to the HCC *via* a pump catheter. The pump catheter is inserted at the proper hepatic artery from the superior mesenteric artery. The distal gastroduodenal artery, right gastric artery and small branches supplying the stomach or duodenum are ligated to prevent gastrointestinal toxicity.

Peer review

The importance of the present study is that HAIC with FUDR could be a promising therapeutic approach for patients with advanced HCC.

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Proteomic analysis of glutathione S-transferase isoforms in mouse liver mitochondria

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Abstract

AIM: To survey glutathione (GSH) S-transferase (GST) isoforms in mitochondria and to reveal the isoforms' biological significance in diabetic mice.

METHODS: The presence of GSTs in mouse liver mitochondria was systematically screened by two proteomic approaches, namely, GSH affinity chromatography/two dimensional electrophoresis (2DE/MALDI TOF/TOF MS) and SDS-PAGE/LC ESI MS/MS. The proteomic results were further confirmed by Western blotting using monoclonal antibodies against GSTs. To evaluate the liver mitochondrial GSTs quantitatively, calibration curves were generated by the loading amounts of individual recombinant GST protein vs the relative intensities elicited from the Western blotting. An extensive comparison of the liver mitochondrial GSTs was conducted between normal and db/db diabetic mice.

Student's *t* test was adopted for the estimation of regression and significant difference.

RESULTS: Using GSH affinity/2DE/MALDI TOF/TOF MS, three GSTs, namely, alpha3, mu1 and pi1, were identified; whereas five GSTs, alpha3, mu1, pi1, kappa1 and zeta1, were detected in mouse liver mitochondria using SDS-PAGE/LC ESI MS/MS, of these GSTs, GST kappa1 was reported as a specific mitochondrial GST. The R^2 values of regression ranged between values of about 0.86 and 0.98, which were acceptable for the quantification. Based on the measurement of the GST abundances in liver mitochondria of normal and diabetic mice, the four GSTs, alpha3, kappa1, mu1 and zeta1, were found to be almost comparable between the two sets of animals, whereas, lower GST pi1 was detected in the diabetic mice compared with normal ones, the signal of Western blotting in control and db/db diabetic mice liver mitochondria is 134.61 ± 53.84 vs 99.74 ± 46.2 , with $P < 0.05$.

CONCLUSION: Our results indicate that GSTs exist widely in mitochondria and its abundances of mitochondrial GSTs might be tissue-dependent and disease-related.

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Key words: Glutathione S-transferase; Mitochondria; Liver; Proteomics; Diabetes

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INTRODUCTION

The glutathione (GSH) S-transferase (GST, EC2.5.1.18) superfamily contains eight subclasses classified by their properties of sequence homology, immunology, substrate specificity and isoelectric point. GSTs catalyze the reactions between reduced GSH and unsaturated aldehydes, quinines, and many other substrates, especially under conditions of oxidative stress^[1-3]. These enzymes are involved in many physiological functions, such as the reduction of free radical damage, detoxification, metabolism, regulation of cell signaling and nitric oxide storage^[2-7]. The abundance of GSTs is closely related with the disease status of the organism^[8-12]. For instance, regarding a cancer biomarker, GST pi was found in higher abundance in several cancer cells and tissues and was believed to be involved in drug resistance^[13-15]. GST alpha has main functions in detoxification in the liver, and when hepatocytes are damaged, GST alpha enters the bloodstream. Therefore, GST alpha in blood and urine is an ideal marker indicating hepatocellular impairment^[16]. The accurate measurement of GSTs in tissues or body fluids is urgently required in biomedicine.

GSTs have been identified mainly in the cytoplasm, but they have also recently been detected in organelles, including the microsome, nucleus, mitochondria and peroxisomes^[17-20]. Mitochondria are the primary intracellular sites of oxygen consumption and reactive oxygen species (ROS) generation. GST kappa1 was identified in mitochondria and peroxisomes but is absent in the cytoplasm, and GST kappa was reported to participate in energetic and lipid metabolism in the mitochondria^[21]. In contrast to the cytoplasmic GSTs, the physiological functions of the mitochondrial GSTs have yet to be explored. Generally, it is accepted that GSTs are able to protect mitochondria from dysfunctions of catalase and superoxide dismutase by maintaining the redox balance^[22-24]. A high abundance of GST pi protected the decrease of the mitochondrial membrane potential induced by rotenone. Raza group observed that under oxidative stress, GST alpha was translocated from the cytoplasm into the mitochondria, but its functions in the mitochondria were not elucidated^[23]. To understand the functions of the mitochondrial GSTs fully, we must first define how many GST isoforms exist in the mitochondria. Although there were several reports regarding the distribution of GSTs in different tissue mitochondria, a systematic investigation in this field has not been undertaken.

Recently, proteomic approaches have provided a good opportunity to survey the members of a protein family, as they usually share similar properties, such as immunofluorescence, ligand binding sites, homology sequences and catalytic substrates. Using chromatography or electrophoresis based on these properties, protein family members could be separated from one another in a mixture. Moreover, mass spectrometry is able to identify the separated proteins. Such a proteomics strategy has been widely used in exploring protein isoforms^[25-27].

In this study, we propose a proteomic strategy to

define the GSTs in mouse tissue mitochondria. With combined separation based on size exclusion and affinity chromatography, we enriched the mitochondrial GSTs from a mitochondria preparation of mouse liver. Using MALDI TOF/TOF MS or ESI MS/MS, five GSTs were identified in the mitochondria. The presence of GSTs was further verified by Western blot using monoclonal antibodies. We constructed calibration curves of the GST quantification and employed a quantitative assay of the immunoblots to estimate the different abundances of the mitochondrial GSTs between normal and diabetic mice. For the first time, we found that GSTs are widely distributed in the mitochondria of many tissues and that mitochondrial GST pi1 is sensitive to the development of diabetes.

MATERIALS AND METHODS

Preparation of mitochondria from mouse tissue

The C57BLKS/J db/db and control mice were provided by Peking University Diabetes Center. The heart, kidney and liver tissues of the mice were minced and homogenized in buffer (25 mmol sucrose, 0.5% protease inhibitor cocktail and 10 mmol HEPES, pH 7.4). The crude mitochondria were prepared by differential centrifugation at $1000 \times g$ for 30 min and at $10\,000 \times g$ for 20 min at 4 °C. The purified mitochondria were extracted from a Nycodenz gradient at the interface of 25%-30% Nycodenz solution after centrifugation at $52\,000 \times g$ for 90 min. The purity and integrity of the mitochondria were determined by Western blotting and transmission electron microscopy (TEM). Mitochondrial proteins were extracted using lysis buffer [7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 40 mmol/L Tris-HCl (pH 7.4) and protease inhibitor cocktail].

The animal experiments described in this article were approved by the Animal Care and Welfare Committee at the Beijing Institute of Genomics, Chinese Academy of Sciences.

GSH-affinity chromatography

We purified the GSTs using GSH-affinity chromatography with GSH-Sepharose 4B (Amersham Biosciences, United States). The GSH-Sepharose 4B was equilibrated with binding buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 1 mmol/L ethylene glycol tetraacetic acid, and 0.1% Triton \times 100]. The mitochondria were re-suspended in 500 μ L binding buffer and were sonicated. After centrifugation, the supernatant was mixed with the equilibrated resin and centrifuged for 30 min 3000 r/min at 4 °C. The affinity resin was washed 3 times with binding buffer, and the proteins were eluted from the resin using 30 mmol/L reduced GSH. A sample of the elution products was retained for two-dimensional electrophoresis (2-DE) separation.

2-DE

The first dimension separation was conducted using an

Ettan IPGphor IEF system with 7 cm (pH 6-11) IPG strips at 20 °C. The proteins isolated by GSH-affinity chromatography were loaded onto strips, and the strips were rehydrated without voltage for 4 h and with 50 V for 8 h. The isoelectric focusing was programmed for 1 h at 500, 1000 and 4000 V, respectively, and was subsequently focused at 4000 V up to a total of 30 kVh. The focused strips were equilibrated in buffer with 6 mol/L urea, 50 mmol/L Tris-HCl, 30% glycerol, 2% SDS and trace bromophenol blue and were subsequently reduced by dithiothreitol and alkylated by iodoacetamide. The treated strips were inserted into a 15% SDS-PAGE gel running in 2.5 W (each gel) for 30 min and 15 W (each gel) thereafter until the bromophenol blue dye reached the bottom of the gels. The gels were stained by silver staining.

Mass spectrometry for protein identification

The proteins were identified by two mass spectrometry methods: MALDI TOF/TOF and LC ESI MS/MS.

The proteins that were separated by GSH-affinity chromatography and 2D gel electrophoresis were excised and in-gel digested with trypsin overnight and identified by MALDI TOF/TOF MS. Briefly, the tryptic digests were co-crystallized with a matrix of α -cyn-4-hydroxycinnamic acid spotted onto the AnchorChip and desalted by 0.1% trifluoroacetic acid. The AnchorChip was analyzed using an Ultraflex TOF/TOF MS mass spectrometer (Bruker Dalton, Bremen, Germany) for protein identification. Positively charged ions were analyzed in the reflector mode. Typically, 100 shots were cumulated per spectrum in the MS mode and 400 shots in the MS/MS mode. The mass spectra and tandem mass spectra obtained were processed using the FlexAnalysis 2.2 and BioTools 2.2 software tools. The protein identification was performed using the Mascot software (<http://www.matrixscience.com>), and the NCBI database was searched using mouse as the taxonomy. The following parameters were used for the database searches: one incomplete cleavage, alkylation of cysteine by carbamidomethylation, oxidation of methionine, and pyro-Glu formation of the N-terminal Gln.

The 20-30 kDa proteins separated by SDS-PAGE were a mixture of many proteins, and the proteins were examined by LC ESI MS/MS after the in-gel trypsin digestion. Briefly, after capillary reversed-phase high-performance liquid chromatography, the separated peptides were analyzed using an ion-trap mass spectrometer LCQ DecaXP ion-trap mass spectrometer (Thermo Finnigan, Ringoes, NJ) with 3.2 kV of spray voltage and 150 °C at the heated desolvation capillary. A mass-to-charge (m/z) range from 400 to 2000 was scanned over 1.2 s, and the ions were detected with a high energy conversion dynode detector. The MS/MS data were converted into DTA-format files, which were further searched for proteins with Sequest software. All of the accepted results had a deltaCn of 0.1 or greater. Furthermore, a singly charged peptide must be tryptic, and the cross-correlation score (Xcorr) had to be more than 1.9. The tryptic peptides with a charge state of

+ 2 must have a Xcorr of more than 2.2. Triply charged tryptic peptides were accepted if the Xcorr was ≥ 3.0 .

Generation of monoclonal antibodies

To generate the specific antibodies against these five GST isoforms, we cloned these five *GST* genes and expressed the recombinant protein by inserting these genes into the prokaryotic expression vector, named pET-28a. The recombinant proteins expressed from the pET-GST plasmids were 6 \times His-tagged. After expression in *E. coli* BL21 (DE3), the proteins were purified using Ni-NTA agarose resin (Qiagen, United States) and metal chelate chromatography.

Six-weeks-old female BALB/c mice were immunized subcutaneously with the recombinant protein in Freund's complete adjuvant. After booster injections, the mice with positive serum immunogenicity against the recombinant protein were used for the monoclonal fusion experiments. The mice's spleens were excised, and single-spleen cell suspensions were fused with Sp2/0 myeloma cells. After several days of fusion, the hybridomas were picked and cultured in complete medium supplemented with 1% HT. Ascitic fluids were collected from the mice after an intraperitoneal injection of the hybridomas, and the antibodies were purified by protein A/G-Sepharose affinity chromatography. The protein concentrations of the purified antibodies were determined using the Bradford method, and the antibodies were diluted to 1 mg/mL and stored in 50% glycerin at -20 °C.

Western blotting

Proteins were separated by 15% SDS-PAGE and were electro-transferred onto polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were blocked with 5% non-fat milk dissolved in Tris-buffered saline with 0.05% Tween-20 (TTBS) at 37 °C for 90 min. The membranes were incubated with the primary antibodies at a dilution of 1:5000 in blocking reagent at room temperature for 2 h. The antibodies against the GSTs were generated by our laboratory, and the anti-ATP synthase β antibody was purchased from BD Biosciences. The membranes were incubated in goat anti-mouse/rabbit IgG conjugated with horseradish peroxidase at a 1:3000 dilution at room temperature for 1 h. The membranes were developed using the Super ECL Plus Detection Reagent kit, and the images were captured using ImageQuant ECL (GE Healthcare, United Kingdom).

Statistical analysis

To quantify the GSTs, calibration curves were constructed using the protein concentrations and immunosignal obtained from the Western blotting. To analyze the quantities of GSTs in the diabetes mouse model statistically, the relative intensity of the Western blotting signals in the samples was quantified using ImageQuant TL software. The three pairs of samples from the control and db/db mice were then randomly paired, and the relative abundance ratios of the five GST proteins were

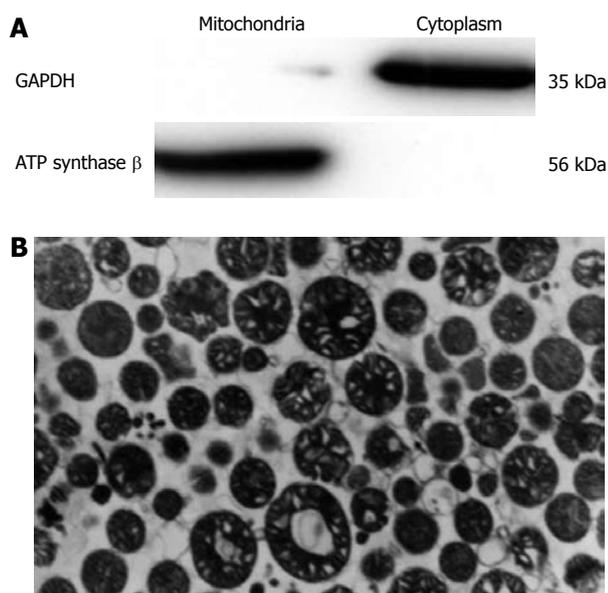


Figure 1 Measurement of mitochondrial purity by Western blotting and transmission electron microscopy. A: Glyceraldehyde-3-phosphate dehydrogenase is an indicator of cytoplasm and ATP synthase an indicator of mitochondria; B: Image of products purified by Nycodenz gradient centrifugation.

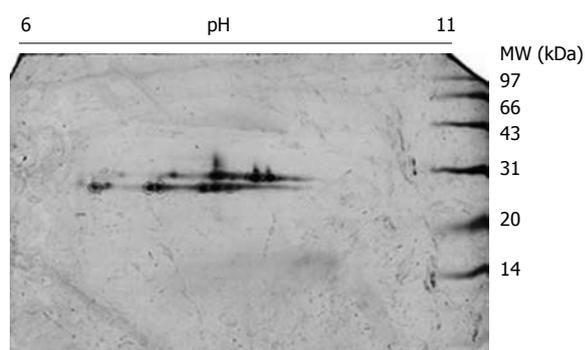


Figure 2 Two dimensional electrophoresis image of proteins purified by glutathione-affinity chromatography. The mitochondrial proteins enriched by glyceraldehyde affinity column were separated in two dimensional electrophoresis with pH 6-11 strips and 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The string spots around molecular weight (MW) 28 kDa appeared on the gels labeled by circles were identified.

statistically analyzed and were normalized using the levels detected in normal mice based on two parallel experiments. All of the values are expressed as the mean \pm SD, and the statistical significance was set to a $P < 0.05$.

RESULTS

Screening of GST isoforms in liver mitochondria

Considering that the purity of the mitochondria is a key element in studying mitochondrial components, Nycodenz gradient centrifugation was employed for the preparation of the mouse liver mitochondria. The mitochondrial integrity and purity were examined by Western blot and TEM. Our data as shown in Figure 1 indicated that the purity and integrity of the mitochondria were satisfactory for further analysis.

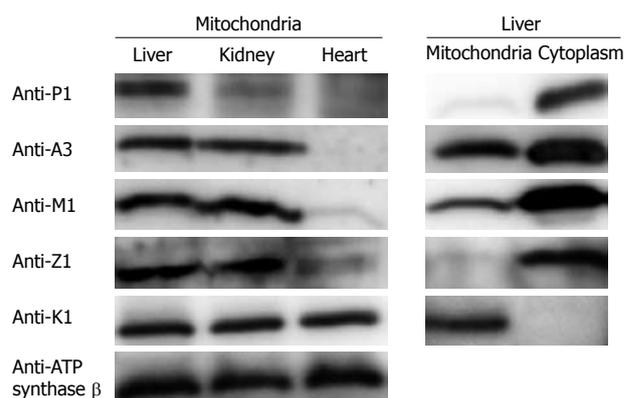


Figure 3 The distribution of glutathione S-transferases in mouse tissue mitochondria, as measured by Western blotting. A total of 20 μ g mitochondrial and cytoplasmic protein from mouse heart, kidney, and liver were loaded in each lane. Left: Glutathione S-transferases (GSTs) in mouse tissue mitochondria; Right: GSTs in liver mitochondria and cytoplasm.

The mitochondrial lysates were loaded onto GSH affinity columns, and the fractions eluted by GSH were collected and subjected to 2DE using pH 6-11 strips and 15% SDS-PAGE. After silver staining, a string spots of approximately 28 kDa and pI 7-10 appeared on the gels (Figure 2), whereas no spots were detected in other parts of the gel, indicating that the affinity enrichment was effective to remove non-GSH-binding proteins. The silver-stained spots were excised and digested with trypsin followed by protein identification using MALDI TOF/TOF MS. In total, three GSTs, GST alpha3, GST mu1 and GST pi1, were found in the liver mitochondria. Because all of the GSTs have similar MWs (about 28 kDa), we attempted to separate the mitochondrial GSTs from the other mitochondrial proteins through size-exclusion methods. The mitochondrial lysates were loaded onto a 15% SDS-PAGE gel, and the portion corresponding to 20-30 kDa was sliced into 19 fractions. These fractions were digested with trypsin, and the digested fractions were further analyzed using LC ESI MS/MS. All of the unique peptide sequences of GSTs are listed in Table 1, indicating five GSTs: GST pi1, GST alpha3, GST mu1, GST kappa1 and GST zeta1. All of the GSTs identified by GSH/2DE/MALDI TOF/TOF MS are included in the list of the identified GSTs by SDS-PAGE/LC ESI MS/MS; therefore, the SDS-PAGE/LC ESI MS/MS approach exhibits a better sensitivity for the detection of GSTs.

The existence of GSTs in the liver mitochondria was further confirmed by Western blotting using monoclonal antibodies against the individual GSTs (the specificity of detection is described below). As depicted in Figure 3, the five GST isoforms detected by the proteomic methods showed positive immunoreactivity; this evidence strongly supports the conclusion reached with the proteomic analysis. Furthermore, we prepared the tissue mitochondria from mouse kidney and heart and conducted Western blotting to evaluate the abundance of the GSTs in the mitochondria from different tissues. The left panel in Figure 3 reveals the wide distribution of GSTs in

Table 1 The unique peptides of glutathione S-transferases identified by LC ESI MS/MS

GST	Peptide sequence	MH +	Location	XC	DeltaCn	Ions
Mu1	MALLEYTDSSYDEK	1593.699	19-31	4.062	0.47	20 24
	ADIVENQVMDTR	1390.664	97-108	4.1055	0.5208	17 22
	MLQIMLCYNPDFEK	1801.811	109-122	4.8593	0.5987	20 26
Zeta1	GIDYEIVPINLIK	1486.852	28-40	3.0229	0.3943	15 24
	VITSGFNALEK	1178.642	134-144	2.6217	0.4564	16 20
Kappa1	FLTTVSMEQPEMLEK	1782.866	102-116	4.944	0.5723	22 36
	AGMSTAQAQHFLEK	1518.737	145-158	2.4224	0.2908	13 16
Alpha3	SDGSLMFQQVPMVEIDGMK	2111.982	46-64	3.9127	0.5378	21 28
	AILNYIASK	992.578	70-78	3.0611	0.3416	17 26
	YFPAFEK	901.446	132-138	2.5832	0.3767	8 12
Pi1	EAAQMDMVNDGVEDLR	1792.785	86-101	3.9673	0.5391	22 30
	YVTLIYTNENYNGK	1577.785	104-116	3.6743	0.4268	18 24

MH+ stands for the mass of ions detected by LC ESI MS/MS. XC stands for the cross-correlation score. GST: Glutathione S-transferase.

the mitochondria from the three tissues with relatively different abundances. As a specific GST only located in mitochondria, GST kappa1 exists in all three tissues with similar abundances. GST pi1 is clearly detected in the mitochondria of all three of the tissues, whereas GST pi1 in the liver mitochondria is in the highest abundance. The three GSTs, GST alpha1, GST mu1, and GST zeta1, are clearly observed in the liver and kidney mitochondria, whereas they are almost undetected in the heart. Furthermore, we examined the cytoplasmic GSTs and the mitochondrial GSTs in the mouse liver using Western blotting. The data illustrated in the right panel in Figure 3 indicate that GST kappa1 is the only isoform undetectable in the cytoplasm, whereas the other GST isoforms are readily detected in the two subcellular fractions. When equal amounts of proteins, either cytoplasmic or mitochondrial, are loaded onto an SDS-PAGE gel, the analysis of the relative intensities demonstrate that the abundances of GSTs in the cytoplasm are commonly higher than those in the mitochondria.

Quantitative analysis of the mitochondrial GST using Western blotting

The five GSTs in liver mitochondria were first discovered by proteomic approach. As a routine assay in laboratory, mass spectrometry is not a common instrument and easy in use. Western blotting is still a widely acceptable and enough sensitive approach to detect proteins. We therefore developed a quantitative assay for the mitochondria GSTs based on immuno-blot. It is well known that specificity and sensitivity of antibody are a key factor for a successful Western blotting. To have the qualified antibodies against GSTs, we generated a set of monoclonal antibodies of GSTs, and performed a strict screening. A qualified antibody should match to criteria: (1) It can recognize 2 ng of the correspondent recombinant protein; and (2) It cannot pick up any signals to 20 ng of unrelated GSTs. The qualified GST antibodies are illustrated in the upper panel of Figure 4.

Selection of the concentration ranges for the recombinant GSTs is an important consideration for quantitative calibration. In our routine experiments, the

maximum loading of mitochondrial proteins are approximately 20 μ g; therefore, we loaded 20 μ g of proteins and different amounts of recombinant GSTs on a relatively large scale onto the same gels and performed the Western blotting analysis. After determining the band intensities, we were able to estimate the proper amounts of the recombinant GSTs for the generation of calibration curves. As shown in Figure 4, five calibration curves are generated corresponding to the multiple assays of our Western blotting. However, Western blotting has a disadvantage in quantitative measurements because the reproducibility of the band intensities is relatively poor. The data shown in Figure 4 is in agreement with the observation, although some spots indeed display a large deviation. However, the regression calculation revealed that the values of R^2 ranged from 0.85-0.98, which are generally accepted in quantitative measurements using Western blot. We further estimated the possible dynamics of the GST abundances in the liver mitochondria. With the exception of GST pi1, measuring approximately 50 ng to 300 ng, the other four GSTs were between 10 ng to 40 ng, indicating that GST pi has the highest abundance in liver mitochondria. According to the regression curves in Figure 4, the mitochondrial GST abundances were quantitatively estimated as follows: $134.61 + 53.84$, $38.83 + 2.33$, $29.25 + 0.29$, $27.28 + 0.27$, $15.84 + 0.16$ ng per μ g mitochondria protein for GST pi1, mu1, alpha3, kappa1, and zeta1, respectively.

Comparison of the GSTs' abundances in liver mitochondria between normal and diabetes mice

The db/db mouse is a well-accepted type II diabetes model, and previous studies have shown that free radicals were increased in most of the tissues in this model. After carefully examining the mice's blood glucose and body weight to ensure diabetes development, we collected the livers from three normal and three db/db mice; the prepared mitochondria were analyzed by Western blot to quantify the contents of the GSTs. Figure 5A shows such a typical Western blotting, suggesting that the immunoreactivity of GST pi1 is significantly attenuated in the mitochondria of the diabetic mice, the signal of Western

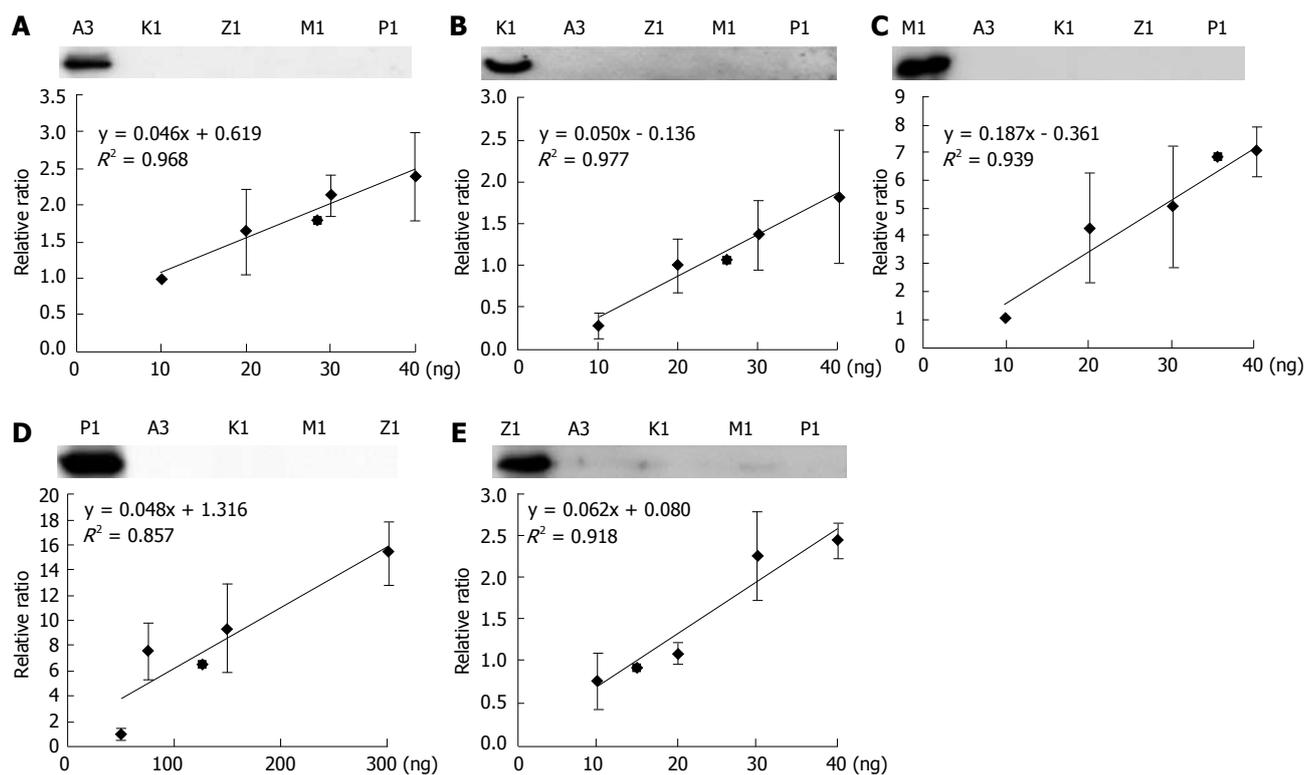


Figure 4 The calibration curves of mitochondrial glutathione S-transferase proteins. Upper panel in A, B, C, D, E: Examination of the specificity of the five glutathione S-transferase (GST) antibodies, 2 ng of the corresponding GST was loaded in all of the left lanes, and 20 ng of the other unrelated GSTs was loaded in the other four lanes; Lower panel in A, B, C, D, E: The X-axis represents the amounts of recombinant GSTs loaded (ng), and the y-axis represents the relative intensity of the Western blotting, taking the signals generated from the lowest GST amount as the reference. The calibration curves were generated by regression. Insert: The linear equations and R^2 were generated by regression.

blotting in control and db/db diabetic mice liver mitochondria is 134.61 ± 53.84 vs 99.74 ± 46.2 , with $P < 0.05$, whereas the other four GST isoforms are nearly comparable between the normal and diabetic mice. Statistically, we performed pairwise comparisons and estimations of the significant differences based on multiple Western blotting. The comparison data depicted in Figure 5B support the conclusion drawn from Figure 5A that the abundance of mitochondrial GST pi1 in diabetic mice is lower than in normal mice, whereas the other mitochondrial GSTs did not exhibit significant changes.

We screened the response of the GSTs to diabetes in the db/db liver mitochondria. As depicted in Figure 5A, which shows representative images of mitochondrial tissue from one pair of mice (control vs db/db), GST pi1 showed a decrease in the db/db mouse when we compared three pairs of mice. In contrast, similar expression levels of GST alpha3, mu1, and zeta1 were observed in the liver mitochondria of the db/db mice and control mice. The statistical analysis indicated in Figure 5B supported these observations.

DISCUSSION

GSTs have been detected as a group of oxidative stress proteins in mitochondria, and they were characterized as associated with the maintenance of mitochondria functions^[22,28]. For example, alpha-class isozymes of

GST translocated into the mitochondria under oxidative stress, and the isozymes showed glutathione peroxidase activity toward phospholipid hydroperoxide in the rat liver cytosol. Chemicals that generate reactive oxygen species, such as rotenone and antimycin A, reduced the cell viability and mitochondrial membrane potential, and the overexpression of GST pi1 diminished these changes.

In general, GSTs were determined by their enzymatic activities and immunological features; however, these methods had limitations in evaluating the presence of GST. The substrate specificity of purified rat liver GSTs has been investigated by a series of gamma-glutamyl-modified GSH analogues, and GST had a different ability to conjugate with the substrates. Furthermore, a product of the purification using GSH could not provide the status of GST *in vivo*. Therefore, the uncertainty of the type of GSTs localized in the mitochondria and the antibody specificity led to the uncertainty of an increase or decrease of GSTs under oxidative stress or in a pathological condition.

In this study, we have taken multiple approaches to identify the GSTs in mouse liver mitochondria. The mitochondrial proteins were separated by SDS-PAGE, and the 20-30 kDa proteins were analyzed by liquid chromatography and subsequently identified by ESI MS/MS. Five GST isoforms were detected in the liver mitochondria. Our strategy has two advantages: more proteins could be resolved regardless of the solubility by SDS-

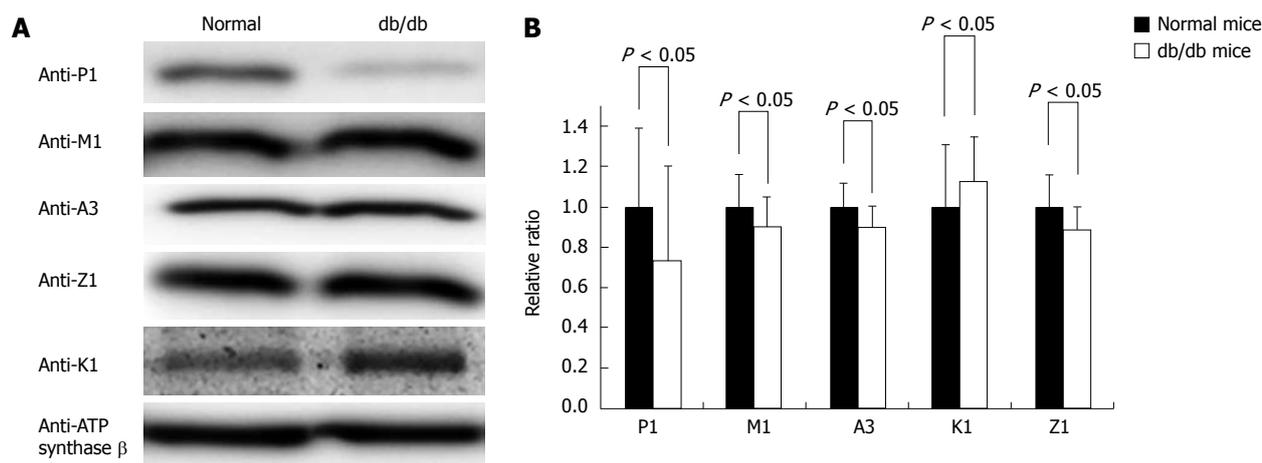


Figure 5 Comparison of mitochondrial glutathione S-transferase abundances between normal and diabetic mice by Western blotting. A: A typical image of a Western blotting comparing a pair of mouse liver mitochondria from normal and diabetic mice. Approximately 20 μ g mitochondrial proteins were loaded in each lane. Anti-ATP synthase β acted as a control to normalize the loading; B: The statistical comparison of the relative ratio of mitochondrial glutathione S-transferases (GSTs) between normal and diabetic mouse liver when normalized by the GSTs in normal mice ($n = 3$).

PAGE, and the sample complexity was reduced when the proteins distributed in the 20-30 kDa range were divided into 19 fractions for digestion and separation by liquid chromatography. Although we could not dismiss the possibility of other GSTs in the mitochondria, our multiple proteomic techniques provided new insights into analyzing GST isoforms, especially for those of low abundance.

GSH is not generated in mitochondria and is instead transferred from the cytoplasm; a GSH concentration of 5-10 mmol is maintained in mitochondria^[29]. GSH is a major defense molecule against ROS, and mitochondrial GST may play an important role in responding to chemical and oxidative stress because GST is the main phase II enzyme that catalyzes the conjugation of GSH with numerous reactive electrophiles. The identification of GSTs would influence the study of the functions of mitochondrial GST.

We discovered GST zeta1 in the mitochondria. GST zeta1 is localized in the cytoplasm and plays a significant role in the catabolism of phenylalanine and tyrosine^[5]. When GST zeta1 is lacking in mice, the inductions of the class alpha, mu, and pi GSTs could be detected by Western blotting and high-performance liquid chromatography analysis of glutathione affinity-purified proteins^[50]. The role of GST zeta1 in the mitochondria will be investigated further.

The expression of GSTs is different in distinct tissues. Our data indicate that mitochondrial GST also has tissue-specific expression. GSTs are abundant in the liver but are scarce in heart tissue. The abundance of GST kappa1 in the heart, kidney, and liver is similar, but this GST is only detected in the mitochondria, indicating that it might perform a conserved function in the mitochondria of different cell types.

We screened these mitochondrial GSTs in the diabetes mouse model and found that the GST response to

oxidative stress varies: only GST pi1 is decreased in the db/db mouse compared to normal mice, and the levels of the other four isoforms did not change. This result indicated that a decrease of GST pi1 might accelerate the pathological progress of diabetes.

COMMENTS

Background

The glutathione (GSH) S-transferase (GST) involve in many physiological functions. They are located mainly in the cytoplasm, but they have also been detected in suborganelles. Because mitochondria are the primary intracellular sites of oxygen consumption and reactive oxygen species generation, the mitochondrial GSTs abundances may relate with disease status. The goal was to survey GST isoforms in mitochondria and to reveal their biological significance in diabetic mice.

Research frontiers

It is reported that GSTs are able to protect mitochondria from oxidation by maintaining the redox balance, but their functions in the mitochondria were not elucidated. To fully understand the functions of the mitochondrial GSTs, new method to monitor the mitochondrial GSTs is urgently required.

Innovations and breakthroughs

The mitochondrial GSTs are measured by the combined quantitative proteomic strategies based on mass spectrum and antibodies. The authors discovered that GSTs are widely distributed in tissue mitochondria and their responses to the diabetes physiology. It is noted the mitochondrial GST pi1 is sensitive to diabetic development.

Applications

This research showed how the proteomics produces meaningful information and offered a relatively easy and simple workflow to detect GSTs in liver mitochondria and to reveal their biological significance of normal and diabetes mouse. At the same time, the application of this strategy provides an alternative tool to analyze how isoforms of protein family response to disease in complex biological systems.

Terminology

Proteomics is the protein map of a biological system and involves the systematic study of proteins in order to provide a comprehensive view of the function and regulation of proteins.

Peer review

This paper provides solid data on systematic analysis of GST isoforms in mouse liver mitochondria.

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Anticoagulation therapy prevents portal-splenic vein thrombosis after splenectomy with gastroesophageal devascularization

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Abstract

AIM: To compare the incidence of early portal or splenic vein thrombosis (PSVT) in patients treated with irregular and regular anticoagulation after splenectomy with gastroesophageal devascularization.

METHODS: We retrospectively analyzed 301 patients who underwent splenectomy with gastroesophageal devascularization for portal hypertension due to cirrhosis between April 2004 and July 2010. Patients were categorized into group A with irregular anticoagulation and group B with regular anticoagulation, respectively. Group A (153 patients) received anticoagulant monotherapy for an undesignated time period or with aspirin or warfarin without low-molecular-weight heparin (LMWH) irregularly. Group B (148 patients) received subcutaneous injection of LMWH routinely within the

first 5 d after surgery, followed by oral warfarin and aspirin for one month regularly. The target prothrombin time/international normalized ratio (PT/INR) was 1.25-1.50. Platelet and PT/INR were monitored. Color Doppler imaging was performed to monitor PSVT as well as the effectiveness of thrombolytic therapy.

RESULTS: The patients' data were collected and analyzed retrospectively. Among the patients, 94 developed early postoperative mural PSVT, including 63 patients in group A (63/153, 41.17%) and 31 patients in group B (31/148, 20.94%). There were 50 (32.67%) patients in group A and 27 (18.24%) in group B with mural PSVT in the main trunk of portal vein. After the administration of thrombolytic, anticoagulant and anti-aggregation therapy, complete or partial thrombus dissolution achieved in 50 (79.37%) in group A and 26 (83.87%) in group B.

CONCLUSION: Regular anticoagulation therapy can reduce the incidence of PSVT in patients who undergo splenectomy with gastroesophageal devascularization, and regular anticoagulant therapy is safer and more effective than irregular anticoagulant therapy. Early and timely thrombolytic therapy is imperative and feasible for the prevention of PSVT.

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Key words: Portal vein hypertension; Splenectomy with gastroesophageal devascularization; Portal or splenic vein thrombosis; Anticoagulation regimen; Thrombolytic therapy

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INTRODUCTION

Although endoscopic surgery has been widely used to treat esophagogastric variceal bleeding (EGVB), splenectomy with gastroesophageal devascularization is still the primary method to treat and prevent recurrent EGVB in East Asia^[1,2]. Portal or splenic vein thrombosis (PSVT) is a common and potentially life-threatening complication of splenectomy with gastroesophageal devascularization for portal hypertension due to cirrhosis. Severe PSVT can reduce hepatopetal blood flow in the portal system and elevate the blood pressure in the visceral side of portal vein, leading to further deterioration of liver function and the recurrence of upper gastrointestinal (GI) bleeding^[3,4]. In serious cases, PSVT can significantly affect patient's life expectancy.

A prospective study with contrast-enhanced computed tomography scan showed that PSVT occurred in 12 (55%) patients of the laparoscopic splenectomy (LS) group, but in only 4 (19%) of the open splenectomy (OS) group, and the difference was significant^[5]. A recent clinical study performed by the same group of authors has emphasized that the incidence of PSVT still reached 51.52% in 33 cases after LS without any anticoagulation therapy^[6]. The different incidences of PSVT between laparoscopic and open splenectomy may be caused by the operative technique of pneumoperitoneum and ligation of splenic hilar vessels. In the OS group, splenic hilar vessels were ligated conventionally, while these vessels were divided with an endoscopic vascular stapler in the LS group^[5]. But in some other studies, the incidence of PSVT varied after splenectomy with gastroesophageal devascularization^[7-9].

The literatures mentioned above focused on splenectomy to treat hematologic and metabolic disorders. The reported incidence of PVST related to these diseases may be different from that related to liver cirrhosis because of the different disease spectrum. Kawanaka *et al*^[10] analyzed 50 consecutive cirrhotic patients who underwent splenectomy, the PVST incidence was 36.0% (9/25) up to postoperative day (POD) 7 without any prophylactic anticoagulation therapy, the PVST incidence of the other 25 patients was only 4.0% (1/25) up to POD 30 who received antithrombin III (AT-III) therapy in the first three POD. Ushitora *et al*^[11] retrospectively examined 38 consecutive cirrhotic patients who underwent splenectomy, the total incidence of PSVT detected by postoperative dynamic computed tomography was 34.2% (13/38) without any prophylactic anticoagulation therapy. Deng *et al*^[12] reported a 33.69% incidence of PVT 7 to 14 d after portal hypertension surgery (splenectomy with gastroesophageal devascularization) in 52 surgically treated patients with portal hypertension due

to hepatitis B virus (HBV)-related cirrhosis. The 10-year survival rate among adults with PSVT was 38%-60%, the mortality rate from variceal bleeding in patients with PSVT with cirrhosis was 30%-70%, significantly higher than 5% from variceal bleeding in patients with PSVT without cirrhosis^[13].

Therefore, PSVT is indeed a common complication of splenectomy with gastroesophageal devascularization with a high incidence and morbidity even though with different disease spectrum. PSVT was considered contraindicated for liver transplantation in the past because of technical difficulties^[14] and currently it still remains as a risk factor^[15]. It makes the surgical procedure more cumbersome, resulting in a higher morbidity and mortality in the PSVT patients^[16,17]. Englesbe *et al*^[18] found that mortality of patients with previous portal vein thrombosis (PVT) after liver transplantation was higher than that of patients without PVT (at 30 d 17.7% *vs* 4.4%, $P = 0.07$; at 1 year 33.0% *vs* 25.0%, $P = 0.354$; and at follow-up 36.7% *vs* 28.4%, $P = 0.371$), even these differences were not statistically significant. And patients with cirrhosis complicated with PVT have significantly increased risks of death after liver transplantation (hazard ratio 7.389, 95% CI: 2.392-22.827).

So, it is very important to prevent the occurrence of PSVT after splenectomy with gastroesophageal devascularization for the better long-term clinical outcomes and following possible liver transplantation. Prophylactic anticoagulation therapy is a prime method to prevent PSVT after splenectomy with gastroesophageal devascularization. The most frequently used drugs are low molecular weight heparin (LMWH), vitamin K antagonist such as warfarin^[5], and aspirin^[19-21]. But the coarse and dosage of these drugs were variable according to different literatures, there was no generally acceptable PSVT prophylactic regimen for all patients after splenectomy with gastroesophageal devascularization^[22,23].

We studied retrospectively the efficacy of regular anticoagulant therapy on preventing early postoperative PSVT in the cirrhotic patients who received splenectomy with gastroesophageal devascularization in Beijing You-An Hospital of Capital Medical University from December 2004 to July 2010.

MATERIALS AND METHODS

Inclusion and exclusion criteria

Inclusion criteria: Patients with liver cirrhosis due to any causes; liver function grade: Child-Pugh A-B; splenomegaly and hypersplenism; severe esophageal varicose confirmed by gastroscopy; and previous histories of recurrent upper gastrointestinal bleeding. All patients signed the informed consent and the study was approved by the Ethics Committee of the hospital. These patients' data within one month after splenectomy with gastroesophageal devascularization were collected and analyzed.

Exclusion criteria: Patients who did not fulfill the inclusion criteria and could not tolerate surgical treatment were excluded.

Clinical examinations

After fasting for 8 h, all patients lied in the supine or left lateral position, and color Doppler ultrasound examination was performed with ACUSON Sequola 512 SIE-MENS Ultrasound system with a probe frequency of 3.5 MHz. The inner diameter of the portal vein and the splenic vein were measured in the sagittal position. Platelets/international normalized ratio (PLT/INR) was also measured before surgery and at day 1, day 3, day 7 and 1 mo after surgery.

Surgical procedure and portal venous pressure measurement

Selective gastroesophageal devascularization was performed using intraoperative free portal venous pressure (FPVP) as guidance^[24]. A 20G antithrombotic catheter (BD Insyte, Becton Dickinson Medical Devices Co, Ltd. Suzhou, China) was inserted into the right gastro-omental vein to test FPVP after laparotomy and splenectomy, respectively. Gastroesophageal devascularization was performed according to the FPVP.

Treatment and grouping

The patients were classified into two groups according to whether regular anticoagulation was administered. In group A, the patients received irregular anticoagulant aspirin or warfarin monotherapy for an undesignated time period without LMWH due to poor blood clotting after surgery and perioperative abdominal or GI bleeding.

Patients in group B had nearly normal blood clotting before and after surgery; 24 h after surgery, they received subcutaneous injection of LMWH routinely, 0.3 mL per 12 h for 5 d and then maintained by oral therapy with warfarin for one month to keep the target prothrombin time/international normalized ratio (PT/INR) at a level between 1.25 and 1.5. If the postoperative platelet level was increased to $100 \times 10^9/L$ or above, aspirin 100 mg daily was added for one month. If the postoperative platelet level was increased to $300 \times 10^9/L$ or above, ticlopidine was added with the dose of 0.25 g daily for one month.

Color Doppler ultrasound examination and PLT/INR measurement were repeated. Once PSVT was confirmed after splenectomy with gastroesophageal devascularization, the patients would receive a thrombolytic therapy. Urokinase was administered *via* the peripheral venous route with a bonus dose of 200 000 units within 30 min, followed by continuous infusion of 20 000-50 000 units/h for 3-5 d *via* a micro-infusion pump. During the thrombolytic treatment, PT/INR and PLT were measured daily. Following the thrombolytic treatment, the patients were administered with oral warfarin (2.5 mg, 1-2 times daily) and aspirin (100 mg daily). The drug doses were adjusted according to PT/INR and PLT levels. If repeated color

Doppler ultrasound examinations showed complete or partial dissolution of target thrombus, the treatment was considered effective and switched to oral warfarin monotherapy (2.5 mg, 1-2 times daily) for one month. If repeated color Doppler ultrasound examinations showed little change or even enlargement of the target thrombus, the thrombolytic therapy was defined as ineffective. The patients would continue to receive oral warfarin and aspirin and followed up regularly.

Statistical analysis

SPSS version 11.5 software (SPSS Inc., Chicago, IL, United States) was used for statistical analyses. Continuous data were presented as mean \pm SD and compared with two-tailed nonpaired Student's *t* test. Categorical data were analyzed with Chi-square or Fisher exact test. Chronologic changes in the laboratory data were analyzed by the analysis of variance for repeated measures. $P < 0.05$ was considered statistically significant.

RESULTS

A total of 301 cirrhotic patients undergoing splenectomy with gastroesophageal devascularization in our department was included in the study, including 202 males and 99 females with an average age of 45.96 years (range, 14-77 years). They were divided into two groups: Group A, 153 patients, including 103 men and 50 women with a mean age of 46.14 ± 10.39 years; and Group B, 148 patients, including 99 men and 49 women with a mean age of 46.47 ± 9.58 years. There were 254 cases of HBV related cirrhosis, 19 cases of hepatitis C virus related cirrhosis, 22 cases of alcoholic cirrhosis, 3 cases of idiopathic portal hypertension and 3 cases of primary biliary cirrhosis.

As for liver function grade, Child-Pugh A was found in 246 patients and Child-Pugh B in 55 patients. Of the 301 patients, 236 patients (78.40%) had a history of upper GI bleeding before surgery. All major clinical parameters were not significantly different between the two groups (Table 1).

The FPVP before splenectomy was 37.79 ± 5.03 cm H₂O in group A and 36.66 ± 5.24 cm H₂O in group B ($P = 0.179$). The FPVP after splenectomy with gastroesophageal devascularization was 30.01 ± 4.58 cm H₂O in group A and 29.59 ± 4.37 cm H₂O in group B ($P = 0.559$).

Sixteen patients (5.31%) had preoperative spontaneous mural PSVT, with an incidence of 3.92 % (6/153) in group A and 6.76 % (10/148) in group B ($\chi^2 = 1.201$, $P = 0.273$).

The total incidence of postoperative mural PSVT was 31.22 % (94/301), 41.17% (63/153) in group A and 20.94 % (31/148) in group B ($\chi^2 = 15.009$, $P = 0.002$). There were 50 (32.67%) cases of mural thrombi in the main trunk of the portal vein in group A and 27 (18.24%) cases in group B ($P = 0.004$). As shown in Table 2, there was no difference in terms of the location of thrombi between the two groups. No single case of thrombosis involved whole portal system. The incidence of throm-

Table 1 Comparison of preoperative data between two groups

	Irregular anticoagulation (group A)	Regular anticoagulation (group B)	P value
Sex (M/F)	103/50	99/49	0.937
Age (yr), mean \pm SD	46.14 \pm 10.39	46.47 \pm 9.58	0.814
Type of disease			
Hepatitis B viral cirrhosis	128	126	0.816
Hepatitis C viral cirrhosis	11	8	
Alcoholic cirrhosis	11	11	
Idiopathic portal hypertension	1	2	
Biliary cirrhosis	2	1	
Child-Pugh classification (grade A/grade B)	121/32	125/23	0.228
MELD index: mean \pm SD, median	7.68 \pm 3.24, 7.70	6.62 \pm 2.76, 6.51	0.055
History of upper GI bleeding	125 (81.69%)	111 (75%)	0.158
History of GI ulcer	18 (11.76%)	23 (15.54%)	0.340
Preoperative portal vein diameter (mm): mean \pm SD, median	12.38 \pm 1.17, 13	12.90 \pm 1.20, 12	0.083
Preoperative splenic vein diameter (mm): mean \pm SD, median	9.85 \pm 1.69, 10	10.10 \pm 1.41, 10	0.551

MELD: Model for end-stage disease; GI: Gastrointestinal.

Table 2 Distribution of thrombi in two groups, *n* (%)

Location	Irregular anticoagulation (group A)	Regular anticoagulation (group B)
Simple PV main trunk mural thrombosis	31 (20.26)	18 (12.16)
Simple splenic vein mural thrombosis	12 (7.84)	4 (2.70)
Complicated portal and splenic vein thrombosis	19 (12.41)	7 (4.72)
Simple SMV thrombosis	1 (0.65)	0
Complicated portal PV and SMV thrombosis	0	2 (1.35)
Total	63	31
Incidence of thrombosis	(41.17)	(20.94)

$\chi^2 = 15.009$, $P = 0.002$. PV: Portal vein; SMV: Superior mesenteric vein.

basis in the portal vein main trunk was significantly different between the two groups ($\chi^2 = 8.236$, $P = 0.004$).

During the anticoagulant therapy, mild GI bleeding occurred in 3 patients, including 1 in group A and 2 in group B. The anticoagulant therapy was terminated immediately and hemostatic agents were administered. Bleeding was successfully controlled, and the patients recovered well.

All 94 patients with postoperative PSVT were treated with thrombolytic drug, urokinase, anticoagulant and antiaggregation agents. Among the 94 patients, 76 achieved complete or partial thrombus dissolution, including 50 (79.37%, 50/63) patients in group A and 26 (83.87%, 26/31) patients in group B ($\chi^2 = 0.272$, $P = 0.602$). No thrombolytic therapy-related complications such as bleeding were noted.

As shown in Figure 1, platelet count was not significantly different between the two groups ($P = 0.981$). However, the PT/INR in group B was gradually increased from day 7 after surgery, which was statistically different from that in group A ($P = 0.020$). We also compared the patients with and without PSVT and found that the PT/INR in patients without PSVT was greater than those with PSVT at day 14 after surgery (1.30 ± 0.21 vs 1.23 ± 0.17 , $P = 0.037$). Similar results were found in patients with and without PSVT in group B (1.28 ± 0.21 without PSVT vs 1.18 ± 0.14 with PSVT, $P = 0.017$) (Figure 2).

DISCUSSION

Selection of thrombosis prevention regimens after splenectomy with gastroesophageal devascularization

Currently, a number of studies have suggested possible mechanisms of PSVT formation following splenectomy with gastroesophageal devascularization, including elevated postoperative platelet count, hemodynamic changes in splenic and portal veins, endothelial damage, spleen size, postoperative release of procoagulant factors, the reduction of anticoagulant factors, and the postoperative use of hemostatic drugs^[10,25,26]. PSVT may lead to severe clinical adverse events or poor outcomes^[3,4]. Therefore, the importance of PSVT prevention has been gradually recognized and emphasized^[19-21,27].

Various prevention protocols have been proposed^[25,28,29], but the effectiveness of these protocols varied^[4,22,30] because the duration and doses of these drugs were variable. Therefore, there was no generally acceptable PSVT prophylactic regimen for all patients^[22,23]. There are many blood coagulation factors involved in the formation of thrombosis^[31]. Xa is a major factor in the procedure of thrombosis^[32,33]. LMWH can suppress factor Xa by combining with ATIII to depress the activation of thrombin and formation of thrombosis^[34]. LMWH is safer than heparin because of its lower molecular weight, weaker inhibition of factor IIa and longer impact on co-

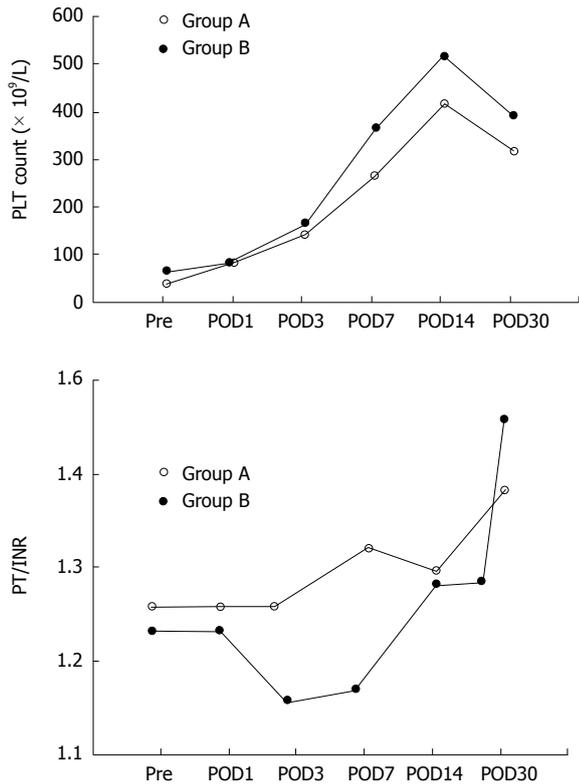


Figure 1 Changes in platelet count and prothrombin time/international normalized ratio in group A and group B. It was not different in platelet (PLT) between the two groups ($P = 0.981$), but it was statistically different in prothrombin time/international normalized ratio (PT/INR) between the two groups ($P = 0.020$). PT/INR in group A had no sequential changes pre- and post-operatively ($P = 0.479$) and PT/INR in group B was increasing gradually from postoperative day 7 with statistical difference ($P = 0.003$). POD: Postoperative day.

agulation system^[35,36].

The increased count and augmented aggregation competence of PLT after operation were important factors related to PSVT^[25]. These factors should be taken into account when selecting anticoagulation drugs. Aspirin has been applied in prevention and treatment of thrombotic diseases with satisfied safety because of its anti-PLT aggregation competence^[37,38]. Also, warfarin is an important antagonist of vitamin K (VK) with powerful anticoagulation effects by inhibiting VK-dependent coagulation factors such as II, VII, IX, X^[39,40]. We selected LMWH, warfarin and aspirin as a regular PSVT prevention regimen, which targets the major factors of mechanisms of PSVT. It was reported that the median interval between splenectomy and PSVT was 1-2 wk after surgery^[5,22,23,41].

Therefore, we used LMWH for 2-5 d followed by oral warfarin and aspirin after surgery in group B. Oral warfarin or aspirin was merely applied to those postoperative patients who were not suitable for LMWH in early postoperative phase as an irregular PSVT prevention regimen in group A.

In this study, we collected and analyzed retrospectively the data about the thrombosis prevention regimens for cirrhotic patients after splenectomy with gastroesophageal devascularization who were treated at our de-

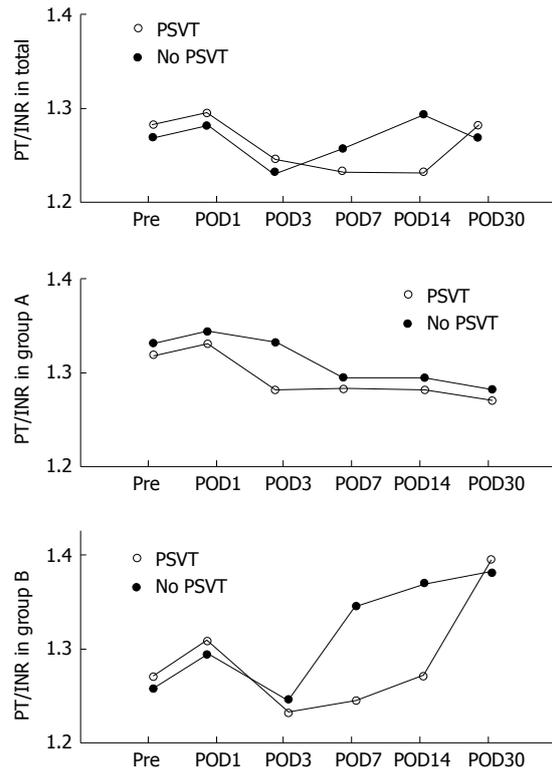


Figure 2 Changes in prothrombin time/international normalized ratio in patients without portal or splenic vein thrombosis and portal or splenic vein thrombosis. In group A, patients with or without portal or splenic vein thrombosis (PSVT) had similar prothrombin time/international normalized ratio (PT/INR), and the difference was not significant between the two subgroups. Patients with or without PSVT presented different PT/INR only at day 14 after surgery, with statistical significance (1.23 ± 0.17 vs 1.30 ± 0.21 , $P = 0.037$) by Student's *t* test. In group B, patients with or without PSVT had different PT/INR only at day 14 after surgery, with statistical significance (1.18 ± 0.14 vs 1.28 ± 0.21 , $P = 0.017$) by Student's *t* test. POD: Postoperative day.

partment over the past six years. Our results showed that the early use of LMWH followed by a long-term maintenance therapy with warfarin and aspirin could reduce the incidence of PSVT in group B (20.94%) without causing major bleeding complications, as compared with group A (41.17%) and other studies (51.5%-55%)^[5,6]. We also compared the patients with and without PSVT and found that the PT/INR in patients without PSVT was greater than those with PSVT at day 14 after surgery. Similar results were found in patients with and without PSVT in group B, suggesting that our anticoagulant regimen with the PT/INR target value set at 1.25-1.5 was reasonable and effective.

Classification and treatment of PSVT

PSVT in patients with portal hypertension due to cirrhosis following splenectomy with gastroesophageal devascularization can have various clinical manifestations, from no symptoms in mild cases to life-threatening recurrence of upper GI bleeding or small bowel necrosis in serious cases^[29]. The difference in clinical consequences stems from the location of PSVT, degree of obstruction, and its impact on portal vein (PV) hemodynamics. In fact, grade IV PSVT (Yerdel's classification^[42]), which involved

PV and superior mesenteric vein (SMV), would result in significant reduction in venous blood return to the liver and elevation in PVP, leading to esophageal and gastric variceal rupture and bleeding. SMV thrombosis can impede venous blood return from the bowels and cause small bowel necrosis, which could be vital for patients.

In this study, all 94 PSVT cases were grade I, which led to almost no clinical manifestations, such as variceal bleeding recurrence and bowel necrosis, and were easy to treat. Thrombolytic therapy has been proven to be effective for acute PSVT. Previous studies have recommended early and timely thrombolytic anticoagulant therapy^[43,44] and commonly used drugs, including urokinase, recombinant tissue plasminogen activator (rt-PA), LMWH, warfarin and dipyridamole. Intervention can be categorized by the administration route, i.e., systemic, portal system, and intravascular interventional treatment^[45,46]. Krauth *et al.*^[23] reported that immediate thrombolytic anticoagulant therapy in PSVT patients can achieve a complete dissolution rate of 63.3% and a partial dissolution rate of 13.3%. In our study, thrombolytic therapy *via* the peripheral venous route was administered in all 94 patients with PSVT, among whom 18 patients showed no sign of thrombus dissolution, but 61 patients (64.89%) achieved complete dissolution and 15 patients (15.95%) had partial dissolution. The overall thrombolytic effectiveness in our study was similar to other studies mentioned above. These phenomena were related to the benefit of regular anticoagulant therapy, which confirmed the clinical value of the anticoagulant regimen.

Our findings suggested that regular anticoagulation and early thrombolytic therapy are safe, effective and rational for PSVT patients who had portal hypertension and underwent splenectomy with gastroesophageal devascularization. At present, splenectomy with gastroesophageal devascularization may only be used as a bridge to liver transplantation in cirrhotic patients, timely prevention and thrombolytic treatment for PSVT offers a significant clinical value in terms of facilitating the portal venous reconstruction of the recipients for liver transplantation^[47].

Dilemma between anticoagulation and bleeding

Theoretically, preventive use of anticoagulants against PSVT in cirrhotic portal hypertensive patients early after surgery would counter the dilemma of bleeding. But in fact, previous studies have demonstrated that early anticoagulant treatment in these patients is a safe and effective protocol to prevent PSVT^[48,49]. Based on our study, the regular monitoring during anticoagulant treatment is necessary, which can guarantee the safety and maintain the PT/INR level between 1.25 and 1.5. Because most of our subjects had end-stage HBV cirrhosis, our suggestion differs from previous studies on non-HBV cirrhotic patients, mostly with hematologic and metabolic disorders and alcoholic cirrhosis, for which, PT/INR value of 2-3 is recommended^[50]. In this study, only three patients presented with mild GI bleeding during the anticoagulant treatment. We immediately terminated it and

shifted to symptomatic treatment such as hemostatic agents. Bleeding was successfully controlled, and the patients were discharged uneventfully. Therefore, our anticoagulant therapy has been proven safe.

On the other hand, there is a lack of large-scale controlled and long-term studies on the prevention of PSVT in cirrhosis patients receiving splenectomy with gastroesophageal devascularization for splenomegaly and hypersplenism. Since a randomized prospective controlled study is difficult to perform, we choose to conduct this retrospective controlled study. However, randomized controlled trial is necessary in the future.

Complications of splenectomy with gastroesophageal devascularization

Overwhelming postsplenectomy infection (OPSI) syndrome is associated with a high mortality, even it is a rare condition. Major risk factors include the age of the patients with splenectomy, the time after splenectomy, the reason for splenectomy, and the overall immune status of the patients. Splenectomy performed for hematological disorders, including thalassemia, hereditary spherocytosis, autoimmune hemolysis, immune thrombocytopenic purpura, or lymphoma, appears to carry a higher OPSI risk than splenectomy performed as a result of other diseases. Treatment of OPSI is generally aggressive due to the serious nature of the condition and associated mortality. The major preventive strategy is the vaccination using the 23-valent pneumococcal polysaccharide vaccine, a 7-valent proteinconjugated pneumococcal vaccine, the hemophilus influenzae type B vaccine, and the meningococcal vaccine^[51]. Fortunately, there was no OPSI occurrence in our study. The reasons may be that all the patients are adults and no splenectomy was performed for hematological disorders, the antibiotic agents were administered for 3-5 d after surgery, and the follow-up period was too short for OPSI.

In summary, the early and regular initiation of anticoagulant treatment using LMWH followed by warfarin and aspirin has been proven safe and effective in early prevention of PSVT in patients with cirrhotic portal hypertension, undergoing splenectomy with gastroesophageal devascularization. The treatment can reduce the incidence of PSVT in the early stage after splenectomy with gastroesophageal devascularization, early and timely thrombolytic therapy is imperative and feasible for the prevention and treatment of PSVT. The protocol presented in our study may benefit the patients not only for approximate and long-term clinical outcome, but also for potential liver transplant candidates in the future. But a better designed randomized prospective study with a longer follow-up period is still needed to clarify our conclusions.

COMMENTS

Background

Portal or splenic vein thrombosis (PSVT) is a common and potentially life-threatening complication of splenectomy with gastroesophageal devascularization for portal hypertension due to cirrhosis, which may lead to further deteriora-

tion of liver function and the recurrence of upper gastrointestinal (GI) bleeding and significantly affects patient's life expectancy. In some severe cases, PSVT may be contraindicated for liver transplantation. It is therefore very important to prevent the occurrence of PSVT after splenectomy with gastroesophageal devascularization to achieve better long-term outcomes and following possible liver transplantation if required.

Research frontiers

Prophylactic anticoagulation therapy using a combination protocol of low molecular weight heparin (LMWH), vitamin K antagonists, such as warfarin and aspirin, is a prime method to prevent PSVT. But the duration and doses of these drugs were variable according to the literature, there was no generally acceptable PSVT prophylactic regimen for all patients after splenectomy with gastroesophageal devascularization. So, a safe and effective prophylactic anticoagulation protocol is needed.

Innovations and breakthroughs

In order to reduce the incidence of PSVT after splenectomy with gastroesophageal devascularization, a regular prophylactic anticoagulation protocol was established with a confirmed monitoring index. This study used the combined and sequential application of LMWH, warfarin, aspirin and ticlopidine according to the regular coagulating function test and color Doppler flow imaging. The incidence and severity of PSVT caused by anticoagulation therapy were reduced, without causing major bleeding complications.

Applications

The study results suggest that prophylactic anticoagulation therapy using LMWH, warfarin and aspirin regularly is a safer and more effective method for PSVT prevention.

Terminology

PSVT is a sort of clinical disease caused by thrombus development in the portal vein system. The major causes of PSVT included the reduced blood flow, the increased platelet count, the injured vessel endothelium and enhanced coagulation function. PSVT is a common complication of abdominal surgery, especially after splenectomy with gastroesophageal devascularization with a high incidence and morbidity.

Peer review

This is a good descriptive study in which authors analyze the preventive effect of prophylactic anticoagulation therapy using low molecular weight heparin, warfarin and aspirin for PSVT. The results are interesting and imply that a regular prophylactic anticoagulation protocol is a safer and more effective method that could be used in preventing PSVT after splenectomy with gastroesophageal devascularization.

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Uncoupling protein 2 regulates glucagon-like peptide-1 secretion in L-cells

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Abstract

AIM: To investigate whether uncoupling protein 2 (UCP2) affects oleic acid-induced secretion of glucagon-like peptide-1 (GLP-1) in L-cells.

METHODS: mRNA and protein expression of UCP2 were analyzed in human NCI-H716 cells, which serve as a model for enteroendocrine L-cells, by quantitative reverse transcription-polymerase chain reaction and Western blotting before and after treatment with oleic acid. Localization of UCP2 and GLP-1 in NCI-H716 cells was assessed by immunofluorescence labeling. NCI-H716 cells were transiently transfected with a small interfering RNA (siRNA) that targets UCP2 (siUCP2) or with a non-specific siRNA using Lipofectamine 2000. The concentrations of bioactive GLP-1 in the medium were measured by enzyme linked immunosorbent assay.

RESULTS: Both GLP-1 and UCP2 granules were expressed mainly in the cytoplasm of NCI-H716 cells. NCI-H716 cells that secreted GLP-1 also expressed UCP2. Time-course experiments revealed that release

of GLP-1 from NCI-H716 cells into the medium reached a maximum at 120 min and remained stable until at least 180 min after treatment with oleic acid (the level of GLP-1 increased about 2.3-fold as compared with the level of GLP-1 in the control cells, $P < 0.05$). In an experiment to determine dose dependence, stimulation of NCI-H716 cells with ≤ 8 mmol oleic acid led to a concentration-dependent release of GLP-1 into the medium; 10 mmol oleic acid diminished the release of GLP-1. Furthermore, GLP-1 secretion induced by oleic acid from NCI-H716 cells that were transfected with si-UCP2 decreased to 41.8%, as compared with NCI-H716 cells that were transfected with a non-specific siRNA ($P < 0.01$).

CONCLUSION: UCP2 affected GLP-1 secretion induced by oleic acid. UCP2 plays an important role in L-cell secretion that is induced by free fatty acids.

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Key words: Glucagon-like peptide-1; L-cell; NCI-H716 cells; Oleic acid; Uncoupling protein 2

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INTRODUCTION

Glucagon-like peptide-1 (GLP-1) which is released from L-cells in the intestine plays an important role in post-prandial glucose homeostasis^[1]. With the recent advent

of anti-diabetic drugs aimed at either mimicking GLP-1 or preventing its degradation, researchers have turned their attention toward the L-cell and have focused on determining whether it would be both possible and beneficial to stimulate the endogenous release of GLP-1. This approach requires an understanding of the mechanisms underlying GLP-1 release from L-cells.

GLP-1 is involved in the regulation of nutrient homeostasis through insulinotropic effects on the β -cell as well as inhibition of glucagon release and gastric emptying and induction of satiety^[1,2]. Most L-cells that secrete GLP-1 are located in the distal small intestine and colon^[3]. GLP-1 release from L-cells is regulated by nutrient ingestion and demonstrates a biphasic pattern of secretion in response to either mixed meals or carbohydrate or fat alone^[4,5]. The first phase of GLP-1 secretion is regulated by neural signals originating in the proximal intestine, within minutes after a meal^[6-8]. In contrast, the second phase is induced by direct nutrient stimulation of the L-cells, resulting in secretion of GLP-1^[9-11]. In addition, *in vitro* studies using primary rat L-cells in culture have shown that the GLP-1 response to fat is highly specific, requiring mono-unsaturated fatty acids (MUFAs) with a chain length of 16 or more carbons (e.g., palmitoleic acid, 16:1 or oleic acid, 18:1). Mono-unsaturated long-chain fatty acids, such as oleic acid, are strong stimulators of GLP-1 secretion from L-cells^[9].

Uncoupling protein 2 (UCP2), a member of the UCP family, is located in the inner mitochondrial membrane and induces proton leakage and regulates the production of reactive oxygen species (ROS)^[12-14]. UCP2 plays an important role in α -cell dysfunction that is induced by free fatty acids (FFAs) *in vitro*, which may be related to its effects on oxidative stress and the insulin signaling pathway^[15]. UCP2 is also involved in the effect of FFAs on β cells, as FFAs stimulate both the expression and activity of UCP2 in α cells^[15-17]. When FFA stimulates UCP2 over-expression, the negative effects of UCP2 on ATP production result in the suppression of insulin secretion in β cells^[18]. Long-term exposure to FFAs also stimulates the release of glucagon and GLPs in a time- and dose-dependent manner in α cell lines^[19]. UCP2 is also believed to be involved in this effect in α cells. A chronic high concentration of FFAs impairs the function of both β and α cells, and this impairment involves UCP2^[16]. It is not, however, known whether UCP2 has similar effects on L-cells. In this study, we investigated the effect of oleic acid on the expression of UCP2 in human NCI-H716 cells, an L-cell model, and the effect of UCP2 down-regulation on GLP-1 secretion in these cells.

MATERIALS AND METHODS

NCI-H716 cell culture and secretion studies

Human enteroendocrine NCI-H716 cells were maintained in suspension culture as described by the American Type Culture Collection. Two days before an experiment, cells were seeded into 12-well culture plates precoated

with Matrigel as described^[20]. On the day of the experiment, the medium was replaced by PBS containing 1 mmol CaCl₂ and dipeptidyl peptidase IV inhibitor, adjusted to pH 7.2 (Millipore Corporation, Bedford, MA, United States). Cells were incubated at 37 °C for 2 h with or without different oleic acid concentrations (0, 2, 4, 6, 8, 10 mmol) and RNA interference targeting UCP2 (see below). GLP-1 was measured by enzyme linked immunosorbent assay (ELISA) (see below).

Small interfering RNA preparation and NCI-H716 cell transfection

The siRNA sequences targeting UCP2 (siUCP2) and a non-specific control small interfering RNA (siRNA) were purchased from Shanghai Shinegene Molecular Biotechnology Co., Ltd. (Shanghai, China). NCI-H716 cells were 70% confluent in 250 μ L of complete RPMI 1640 medium. Transfections of siRNA (at a final concentration of 300 nmol) were performed in NCI-H716 cells using Lipofectamine 2000 according to the manufacturer's protocol (Life Technologies, Carlsbad, CA, United States). Cells were usually examined 48 h after transfection.

Localization of uncoupling protein-2 and GLP-1 in NCI-H716 cells

The antibodies used for immunofluorescence staining in NCI-H716 cells were anti-GLP-1 (C-17; Santa Cruz) and anti-UCP2 (LS-B1911; LifeSpan Bio-Sciences). Cells were grown on coverslips, fixed in 4% paraformaldehyde in PBS for 10 min, washed with PBS, and cooled in 100% methanol at -20 °C for 20 min. Cells were then washed with PBS and permeabilized with 0.1% Triton X-100 for 10 min. After blocking with Dako blocking solution, primary antibody (anti-GLP-1; 1:100) was added and incubated at 4 °C overnight. For the secondary antibody, Cy3-conjugated donkey anti-goat (1:200; red, Beyotime Institute of Biotechnology) was incubated for 30 min at room temperature in the dark. The cells were washed with phosphate buffer saline (PBS), and anti-UCP2 (1:50) was added and incubated at 4 °C overnight. For the secondary antibody, fluorescein isothiocyanate-conjugated goat anti-rabbit (1:200; green, Beyotime Institute of Biotechnology) was added for 30 min at room temperature in the dark. Cells were then incubated for 45 min with Hoechst stain to counterstain nuclei. After a final wash with PBS, the coverslips were mounted with Slow-Fade Antifade kit (Molecular Probes).

RNA isolation and quantitative reverse transcription-polymerase chain reaction

Reagents used for RNA isolation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) included TRIzol Reagent, oligo(dT)₁₈ primers, and AMV reverse transcriptase (all from Invitrogen, Carlsbad, CA, United States) and primers and TaqMan probes (Shanghai Shinegene Molecular Biotechnology). Total cellular RNA was extracted with TRIzol Reagent. The purity and concentration of RNA was determined by measuring

the absorbance at 260 and 280 nm, with $A_{260}/A_{280} > 1.7$ considered sufficient purity^[21]. RNA (1 μ g) was reverse-transcribed into cDNA using oligo(dT)₁₈ primers at 42 °C for 1 h. Quantitative RT-PCR was performed with the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, United States) using TaqMan probes; threshold cycle numbers were obtained by ABI Prism 7000 SDS software, version 1.0. The primer and probe sequences used in this study were as follows: *UCP2* gene (sense primer, 5'-CCAATGTTGCCCGWAATG-3'; antisense primer, 5'-TGAGGTTGGCTTTCAGGAG-3'; probe, 5'-FAM+CTGGTGACCTATGACCTCATCAAAG-3'); β -*actin* gene (sense primer, 5'-GGCACCACA-CYTTCTACAATG-3'; antisense primer, 5'-GGGGGTGTGAAGGTCTCAAAC-3'; probe, 5'-FAM+TGTGGCCCTGAGGAGCACCC-3').

Amplification conditions were one cycle at 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s and 60 °C for 1 min, and one final cycle at 72 °C for 4 min.

Western blot analysis

Mitochondrial proteins from NCI-H716 cells were isolated with 1 mL of extraction buffer (250 mmol sucrose; 1 mmol ethylenediaminetetraacetic acid; 10 mmol Tris, pH 7.4) supplemented with protease inhibitors (Sigma). The mixture was centrifuged at $800 \times g$ for 10 min. The supernatant was centrifuged at $10\,000 \times g$ for 10 min, and the mitochondrial pellet was resuspended in 25 μ L of N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer. Mitochondrial protein concentration was determined colorimetrically with the BCA Protein Assay (Pierce, Rockford, IL, United States). Mitochondrial proteins (15 μ g) were mixed with $3 \times$ sample buffer [0.5 mol phosphate buffer, pH 7.0; 30% (w/v) glycerol; 7.5% (w/v) SDS; 0.75 mmol bromophenol blue], boiled for 5 min, and electrophoresed on an SDS-PAGE gel (12.5% acrylamide). Proteins were then transferred to Immobilon PVDF membranes (Millipore). UCP2 proteins were detected with polyclonal anti-UCP2 (LifeSpan Bio-Sciences) at a dilution of 1:600 followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz) at a dilution of 1:2000 and detection with enhanced chemiluminescence (ECL detection system; NEN, Boston, MA, United States). To validate equal protein loading across various lanes, PVDF membranes were stripped and re-probed with polyclonal anti-cytochrome c (Santa Cruz Biotechnology) at a dilution of 1:1000.

GLP-1 measurement

The level of active GLP-1, GLP-1 (7-36) amide, was measured with an ELISA kit (Linco). This assay relies on a monoclonal antibody fixed in a coated micro-well plate that binds to the N-terminal region of active GLP-1. The concentration of active GLP-1 is proportional to the fluorescence generated by umbelliferone, which is produced by alkaline phosphatase-catalyzed hydrolysis of methyl umbelliferyl phosphate (conjugated to GLP-1 monoclonal

antibodies). Samples of the cell culture medium were collected, and dipeptidyl peptidase IV inhibitor (10 μ L/mL) was added to prevent GLP-1 degradation. Samples (100 μ L each) were added to individual assay wells. The ELISA has a working range of 2 to 100 pmol/L (according to Linco GLP-1 ELISA kit). Each sample was replicated three times.

Statistical analysis

Data are presented as the mean \pm SE. Statistical significance was calculated by a one-way ANOVA and unpaired two-tailed *t* test. *P* values < 0.05 were regarded as significant.

RESULTS

Localization of uncoupling protein-2 and GLP-1 in NCI-H716 cells

The localization of UCP2 and GLP-1 was studied in the human cell line NCI-H716, which serves as a model for intestinal L-cells. Immunofluorescence staining for GLP-1 was observed in the cytoplasm in NCI-H716 cells (Figure 1A1 and A2). Immunofluorescence staining for UCP2 was also observed in the cytoplasm in human NCI-H716 cells (Figure 1B1 and B2). Both GLP-1 and UCP2 granules were expressed mainly in the cytoplasm. Thus L-cells that expressed GLP-1 were also able to express UCP2. Merged picture, yellow staining shows co-expression of GLP-1 and UCP2 (Figure 1C1 and C2).

Oleic acid increases GLP-1 secretion in NCI-H716 cells

The possible effect of UCP2 on the secretion of GLP-1 which is induced by oleic acid in the intestinal enteroendocrine L-cell was investigated. Time-course experiments revealed that GLP-1 release from NCI-H716 cells into the medium peaked 120 min after treatment with oleic acid (the release of GLP-1 increased approximately 2.3-fold of the baseline value by treatment with oleic acid; $P < 0.05$) and maintained a stable plateau until at least 180 min after treatment (Figure 2A). In a dose-dependent experiment, oleic acid significantly increased GLP-1 secretion to a maximum of 2.5 ± 0.3 -fold of baseline levels at 8 mmol ($P < 0.01$); 10 mmol oleic acid led to a less robust increase in GLP-1 expression (Figure 2B). These results indicated that oleic acid led to a concentration-dependent release of GLP-1 from NCI-H716 cells (final concentration of oleic acid ≤ 8 mmol).

Oleic acid increases the expression of uncoupling protein-2 in NCI-H716 cells

We then determined the effect of oleic acid on the expression of UCP2 in NCI-H716 cells. Based on our time-course and dose-dependency results for GLP-1 expression, we pretreated NCI-H716 cells with 8 mmol oleic acid for 2 h and then collected the cells. qRT-PCR for UCP2 mRNA transcripts and western blot analysis for UCP2 protein were carried out on the isolated total RNA and mitochondrial proteins from these cells, respectively. The UCP2 mRNA level in NCI-H716 cells increased 3-fold after

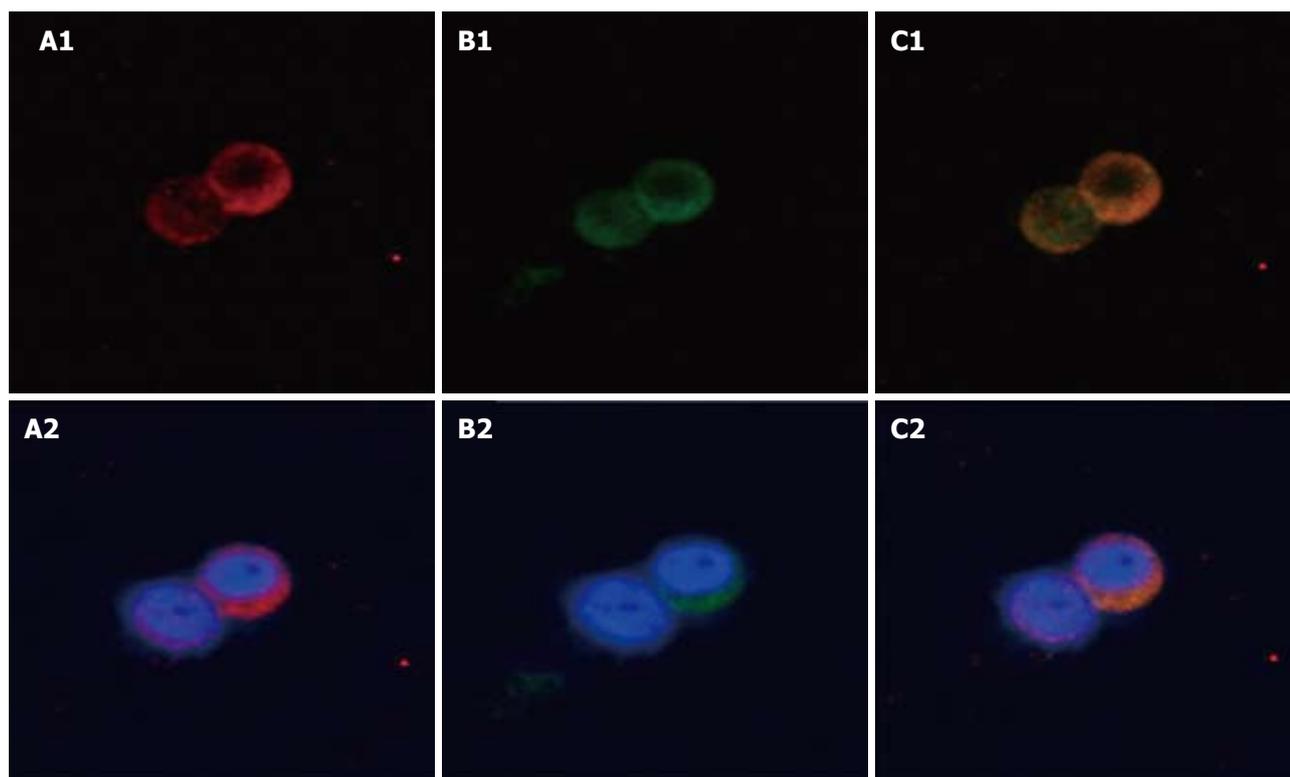


Figure 1 Localization of uncoupling protein-2 and glucagon-like peptide-1 in NCI-H716 cells. Immunofluorescence staining in human NCI-H716 cells which serve as a model for L-cells. A1, A2: Glucagon-like peptide-1 (GLP-1) antibody staining (red); B1, B2: Uncoupling protein-2 (UCP2) antibody staining (green); C1, C2: Merged image shows co-expression of UCP2 and GLP-1. Blue (Hoechst) staining indicates the nuclei. Original magnification, $\times 400$.

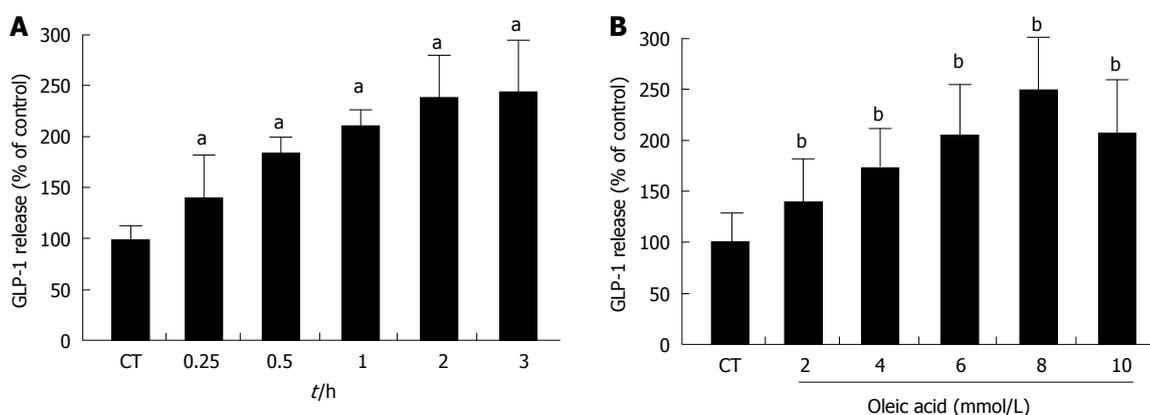


Figure 2 Glucagon-like peptide-1 secretion in NCI-H716 cells after treatment with oleic acid. A: Time course of oleic acid-induced release of glucagon-like peptide-1 (GLP-1) from NCI-H716 cells. NCI-H716 cells were incubated with 8 mmol oleic acid, and the culture medium was collected at 0.25, 0.5, 1, 2, and 3 h to detect GLP-1 levels; B: Dose-dependent oleic acid-induced release of GLP-1 from NCI-H716 cells. NCI-H716 cells were incubated with 2, 4, 6, 8, and 10 mmol oleic acid for 2 h. The culture medium was then analyzed for GLP-1 concentration. NCI-H716 cells without oleic acid exposure. ^a $P < 0.05$, ^b $P < 0.01$ vs baseline value.

treatment with 8 mmol oleic acid (Figure 3A). The UCP2 protein level also increased 1.8-fold *vs* the control amount (Figure 3B). These results indicated that oleic acid increased the expression of UCP2 in NCI-H716 cells at the transcriptional level.

Knocking down uncoupling protein-2 expression decreases oleic acid-induced secretion of GLP-1

To study the function of UCP2, a siRNA targeting UCP2 was used to specifically suppress UCP2 mRNA transla-

tion. Human NCI-H716 cells were left untransfected (blank), were mock transfected (no siRNA), or were transfected with siUCP2. After 48 h, mRNA levels of UCP2 were determined by qRT-PCR. The UCP2 mRNA level was reduced approximately 50% by siUCP2 as compared with the control level (Figure 4, up panel). There were no observable changes in the UCP2 mRNA level in mock-transfected or untransfected cells. The result from the western blot analysis also demonstrated that UCP2 protein was markedly reduced (Figure 4, up panel). Next,

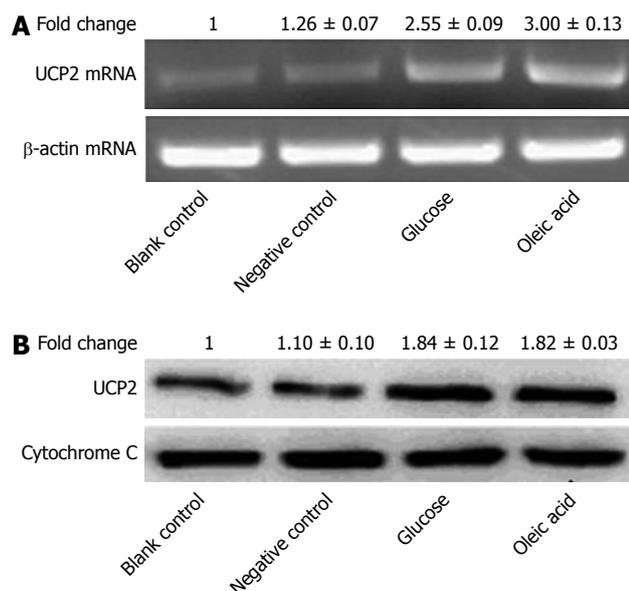


Figure 3 Expression of uncoupling protein-2 in NCI-H716 cells after treatment with oleic acid. A: Total RNA was extracted, and relative mRNA levels of uncoupling protein-2 (UCP2) were determined by quantitative reverse transcriptase polymerase chain reaction with normalization to β-actin ($P < 0.05$); B: UCP2 protein was detected by western blotting. Cytochrome C was used as the loading control ($P < 0.01$); Individual data points in this figure represent the mean ± SE of 12 determinants from four independently prepared samples each with three measurements. NCI-H716 cells were incubated with medium alone (blank control), medium containing 1% dimethyl sulfoxide (negative control), medium with 30 mmol glucose (glucose), and medium with 8 mmol oleic acid (oleic acid).

we treated NCI-H716 cells transfected with siUCP2 with 8 mmol oleic acid for 2 h. Cells that were incubated with medium alone were used as the blank control to determine baseline GLP-1 expression. Oleic acid-stimulated GLP-1 secretion decreased to 41.8% in NCI-H716 cells with siUCP2 compared to NCI-H716 cells with a non-specific siRNA ($P < 0.01$) (Figure 4, down panel).

DISCUSSION

The “incretin effect” of GLP-1 has been known for many years, and its role in the overall regulation of insulin release *in vivo* is well established. The regulation of GLP-1 secretion itself is, however, not well understood. GLP-1 is secreted from isolated perfused ileum preparations when glucose or fat is infused through the gut lumen^[22,23]. Nutrients that infuse directly into the ileal lumen can lead to the release of GLP-1^[24], indicating that direct stimulation of L-cell secretion is possible in humans. Oral glucose^[25-29], sucrose^[30], triglycerides^[27], and mixed meals also lead to an increase in plasma GLP-1^[31-34].

The mechanism of GLP-1 release is believed to involve a proximal-distal loop as well as a glucose sensor. Holst *et al.*^[35] proposed a proximal-distal loop to explain the phenomenon of GLP-1 release. This regulatory pathway involves nutrient detectors located in the upper regions of the gastrointestinal tract, which would control the release of GLP-1 from distal L-cells by neural or hormonal circuitry^[36]. Theodorakis *et al.*^[37] reported that the density

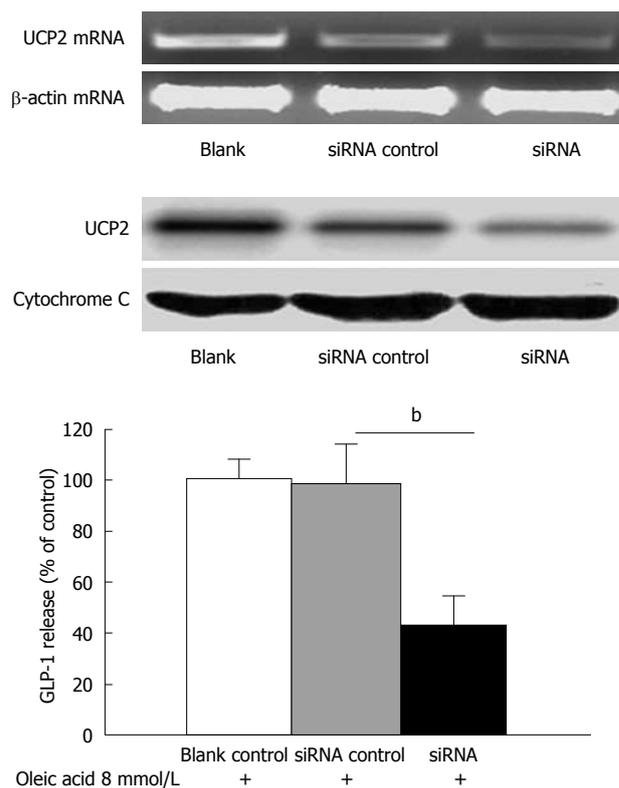


Figure 4 Knockdown of uncoupling protein-2 expression decreased oleic acid-induced secretion of glucagon-like peptide-1 in NCI-H716 cells. Up panel: The knock-down efficiency of small interfering RNA which targets UCP2 (siUCP2) in NCI-H716 cells. NCI-H716 cells were left untransfected (blank), were mock transfected (no siRNA), or were transfected with siUCP2. After 48 h, the mRNA levels of uncoupling protein-2 were determined as in Figure 4. Protein levels were determined as in Figure 4; Down panel: Effects of oleic acid on levels of secreted glucagon-like peptide-1 (GLP-1). NCI-H716 cells were transfected as described above and were then incubated with 8 mmol oleic acid for 2 h. GLP-1 concentrations were determined as in Figure 4. ^b $P < 0.01$ vs siRNA control.

of L-cells in the human duodenum may be sufficiently high to account for the early phase of GLP-1 secretion. With respect to the glucose sensor, a variety of signaling mechanisms have been proposed to explain how L-cells might sense glucose, including ATP-sensitive potassium channel closure, sodium glucose cotransporter activity, and activation of sweet taste receptors^[19,36]. Fats and protein are other well-known stimuli of GLP-1 release *in vivo*^[5,27].

G protein-coupled receptors (GPCRs) are regarded as potentially important components of signaling pathways in L-cells, as they may underlie the regulation of L-cells by certain neurotransmitters and hormones^[23,37,38], as well as by some luminal nutrients. One potential mechanism by which lipids, fatty acids, and bile acids might stimulate GLP-1 release is *via* activation of specific GPCRs on L-cells, such as GPR40, GPR120, GPR119, and TGR5. GPRs 40 and 120 are responsive to long-chain unsaturated fatty acids^[39,40]. GPR119 and TGR5 are responsive to oleoylethanolamide and bile acids, respectively^[41,42]. There is also convincing evidence that activation of protein kinase C-zeta contributes to the stimulatory action of fatty acids on GLP-1 release^[43]. These do not, however, explain the mechanism of GLP-1 release completely. Thus,

there may be other fatty acid-responsive pathways.

Chronic exposure of pancreatic islet cells to FFAs blunts glucose-stimulated insulin secretion and is accompanied by elevated levels of UCP2^[12]. UCP2 expression is regulated in tandem with the level of circulating FFAs^[44]. FFAs that increase as a result of fasting or a high-fat diet lead to UCP2 expression in adipose tissue and muscle^[45-47]. In isolated rat islets and INS-1 pancreatic β cells, long-term treatment with FFAs can increase UCP2 mRNA^[48,49].

We found that the human L-cell model, NCI-H716 cells, also expressed UCP2 in the cytoplasm. Oleic acid stimulated GLP-1 secretion in NCI-H716 cells. Time-course experiments revealed that release of GLP-1 from NCI-H716 cells into the medium peaked at 120 min after treatment with oleic acid and maintained a stable plateau at least until 180 min after treatment (Figure 2A).

Whereas the stimulation of NCI-H716 cells with \leq 8 mmol oleic acid led to a concentration-dependent increase in GLP-1 secretion into the medium, this increase was not maintained at 10 mmol (Figure 2B). It seemed that the higher concentration of oleic acid inhibited GLP-1 release from NCI-H716 cells. Lauffer *et al.*^[50] indicated that higher concentrations of oleoylethanolamide are associated with diminished GLP-1 release in all L-cell models which is suggestive of desensitization. Our results may also be related to desensitization.

Oleic acid also increased UCP2 mRNA levels in NCI-H716 cells. Furthermore, reduced UCP2 expression as a result of RNA interference decreased oleic acid-induced GLP-1 secretion (Figure 4). These results suggest that UCP2 is intimately involved in oleic acid-stimulated secretion of GLP-1 from intestinal endocrine L-cells. Understanding the mechanism responsible for this effect of UCP2 on GLP-1 secretion will require further investigation.

COMMENTS

Background

A chronic high concentration of free fatty acids (FFAs) impairs β cell function, as well as α cell function. This impairment involves uncoupling protein-2 (UCP2). Whether UCP2 has similar effects on intestinal L-cells is not clear. In this study, we investigated the effect of oleic acid, a FFA, on the expression of UCP2 in human NCI-H716 cells, which are a model for L-cells. We also analyzed the effect of UCP2 down-regulation on glucagon-like peptide-1 (GLP-1) secretion in these cells.

Research frontiers

Recent therapeutic successes of antidiabetic drugs aimed at either mimicking GLP-1 or preventing its degradation have focused attention on the L-cell and on addressing whether it would be both possible and beneficial to stimulate the endogenous release of GLP-1. However, the mechanism underlying GLP-1 secretion *in vivo* is still not fully understood.

Innovations and breakthroughs

To our knowledge, this is the first study to investigate the effect of oleic acid on the expression of UCP2 and the effect of UCP2 down-regulation on GLP-1 secretion in a model for human L-cells.

Applications

We have shown that UCP2 might be involved in a novel mechanism for regulating the secretion of GLP-1. This finding has potential benefit in finding new therapies for diabetes.

Terminology

UCP2 played an important role in α cell and β cell dysfunction. A chronic high

concentration of FFAs impairs the function of both β and α cells, and this impairment involves UCP2. The pancreatic endocrine β -cells are glucose-sensing cells and the intestinal endocrine L cells secreting GLP-1 are also glucose-sensing cells.

Peer review

The manuscript is a very interesting topic. The article is experimentally well prepared and effectively addresses the relationship between oleic acid and UCP2 and GLP-1. The research is very well described; the conclusions are applicable.

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Phosphoinositide-3-kinase, catalytic, alpha polypeptide RNA interference inhibits growth of colon cancer cell SW948

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Abstract

AIM: To investigate the gene knock-down effect by the phosphoinositide-3-kinase, catalytic, alpha polypeptide (*PIK3CA*)-targeted double-stranded RNA (dsRNA) and its effect on cell proliferation and cycle distribution in SW948.

METHODS: Two *PIK3CA*-targeted dsRNAs were constructed and transfected into SW948 cells. Transfections were performed using lipofectamine™ 2000. The transfection effectiveness was calculated basing on the rate of fluorescence cell of SW948 at 6 h after transfection. Total messenger RNA was extracted from these cells using the RNeasy kit, and semiquantitative reverse transcription polymerase chain reaction was performed

to detect the down-regulation of *PIK3CA*, *AKT1*, *MYC*, and *CCND1* gene expression. Cells were harvested, proteins were resolved, and western blot was employed to detect the expression levels of *PIK3CA*, *AKT1*, *MYC*, and *CCND1* gene. Cell proliferation was assessed by 3-(4,5)-dimethylthiazoliazolo-2-yl-2,5-diphenyltetrazolium bromide assay and the inhibition rate was calculated. Soft agar colony formation assay was performed basing on colonies greater than 60 μm in diameter at $\times 100$ magnification. The effect on cell cycle distribution and apoptosis was assessed by flow cytometry. All experiments were performed in triplicate.

RESULTS: Green fluorescence was observed in SW948 cell transfected with plasmid Pgenesil-1, and the transfection effectiveness was about 65%. Forty-eight hours post-transfection, mRNA expression of *PIK3CA* in SW948 cells was 0.51 ± 0.04 vs 0.49 ± 0.03 vs 0.92 ± 0.01 vs 0.93 ± 0.03 ($P = 0.001$) in Pgenesil-CA1, Pgenesil-CA2, negative and blank group respectively. mRNA expression of *AKT1* was 0.50 ± 0.03 vs 0.48 ± 0.01 vs 0.93 ± 0.04 vs 0.92 ± 0.02 ($P = 0.000$) in Pgenesil-CA1, Pgenesil-CA2, negative and blank group respectively. mRNA expression of *MYC* was 0.49 ± 0.01 vs 0.50 ± 0.04 vs 0.90 ± 0.02 vs 0.91 ± 0.03 ($P = 0.001$) in the four groups respectively. mRNA expression of *CCND1* was 0.45 ± 0.02 vs 0.51 ± 0.01 vs 0.96 ± 0.03 vs 0.98 ± 0.01 ($P = 0.001$) in the four groups respectively. The protein level of *PIK3CA* was 0.53 ± 0.01 vs 0.54 ± 0.02 vs 0.92 ± 0.03 vs 0.91 ± 0.02 ($P = 0.001$) in Pgenesil-CA1, Pgenesil-CA2, negative and blank group respectively. The protein level of *AKT1* in the four groups was 0.49 ± 0.02 vs 0.55 ± 0.03 vs 0.94 ± 0.03 vs 0.95 ± 0.04 , ($P = 0.000$). The protein level of *MYC* in the four groups was 0.51 ± 0.03 vs 0.52 ± 0.04 vs 0.92 ± 0.02 vs 0.95 ± 0.01 ($P = 0.000$). The protein level of *CCND1* in the four groups was 0.54 ± 0.04 vs 0.56 ± 0.03 vs 0.93 ± 0.01 vs 0.93 ± 0.03 ($P = 0.000$). Both Pgenesil-CA1 and Pgenesil-CA2 plasmids significantly suppressed the growth of SW948 cells when compared with the negative or blank group at 48 h after transfection.

tion (29% vs 25% vs 17% vs 14%, $P = 0.001$), 60 h after transfection (38% vs 34% vs 19% vs 16%, $P = 0.001$), and 72 h after transfection (53% vs 48% vs 20% vs 17%, $P = 0.000$). Numbers of colonies in negative, blank, CA1, and CA2 groups were 42 ± 4 , 45 ± 5 , 8 ± 2 , and 10 ± 3 , respectively ($P = 0.000$). There were more than 4.5 times colonies in the blank and negative control groups as there were in the CA1 and CA2 groups. In addition, the colonies in blank and negative control groups were also larger than those in the CA1 and CA2 groups. The percentage of cells in the CA1 and CA2 groups was significantly higher in G₀/G₁ phase, but lower in S and G₂/M phase when compared with the negative and control groups. Moreover, cell apoptosis rates in the CA1 and CA2 groups were 5.11 ± 0.32 and 4.73 ± 0.32 , which were significantly higher than those in negative (0.95 ± 0.11 , $P = 0.000$) and blank groups (0.86 ± 0.13 , $P = 0.001$). No significant difference was found between CA1 and CA2 groups in cell cycle distribution and apoptosis.

CONCLUSION: *PIK3CA*-targeted short hairpin RNAs can block the phosphoinositide 3-kinase-Akt signaling pathway and inhibit cell growth, increase apoptosis, and induce cell cycle arrest in the *PIK3CA*-mutant colon cancer SW948 cells.

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Key words: Phosphoinositide-3-kinase, catalytic, alpha polypeptide; RNA interference; Colon cancer; Phosphoinositide-3-kinase pathway

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INTRODUCTION

Colon cancer is the fifth most common cancer in the United States, and the third leading cause of cancer-related death in the Western world. The phosphoinositide 3-kinase (PI3K)/Akt signaling transduction pathway is believed to play an important role in carcinogenesis. PI3K is a major signaling component downstream of growth factor receptor tyrosine kinases (RTKs), which may serve as a potential target for colon cancer therapy^[1,2]. PI3K is a heterodimer consisting of a regulatory subunit (p85) and a catalytic subunit (p110). The subtype p110a encoded by the gene phosphoinositide-3-kinase, catalytic, alpha

polypeptide (*PIK3CA*) is very important in phosphorylating phosphatidylinositol (4,5)-bisphosphate (PIP₂) to the lipid second messenger PIP₃, and PIP₃ in turn functions in the recruitment and activation of a wide range of downstream targets, including the serine-threonine protein kinase Akt. Gene reactions distal to the *PI3K*-Akt signaling pathway contribute to tumor cell proliferation, cell cycle progression, energy metabolism, and resistance to apoptosis^[3-6]. Troxell *et al*^[7] found *PIK3CA* mutations were identified in 13/24 breast columnar cell lesions (54%) and 3/8 invasive carcinomas (37%). The high prevalence of *PIK3CA* mutations in breast cancer is an emerging tumor marker which might become used in treatment-choosing process^[8]. Higgins *et al*^[9] detected the *PIK3CA* status in metastatic breast cancer using peripheral blood, and found plasma level of *PIK3CA* presented predictive biomarkers of response to targeted therapies. *PIK3CA* mutations were detected in 18% patients with breast and gynecologic malignancies, and cases with *PIK3CA* mutations treated with its inhibitors demonstrated a higher response rate than patients without mutations^[10]. Studies demonstrated *PIK3CA* mutations were not common, but its amplification was very common in gastric cancer, Akt (p-Akt) was often functionally linked to tumor progression and metastasis in gastric cancer, and double-stranded RNA (dsRNA) mediated targeting of *PIK3CA* may specifically knockdown the expression of *PIK3CA*, providing a potential implication for therapy of cancer^[11-13].

RNA interference (RNAi) is a potent gene-silencing tool, which is triggered by the introduction of dsRNA. Degradation of mRNA homologous to dsRNA can cause a post-transcriptional gene-silencing effect. Recently, the vector-based RNAi has been developed in order to achieve long-term and stable effects. Short hairpin RNA (shRNA) is formed by hairpin structures and stretches of dsRNA. After being cleaved by a ribonuclease dicer, it becomes mature microRNA (miRNA) inside the targeted cells^[14-17].

In our study, two shRNA plasmid vectors were constructed against the gene *PIK3CA*, and were transfected into the colon cancer cell line SW948. Their effects on cell proliferation and cycle distribution in this cell line were investigated.

MATERIALS AND METHODS

Cell line and culture

The human colon carcinoma cell line SW948 was routinely grown in Leibowitz's L-15 medium supplemented with 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37 °C in a humidified 5% CO₂/95% air atmosphere.

RNA silencing

Two small interference RNA (siRNA) for *PIK3CA* were designed according to the consensus sequence of the *PIK3CA* gene (GeneBank NM_006218) obtained from the online database of the National Center for Biotech-

nology Information, and then were cloned separately into the vector plasmid Pgenesil-1. One sequence, 5'-GCTAT-CATCTGAACAATTA-3' was designated Pgenesil-CA1, and the other 5'-GGATAGAGGCCAAATAATA-3' was designated Pgenesil-CA2. Both were verified in a basic local alignment search tool search of the database. An siRNA scrambled sequence 5'-GACTTCATAAGGCG-CATGC-3', designated Pgenesil-Neg, was used as a negative control. All siRNA sequences were synthesized by Invitrogen (Carlsbad, CA). The multicloning sites of plasmid Pgenesil-1 containing enhanced green fluorescent protein gene were as follows: *Hind*III-*ShRNA-Bam*H I -U6Promotor-*Eco*R I -*Sal*I -*Xba*I -*Dra*III. All the above RNAi sequences were transcribed with DNA polymerase III U6 promoter. Cells at 80%-90% confluency were transfected with the three shRNA vectors CA1, CA2, and Neg group, described above. Transfections were performed using lipofectamine TM2000 (Invitrogen, United State) according to the manufacturer's instructions. The Blank group was also treated with only LipofectamineTM2000, but without vector. Six hours post-transfection, 500 μ L of fetal bovine serum (FBS) was added per well. Twenty-six hours after transfection, the medium was replaced by normal medium containing 10% FBS and antibiotics up to 72 h post-transfection. The transfection effectiveness was calculated basing on the rate of fluorescence cell of SW948 at 6 h after transfection.

RNA isolation and semiquantitative reverse transcription polymerase chain reaction

Cultured cells (described above) were harvested 48 h post-transfection. Total messenger RNA was extracted from these cells using the RNeasy kit. Reverse transcription polymerase chain reaction was performed using 500 ng of total RNA samples with oligo dT primers (Fermentas, United States). β -actin mRNA was included as an internal standard for quantitative analysis. The primer pairs used in this study are listed in Table 1. Samples in each group were run in triplicate. The photodensity of the goal gene product was normalized with respect to β -actin content.

Western blotting

Cells were harvested 48 h post-transfection, and were washed twice in ice-cold 1 \times phosphate-buffered saline (PBS). Cell pellets were treated with the lysis buffer, and whole cell extracts were isolated. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%), then transferred onto a polyvinylidene fluoride membrane, and subjected to immunohybridization analysis using a monoclonal antibody against the targeted proteins (Santa Cruz Biotechnology, Inc., United States). Peroxidase-conjugated secondary antibody was added later (Bolster, China). Hybridized protein bands were displayed *via* chemiluminescence reagents (Santa Cruz Biotechnology, Inc., United State) according to the manufacturer's instructions. β -actin staining was used as an internal standard. All experiments were performed in triplicate.

3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

The transfected cells were seeded in 96-well plates (1 \times 10⁴/mL), and were allowed to attach for 24 h. Thiazolyl blue tetrazolium bromide (MTT, Sigma, United States) was dissolved in 1 \times PBS at a concentration of 5 mg/mL, and filtered as a stock solution. Ten microlitres of stock solution were added to 100 μ L of medium in each well. The plates were incubated for 4 h at 37 $^{\circ}$ C. After loading of 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), medium was replaced with 100 μ L dimethyl sulfoxide, and was incubated for 30 min at room temperature for color development. Results were read by an enzyme-linked immunosorbent assay reader (570 nm, DG-3022A, United States) to determine absorbance values (*A*). In each group, time points for detection of absorbance values were 36, 48, 60 and 72 h after transfection. The inhibition rate was calculated as follows: Inhibition rate (IR) = [1 - *A*₁/*A*₂] \times 100%, where *A*₁ is the absorbance value of the observation group, and *A*₂ is the absorbance value of the control group.

Soft-agar colony-formation assays

Different groups of cells were mixed with culture medium containing agar at a final concentration of 0.4% 24 h after transfection. One milliliter of cell suspension was seeded onto 6-well plates coated with 0.5% agar in culture medium. Colonies greater than 60 μ m in diameter at \times 100 magnification were counted in each plate to measure the colony efficiency after 10 d incubation. The assays were performed in triplicate.

Flow cytometry

Flow cytometry was used to assess cell cycle and apoptosis. Cells were harvested 72 h after incubation, and then were washed with cold 1 \times PBS and fixed with 80% ethanol overnight at -20 $^{\circ}$ C. Next, cells were treated with RNase A (Sigma, United States), and were stained with propidium iodide (PI, Sigma, United States). All samples underwent analysis using flow cytometry (Becton Dickinson, United States). The experiments were performed three times.

Statistical analysis

Results of experimental data are reported as mean \pm SE. Significance levels were determined by one-way analysis of variance and Student's *t* test using SPSS 11.5 Statistics software. A statistically significant result is indicated by a *P* < 0.05.

RESULTS

PIK3CA-specific shRNA inhibited mRNA and protein expression of target genes

SW948 transfected with plasmid Pgenesil-1 presented green fluorescence, and the transfection effectiveness was about 65% (Figure 1). Forty-eight hours post-transfection, the expression of mRNA and protein from targeted genes was tested in SW948 cells. The results are shown in Figure 2, Tables 1 and 2. All mRNA and protein expres-

Table 1 Oligonucleotides sequences of primer pairs

Goal gene	Upstream primer	Downstream primer	PCR frag (bp)
<i>β-actin</i>	5'-TCCTGTGGATCCACGAAACT-3'	5'-GAAGCATTGCGGTGGACGAT-3'	330-bp
<i>PIK3CA</i>	5'-CCCTGCTCATCAACTAGGAAACC-3'	5'-TTGCCGTAAATCATCCCCATT-3'	160-bp
<i>Akt</i>	5'-GGACAACGCCATCCAGACT-3'	5'-GCCAGGGACACCTCCATCTC-3'	121-bp
<i>c-myc</i>	5'-TACCCTCTCAACGACAGCAG-3'	5'-TCTTGACA TTCTCCTCGGTG-3'	477-bp
<i>cyclinD1</i>	5'-GCCAACCTCTCAACGACCGG-3'	5'-GTCCATGTTCTGCTGGGCTG-3'	744-bp

PCR: Polymerase chain reaction; *PIK3CA*: Phosphoinositide-3-kinase, catalytic, alpha polypeptide.

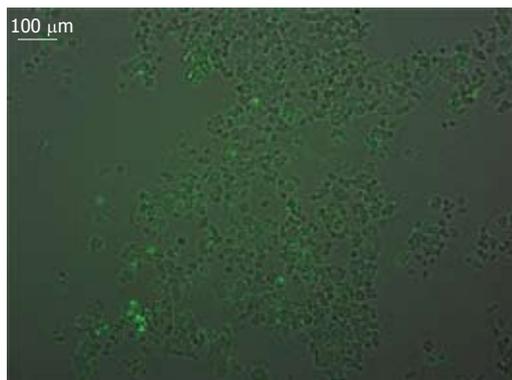


Figure 1 Fluorescence of SW948 cell transfected with plasmid Pgenesil-1 ($\times 100$).

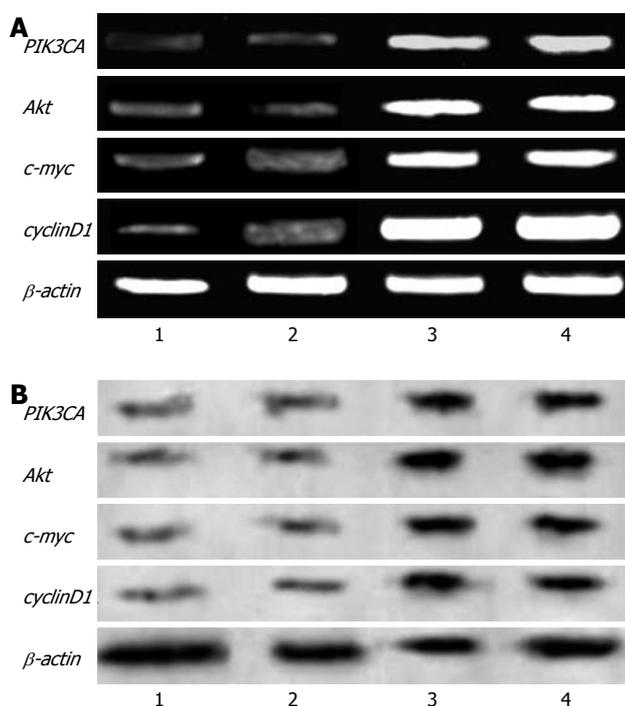


Figure 2 Phosphoinositide-3-kinase, catalytic, alpha polypeptide specific short hairpin RNA inhibited target genes mRNA expressions (A) and protein expressions (B) in SW948 cells. Lane 1: CA1 group; Lane 2: CA2 group; Lane 3: Negative group; Lane 4: Blank group. *PIK3CA*: Phosphoinositide-3-kinase, catalytic, alpha polypeptide.

sion from targeted genes in SW948 cells was down-regulated significantly after transfection with either plasmids

Table 2 Levels of mRNA expression and protein expression of target genes in different groups normalized by β -actin

Goal gene	CA1	CA2	Negative	Blank
mRNA expression				
<i>PIK3CA</i>	0.51 \pm 0.04 ^a	0.49 \pm 0.03 ^a	0.92 \pm 0.01	0.93 \pm 0.03
<i>Akt</i>	0.50 \pm 0.03 ^b	0.48 \pm 0.01 ^b	0.93 \pm 0.04	0.92 \pm 0.02
<i>c-myc</i>	0.49 \pm 0.01 ^a	0.50 \pm 0.04 ^a	0.90 \pm 0.02	0.91 \pm 0.03
<i>cyclinD1</i>	0.45 \pm 0.02 ^a	0.51 \pm 0.01 ^a	0.96 \pm 0.03	0.98 \pm 0.01
Protein expression				
<i>PIK3CA</i>	0.53 \pm 0.01 ^a	0.54 \pm 0.02 ^a	0.92 \pm 0.03	0.91 \pm 0.02
<i>Akt</i>	0.49 \pm 0.02 ^b	0.55 \pm 0.03 ^b	0.94 \pm 0.03	0.95 \pm 0.04
<i>c-myc</i>	0.51 \pm 0.03 ^b	0.52 \pm 0.04 ^b	0.92 \pm 0.02	0.95 \pm 0.01
<i>cyclinD1</i>	0.54 \pm 0.04 ^b	0.56 \pm 0.03 ^b	0.93 \pm 0.01	0.93 \pm 0.03

PIK3CA: Phosphoinositide-3-kinase, catalytic, alpha polypeptide. ^a*P* = 0.001, ^b*P* = 0.000 *vs* negative or blank group.

Table 3 Phosphoinositide-3-kinase, catalytic, alpha polypeptide short hairpin RNA suppressed SW948 cell proliferation (%)

	Blank	Negative	CA1	CA2
36 h	13	14	17	15
48 h	14	17	29 ^a	25 ^a
60 h	16	19	38 ^a	34 ^a
72 h	17	20	53 ^b	48 ^b

^a*P* = 0.001, ^b*P* = 0.000 *vs* negative or blank group.

Pgenesil-CA1 or Pgenesil-CA2 (*P* < 0.001, *vs* negative or blank group). No significant difference was found between the CA1 and CA2 groups. Pgenesil-Neg plasmid had no significant inhibitory effect on the expression of mRNA or protein.

PIK3CA RNA silencing inhibited SW948 cell proliferation

The effect of *PIK3CA* silencing on proliferation of SW948 cells was analyzed by the MTT assay. As demonstrated in Table 3, both Pgenesil-CA1 and Pgenesil-CA2 plasmids significantly suppressed the growth of SW948 cells when compared with the negative or blank group.

Soft-agar colony-formation assays

Soft-agar colony-formation assays were used to analyze the anchorage-independent proliferation of SW948 cells. Numbers of colonies in negative, blank, CA1, and CA2 group were 42 \pm 4, 45 \pm 5, 8 \pm 2, and 10 \pm 3, respectively. There were more than 4.5 times more than colonies in the blank and negative control groups as

Table 4 Effect of short hairpin RNA interference on cell cycle distribution and apoptosis (%)

Group	G ₀ /G ₁	S	G ₂ /M	Apoptosis
Blank	35.41 ± 2.63	47.54 ± 3.87	16.74 ± 1.59	0.86 ± 0.13
Negative	40.12 ± 2.54	41.71 ± 2.16	17.57 ± 1.26	0.95 ± 0.11
CA1	79.72 ± 4.63 ^a	15.24 ± 1.28 ^a	4.37 ± 0.43 ^b	5.11 ± 0.32 ^a
CA2	77.93 ± 4.31 ^a	16.71 ± 0.89 ^a	5.17 ± 0.29 ^a	4.73 ± 0.32 ^b

^aP = 0.001, ^bP = 0.000 vs negative or blank group.

there were in the CA1 and CA2 groups. In addition, the colonies in blank and negative control groups were also larger than those in the CA1 and CA2 groups.

Effect of PIK3CA shRNA on cell cycle distribution and apoptosis

As shown in Table 4, the percentage of cells in the CA1 and CA2 groups was significantly higher in G₀/G₁ phase, but lower in S and G₂/M phase when compared with the negative and control groups. Moreover, cell apoptosis rates in the CA1 and CA2 groups were around 5%, which was significantly higher than those in negative and blank groups. No significant difference was found between CA1 and CA2 groups in cell cycle distribution and apoptosis.

DISCUSSION

One of the areas of greatest interest in cancer research concerns the coordination of cancer cell proliferation and apoptosis. The balance between cell proliferation and apoptosis, and the distribution of cell cycles play very important roles in the process of carcinogenesis in colon cancer^[18]. Abnormal activation of the PI3K signaling pathway can cause hyper-proliferation of intestinal crypt progenitors, and promote the transformation of a normal cell to a cancer cell. This process involves a series of complicated molecular activities. The PI3K signaling cascade begins with the phosphorylation of PIP2 to PIP3, which is largely regulated by the balance of activity of PTEN and PI3Ks. Many investigators have reported that mutations of PI3Ks contribute to the process of carcinogenesis in colon cancer^[2,5]. Liao *et al.*^[19] reported that co-existence of PIK3CA exon 9 and 20 mutations, but not PIK3CA mutation in either exon 9 or 20 alone, was associated with poor prognosis of colorectal cancer patients. Thus, PI3K has become the focus of considerable attention for research into the treatment of colon cancer. Small-molecule inhibitors targeting PI3K are important to investigate, but their toxicity, drug resistance, and side-effects have prevented widespread use of these compounds^[20]. Therefore, more efficient and safer new methods have been developed to solve those problems.

RNA interference is a ubiquitous mechanism of eukaryotic gene regulation, and an excellent strategy for specific gene silencing. Recently the vector-based approach of shRNA interference has been developed in order to achieve highly specific suppression of gene ex-

pression in mammalian cells. ShRNA is formed by hairpin structures and stretches of double-stranded RNA, which determine the specificity of RNA interference. Previous reports about ShRNA interference have demonstrated their easy and stable introduction into cells, and more importantly, their consistently efficacy, which has potential as a new method for cancer therapy^[15-17,21].

PI3K is a heterodimer composed of a regulatory subunit (p85) and a catalytic subunit (p110), which has three isoforms. Among them, p110a (PIK3CA), is an important catalytic subunit that is implicated in a wide range of cancers including colon cancer. Beuers *et al.*^[22] reported strong associations were found between KRAS, PIK3CA mutations and colorectal cancers. Dukes' staging, and KRAS and PIK3CA bi-mutations were more likely to develop into liver metastasis. It naturally serves as a potent potential target for colon cancer therapy^[2-6]; however, the use of an RNA interference technique for PIK3CA gene silencing in established colon cancer cell lines has rarely been reported. In a previous study, Wee *et al.*^[23] reported successful PIK3CA gene depletion by RNA interference and consequential inhibition of proliferation in the colon cancer cell lines HCT116 and DLD1; however, the down-regulation of PIK3CA gene expression by RNAi in other colon cancer cell line has not been reported, due to the limited availability of colorectal carcinoma cell lines with PIK3CA gene mutation. Herein we describe our experience with the effects of two PIK3CA-specific shRNAs on cell proliferation and apoptosis in the colon cancer cell line SW948, which harbors mutation in the PIK3CA gene.

In this study we used plasmid Pgenesil-1 as vector into which two PIK3CA-specific interference sequences were successfully inserted. The vector Pgenesil-1 could transcribe and generate interfering RNAs continually under the control of U6 promoter. Thus, a persistent gene knock-down effect could be achieved by successfully transfecting the vectors into SW948 cells. Our results demonstrated that these two PIK3C-specific shRNAs both showed evident effects on silencing the PIK3CA gene either at the mRNA or at the protein level. At the same time, the expression of downstream genes, namely AKT1, MYC, and CCND1 was also significantly suppressed. MTT assays demonstrated that cell proliferation in the PIK3CA knockdown groups was significantly inhibited from 48 to 72 h after transfection. Similar results were achieved in detecting anchorage-independent cell proliferation by soft agar colony formation assays, in which significant reduction in either the number or the size of colonies was found in both CA1 and CA2 groups. These results suggest that PIK3CA knockdown by specific shRNAs decreased the abilities of SW948 cells to form colonies in soft agar, which was consistent with Wee's report^[23]. Our results revealed that these two PIK3CA-specific shRNAs expressed an anti-proliferation function in the PIK3CA-mutant cancer cells, and suggest that they may have therapeutic potential for colon cancer.

Our flow cytometry results showed cell cycle arrest

at G₁/S, and an increase in apoptosis in these two *PIK3CA*-specific shRNAs-transfected SW948 cells, which may have caused the inhibition of cell growth. This finding is consistent with previously published results in the HCT116 cell line^[23]. Akt, a major downstream effector of PI3K, is activated by phosphoinositide-dependent protein kinase 1, and plays an important role in cell proliferation, apoptosis, cell motility and invasion^[12]. Lots of studies have shown that Akt is activated in a variety of malignances, and is often functionally linked to cancer progression and metastasis^[13]. Another possible mechanism is that *AKT* down-regulation induced by *PIK3CA* gene silencing resulted in the degradation of cyclinD1 and down-regulation of c-myc expression *via* GSK3 β activity. CyclinD1 is a regulatory kinase that is critical in modulating the cell cycle through G₁ to S phase. C-myc is a positive regulator of G₁-specific cyclin-dependent kinases. Over-expression of these two genes may stimulate cells to overcome the cell cycle checkpoints, and thus enhance cell proliferation^[24,25]. Inhibition of these two genes may result in cell cycle arrest and increased apoptosis, and thus inhibit cell growth. Migliardi *et al*^[26] reported that PI3K/mTor inhibitor BEZ235 could produce prevalent growth-suppressive effects in patient-derived xenografts of RAS-mutant colorectal carcinomas.

In conclusion, our experiment showed that the shRNA targeted against *PIK3CA* could specifically silence the *PIK3CA* gene and consequentially suppress the expression of its downstream genes including, *AKT1*, *MYC*, and *CCND1*. This silencing effect on these genes could efficiently inhibit the growth of *PIK3CA*-mutant colon cancer SW948 cells, and might be a potential approach for treating human *PIK3CA*-mutant colon cancer.

COMMENTS

Background

The phosphoinositide-3-kinase (PI3K)/Akt signaling transduction pathway is believed to play an important role in carcinogenesis. PI3K is a major signaling component, which is a heterodimer consisting of a regulatory subunit (p85) and a catalytic subunit (p110). The subtype p110a encoded by the gene phosphoinositide-3-kinase, catalytic, alpha polypeptide (*PIK3CA*) is very important in phosphorylating, and may serve as a potential target for colon cancer therapy.

Research frontiers

The coordination of cell proliferation and apoptosis in cells is one of the hotspots in cancer research. The balance between cell proliferation and apoptosis, and the distribution of cell cycles play very important roles in the carcinogenesis process of many cancers. *PIK3CA* is the key component in this process. Thus *PIK3CA* might be a gene therapy target for lots of cancers include colon carcinoma.

Innovations and breakthroughs

In this study, two *PIK3CA* specific shRNAs showed their evident effect on silencing the *PIK3CA* gene. The results also indicated that *PIK3CA* shRNA inhibits cell growth and induces apoptosis of SW948 cell.

Applications

The shRNA interference targeted against *PIK3CA* may have potential therapeutic utility in human *PIK3CA*-mutant colon cancer.

Terminology

The official name of *PIK3CA* is "phosphoinositide-3-kinase, catalytic, alpha polypeptide". It is located on the long(q) arm of chromosome 3 at position 26.3. Phosphatidylinositol 3-kinase is composed of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit. The protein encoded by *PIK3CA* gene represents the catalytic subunit. The *PIK3CA* gene has been found to be oncogenic and is

mutated in a range of human cancers. Due to the association between *PIK3CA* and cancer, it is believed to be a promising drug target.

Peer review

The *PIK3CA* knock-down effect on cells was investigated in the present study. The results showed that shRNAs transfection down-regulated *PIK3CA* and its downstream genes such as *Akt1*, *MYC* and *CCND1*, and inhibited cell growth. Further, it was demonstrated that cell cycle arrest and apoptosis were induced in *PIK3CA* shRNAs-transfected SW948 cells. The study appears to be interesting and may have therapeutic implication.

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Endoscopic therapy for gastric stromal tumors originating from the muscularis propria

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Abstract

AIM: To explore endoscopic therapy methods for gastric stromal tumors originating from the muscularis propria.

METHODS: For 69 cases diagnosed as gastric stromal tumors originating from the muscularis propria, three types of endoscopic therapy were selected, based on the size of the tumor. These methods included endoscopic ligation and resection (ELR), endoscopic submucosal excavation (ESE) and endoscopic full-thickness resection (EFR). The wound surface and the perforation of the gastric wall were closed with metal clips. Immunohistostaining for CD34, CD117, Dog-1, S-100 and smooth muscle actin (SMA) was performed on the resected tumors.

RESULTS: A total of 38 cases in which the tumor size was less than 1.2 cm were treated with ELR; three cases were complicated by perforation, and the perforations were closed with metal clips. Additionally, 18 cases in which the tumor size was more than 1.5 cm were treated with ESE, and no perforation occurred. Finally, 13 cases in which the tumor size was more

than 2.0 cm were treated with EFR; all of the cases were complicated by artificial perforation, and all of the perforations were closed with metal clips. All of the 69 cases recovered with medical treatment, and none required surgical operation. Immunohistostaining demonstrated that among all of the 69 gastric stromal tumors diagnosed by gastroscopy, 12 cases were gastric leiomyomas (SMA-positive), and the other 57 cases were gastric stromal tumors.

CONCLUSION: Gastric stromal tumors originating from the muscularis propria can be treated successfully with endoscopic techniques, which could replace certain surgical operations and should be considered for further application.

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Key words: Gastrointestinal stromal tumors; Therapy; Endoscopy; Muscularis propria; Resection

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INTRODUCTION

Gastric stromal tumors are the most common mesenchymal tissue-originated tumors in the digestive tract; these tumors are often found at the gastric fundus, the front wall of the gastric corpus and the antrum and can be sorted based on tissue of origin as originating from the muscularis mucosae or the muscularis propria. As the location of the tumors originating from the muscularis

mucosae is superficial, endoscopic resection or ligation has been widely performed to treat these tumors. In contrast, the location of the tumors originating from the muscularis propria is deep, especially when the tumors grow outside the gastric wall, so perforation often occurs during endoscopic therapy and the tumor cannot be resected easily and thoroughly. Therefore, this tissue of origin has often been considered to be a contraindication for endoscopic therapy, and the traditional treatment for such tumors is a surgical or laparoscopic operation^[1-5]. In recent years, on the basis of sufficient clinical practice, we treated gastric stromal tumors originating from the muscularis propria with endoscopic techniques and obtained satisfactory therapeutic effects, which are reported as follows.

MATERIALS AND METHODS

Patients

A total of 69 patients (36 males and 33 females, age range 24-68 years, mean age 42.5 years) were diagnosed with gastric stromal tumors originating from the muscularis propria by gastroscopy and endoscopic ultrasonography from January 2008 to March 2011. Among the 69 cases, 19 tumors were located at the gastric antrum, 24 tumors were located at the gastric corpus, and 26 tumors were located at the gastric fundus. Each patient exhibited only one tumor, and no metastasis was detected *via* computed tomography (CT) examination. Routine blood tests, tests of blood coagulation function and hepatic and renal function, electrocardiograms and abdominal CTs were performed before the endoscopic therapy, and each patient's written informed consent was obtained.

Instruments

The following instruments were used: Electronic gastroscopy (Olympus GIF-Q260J, Olympus company, Japan), hyaline cap (D-201-11304, Olympus company, Japan), spiculiform cutting knife (KD-1 L-1, Olympus company, Japan), IT knife (KD-611 L, Olympus company, Japan), hook knife (KD-620 LR, Olympus company, Japan), injection needle (NM-200 L-0525, Olympus company, Japan), snare (AS-1-S, ASJ-1-S, COOK company, United States), hot biopsy forceps (FD-410 LR, Olympus company, Japan), hemostatic clip (HX-610-90, Olympus company, Japan; HX-600-135, Olympus company, Japan; Boston Resolution™, Boston company, United States), high frequency electric knife (ERBE VIO 200S, ERBE company, Germany) and Argon Plasma Coagulation instrument (ERBE APC₂, ERBE company, Germany).

Endoscopic therapeutic methods

Endoscopic ligation and resection: Endoscopic ligation and resection (ELR) was used for gastric stromal tumors of which the size was less than 1.2 cm. A COOK ligator was assembled on the tip of the gastroscopy, and the gastroscopy was inserted into the stomach. The ligator was aimed at the tumor, and sufficient aspiration

was applied to the tumor that the whole tumor entered the ligator, at which time the rubber band on the ligator was released to ligate the tumor. The purpose of the ligation was to manipulate the gastric stromal tumor into the shape of a polypoid, after which a snare was used to hitch the tumor, and it was resected. The resected tumor was removed for pathologic diagnosis, and the stump was closed with metal clips. If the procedure was complicated by perforation, the perforation was generally small and could be closed with metal clips (Figure 1).

Endoscopic submucosal excavation: Endoscopic submucosal excavation (ESE) was performed for gastric stromal tumors originating from the muscularis propria by the following steps. (1) Marking: Argon plasma coagulation (APC) was used to mark the border of the gastric stromal tumor; (2) Submucosal injection: A mixture of indicarminum, adrenalin and physiological saline (2-3 mL of indicarminum, 1 mL of adrenalin and 100 mL of physiological saline) was injected at the lateral of the mark; (3) Dissection: the mucosa was dissected with a needle knife; (4) Excavation: the submucosa was dissected with a needle knife; when the tumor was exposed, dissection was performed around the margin of the tumor, and when the dissection was finished, the tumor could be resected wholly with a snare; and (5) Wound surface handling: for small vessels bleeding on the surface, hot biopsy forceps or APC could be used for hemostasis, and if necessary, metal clips could be used (Figure 2).

Endoscopic full-thickness resection: A hyaline cap was assembled on the tip of the gastroscopy before the endoscopic full-thickness resection (EFR) procedure, and intravenous anesthesia was performed using propofol for patients. EFR was then performed using the following steps. (1) Pre-cutting the mucosa and submucosa around the gastric stromal tumor: APC was used to mark the margin of the tumor; a mixture of indicarminum, adrenalin and physiological saline (2-3 mL of indicarminum, 1 mL of adrenalin and 100 mL of physiological saline) was injected at the lateral portion of the mark, using approximately 2-3 mL of the mixture for each injected point; the mucosa and submucosa around the gastric stromal tumor were pre-cut with a hook knife, and thus the tumor was exposed; (2) Dissection of the muscularis propria around the gastric stromal tumor: a hook knife or IT knife was used to dissect the tumor from the muscularis propria to the serous membrane; (3) Dissection of the serous membrane around the tumor: typically, the tumor was in compact adhesions with the serous membrane; thus the tumor could not be directly dissected with an IT knife, so instead, the serous membrane was cut around the tumor with a needle knife or hook knife, and an "artificial" perforation was made; (4) Full resection of the tumor: the liquid in the stomach was aspirated completely, and an IT knife or hook knife was used to cut the serous membrane around the tumor, after which the tumor was completely resected; and (5)

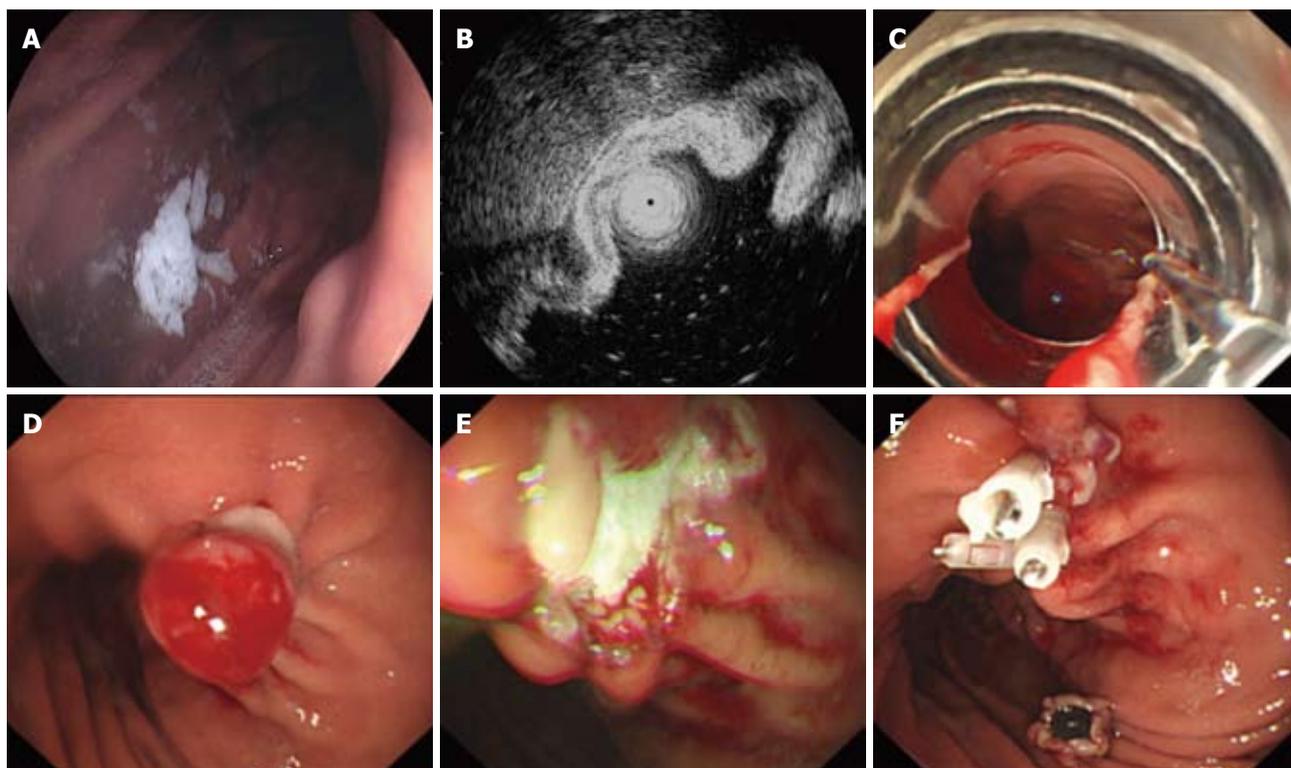


Figure 1 Endoscopic ligation and resection treatment for a gastric stromal tumor less than 1.2 cm in size originating from the muscularis propria. A: Submucosa lesion at the posterior wall of the gastric corpus; B: Endoscopic ultrasound shows that the lesion originates from the muscularis propria; C: COOK ligator aimed at the lesion, ready to ligate; D: The ligated stromal tumor was in the shape of a polypoid with deuto-stem; E: A snare was used to cut the tumor above the rubber band; F: The wound surface was closed with metal clips.

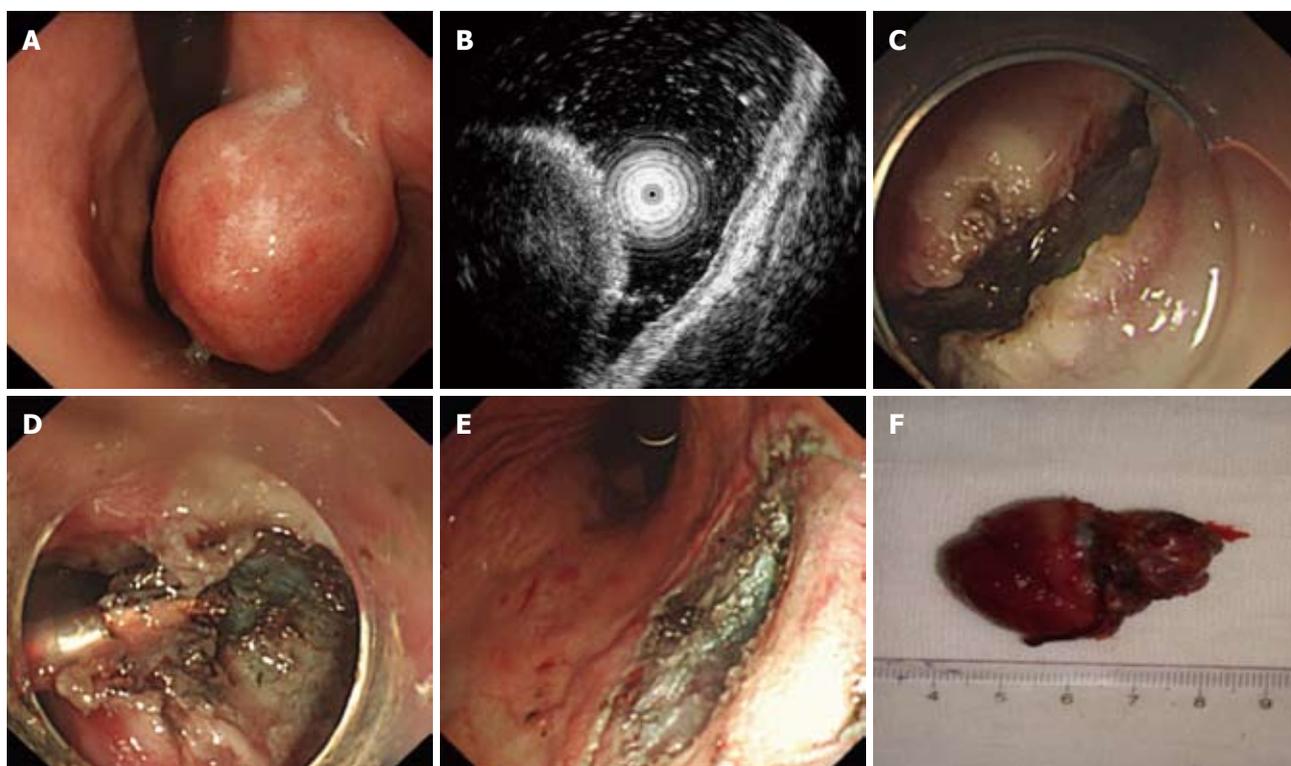


Figure 2 Endoscopic submucosal excavation treatment for a gastric stromal tumor originating from the muscularis propria that is larger than 1.2 cm. A: Submucosa lesion at the gastric corpus; B: Endoscopic ultrasound shows that the lesion originates from the muscularis propria; C: The mucosa of the stromal tumor was cut after submucosal injection; D: Dissection with an IT knife; E: The excavated wound surface, showing that no perforation occurred; F: The resected stromal tumor (4 cm in size).

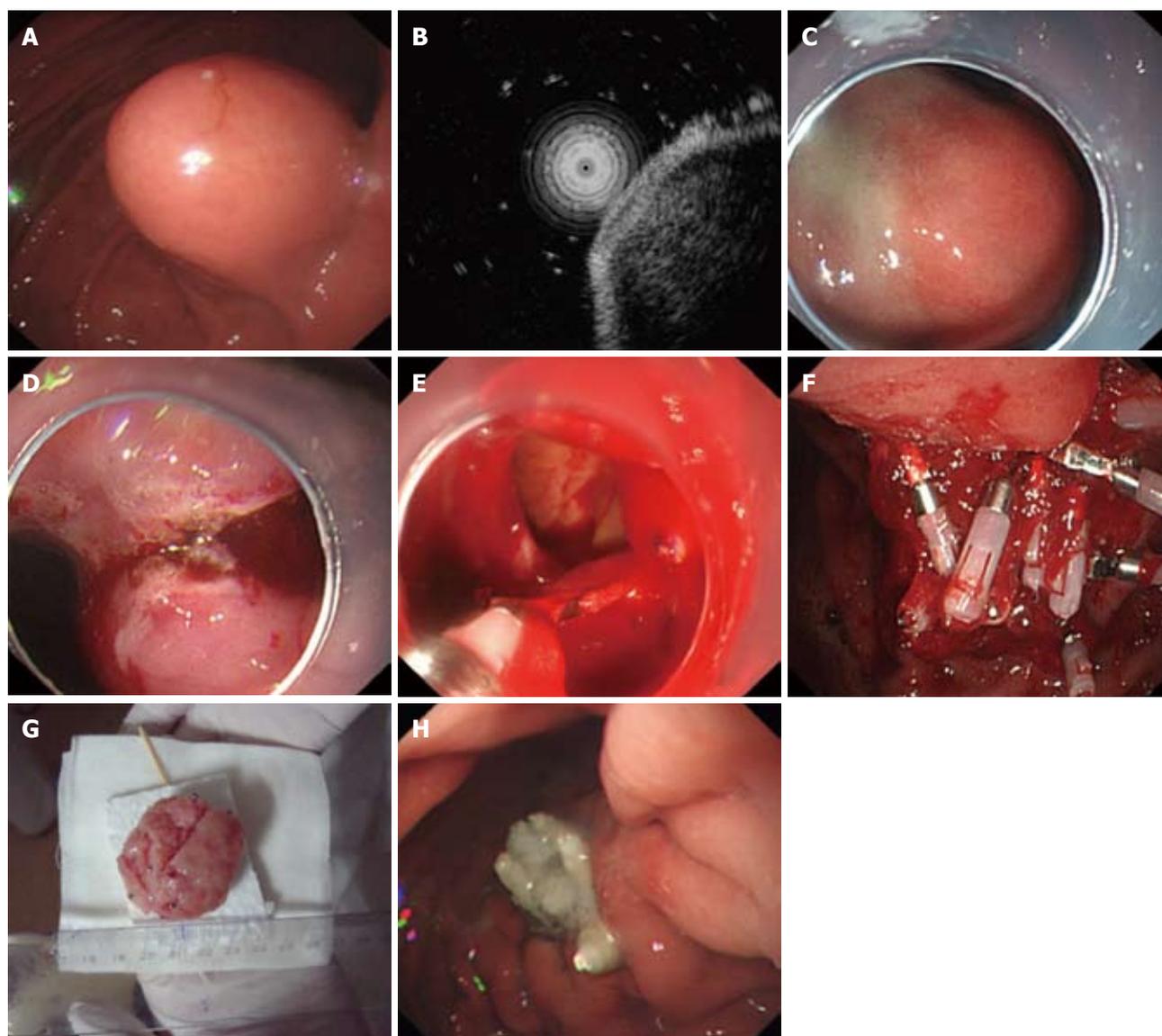


Figure 3 Endoscopic full-thickness resection treatment for a gastric stromal tumor originating from the muscularis propria larger than 1.2 cm. A: Submucosa lesion at the gastric corpus; B: Endoscopic ultrasound shows that the lesion originates from the muscularis propria; C: Submucosal injection of the mixture of indicarninum, adrenalin and physiological saline; D: Dissection with an IT knife; E: “Artificial” perforation after gastric stromal tumor resection, closed with metal clips; F: Many clips used to close the wound defect; G: The resected tumor without mucosa (5 cm in size); H: The perforation healed 9 d after endoscopic full-thickness resection.

Closure of the wound surface of the stomach: a metal clip was used to close the wound surface from the rim to the center. If the wound surface defect was too large to be closed with a metal clip directly, negative pressure aspiration was used to aspirate the omentum majus into the stomach, and then a metal clip was used to close the wound surface defect (Figure 3).

Specimen handling

The resected specimens were fixed in neutral formalin for pathologic diagnosis. Immunohistostaining of CD34, CD117, Dog-1, S-100 and smooth muscle actin (SMA) was performed for specimens suspected of being gastric stromal tumors^[6-10].

Postoperative handling

For patients without perforation, the measures of fast-

ing, infection prevention and limiting acid with proton pump inhibitors were sufficient. For patients with “artificial” perforation, if pneumoperitoneum was serious, evacuation was performed during and after the operation. The evacuating needle was inserted into the lower right quadrant to release abdominal distension. A semi-reclining position was recommended after the operation, along with the use of fasting and gastrointestinal decompression, to allow the perforation to heal. Abdominal pain, abdominal distension and signs of peritoneal irritation after the operation were carefully observed. Meglumine diatrizoate was recommended to be taken orally three days after the operation. To confirm whether there was extravasation of the contrast media and to observe stomach motivation, ultrasonic examination was conducted to observe whether there was hydrops in the abdominal cavity and pelvic cavity. Gastroscopy was

performed one month after the operation to observe the conditions of the wound surface and whether there was residual or recurrence.

RESULTS

Effects of endoscopic therapy

ELR: Thirty-eight patients with gastric stromal tumors with a size of less than 1.2 cm underwent ELR, in which the tumor was ligated wholly and then resected. The procedure was complicated by perforations in 3 patients, and all of the wound surfaces and perforations were closed with metal clips. The patients recovered with medical treatment, and no patient needed surgical operation.

ESE: Eighteen patients with gastric stromal tumors in which the size of the specimen resected was more than 1.5 cm underwent ESE. The tumors were resected successfully, and no perforations complicated the procedure.

EFR: Thirteen patients with gastric stromal tumors, for which the size of the specimen resected was more than 2.0 cm, underwent EFR. The tumors were resected successfully, but “artificial” perforation occurred in all of the patients. All of the perforations were closed with metal clips, and meglumine diatrizoate upper gastroenterography three days after the operation showed that there was no extravasation of the contrast media. Gastroscopy showed that the wound surface of the stomach was nearly healed on the 9th day after the operation (Figure 3H).

Immunohistostaining

The positive expression rates of CD34, CD117, Dog-1, S-100 and SMA in the 69 patients who were diagnosed with gastric stromal tumors by gastroscopy were 81.2% (56/69), 82.6% (57/69), 82.6% (57/69), 0% and 17.4% (12/69), respectively, which demonstrated that, among the 69 patients, there were 12 leiomyomas, which express SMA; the other 57 patients had gastric stromal tumors.

Average stay

The average stay for all of the patients was 8.4 ± 2.4 d.

Residual or recurrence of the tumor

Gastroscopy and abdominal CT scans were performed one month after the operation; these procedures showed that the wound surfaces had healed, and there was no residual or recurrence.

DISCUSSION

Stromal tumor is the most common mesenchyme-originated tumor; as it has potential cancerous tendencies, gastric stromal tumor is recommended to be resected^[11-15]. For gastric stromal tumors originating from the muscularis propria in which the size was less than 1.2 cm, ELR was the ideal method^[16]. After ligation, each

tumor was in the shape of a polypoid with a deuto-stem, which made snare resection easy; additionally, a pathologic diagnosis could be obtained with the resected tumor. Because the wound surface in the stomach was not large, perforation would not generally occur; however, if perforation did occur, it could be closed with metal clips. With the medical treatment of fasting, gastrointestinal decompression and proton pump inhibitors, the patients would recover. There were 38 patients in this study in whom the tumor size was less than 1.2 cm; these patients were treated with ELR. Moreover, 3 of these patients were complicated by perforation; the perforations were closed with metal clips, and no surgical assistance was required. Because the texture of the stromal tumor was hard, it could not be as easily aspirated as mucosa or vascular tissue, so the ligation for the stromal tumor required a long period of aspiration. According to our experience, when the stromal tumor was ligated, a red-colored sign occurred when the tumor was aspirated, and at least another one-minute maximum aspiration was necessary to ensure that the rubber band could fully hitch the tumor.

ESE is another effective measure for the treatment of tumors originating from the submucosa or muscularis propria. For tumors of 3.0 cm or less in size originating from the muscularis propria, the achievement ratio of ESE was high and the patient suffered less, whereas the complication rate was low. ESE was able to excavate the whole tumor successfully, and a pathologic diagnosis could be performed using the resected specimen, which was able to provide a systemic and safe therapeutic method for each patient. In this study, there were 18 patients with gastric stromal tumors originating from the muscularis propria who underwent ESE; all of the tumors were excavated wholly, and no perforations occurred.

For patients with gastric stromal tumors originating from the muscularis propria, the perforation rate of endoscopic therapy is high; therefore, the traditional treatment method is surgical operation or therapeutic laparoscopy, especially for stromal tumors larger than 2 cm^[17-20]. A total of 13 patients in this study underwent EFR, and perforation occurred in all of the patients. The sizes of the 13 tumors were all larger than 2 cm, all of the perforations were closed with metal clips, the abdominal cavity pressure decreased with the use of abdominal paracentesis, all of the patients recovered with medical treatment, and no patients needed surgical help. The key point in avoiding complications was the use of endoscopic metal clips for oversewing; according to our experience, for larger perforations, the perforation should be closed with metal clips from rim to center, known as oversewing, and even some normal mucosa should be sewed up to decrease the perforation. The key point for successful EFR is to mend the perforation successfully, avoiding surgical mending and postoperative peritonitis^[21,22]. The most widely used method to mend perforation is metal clip sutura^[23,24]. For small perforations, one or several metal clips could oversee the per-

foration completely. For larger perforations, as the span of the metal clip is limited, the gas in the stomach must be aspirated properly to reduce the perforation, and several metal clips should be used to close the perforation. If the perforation is too large to be oversewn with metal clips directly, the retina mending method is recommended^[25,26]. The gas in the stomach should be aspirated with continuous negative pressure until the adipose tissue outside the gastric wall covers the perforation, at which point the perforation is closed with metal clips. The other essential point for EFR is to avoid too much gastric juice entering the abdominal cavity; to prevent postoperative infection, strict hemostasis for the wound surface should be performed, but repeat rinse hemostasis should be avoided during the procedure^[27]. The gas and liquid in the stomach should be aspirated fully before cutting the serous membrane. Postoperative gastrointestinal decompression, proton pump inhibitors and antibiotics were effective measures to prevent abdominal cavity infection, and no complications of peritonitis or peritoneal abscesses occurred in this study.

Pneumoperitoneum caused by perforation after EFR can influence the field of vision in the stomach, making endoscopic operation difficult; thus, during EFR, repeated abdominal touch was necessary. If the pressure in the abdominal cavity increased, prompt evacuation was needed; the puncture site was in the right lower quadrant, and the needle was a commonly used 20 mL injection needle. After puncture, the abdomen was compressed to exhaust the air, the needle was detained until the perforation was closed and the pneumoperitoneum had clearly improved, and the needle was removed when it was confirmed that there was no further gas exhausting from the abdominal cavity.

It is difficult to distinguish gastric stromal tumors from leiomyomas and schwannomas based on cell shape. The clinical diagnosis of this tumor type mainly depends on histopathology, immunohistochemistry (CD34- and CD117-positive) and protocols in molecular biology^[28,29]. CD34 is the antigen marker of myeloid stem cells; it resides in the marrow hematopoietic stem cell tissue. CD117 is the product of the *c-kit* oncogene; it is the receptor of tyrosine kinase growth factor. The *Dog-1* gene is located at chromosome 11q¹³; it has 26 exons and is 114 Kb in length; the protein has 8 trans-membrane functional areas; it may be a chloride channel regulated by calcium ions, and it is currently recognized as the most sensitive and specific marker for gastrointestinal stromal tumors (GIST)^[30,31]. Typical leiomyomas and schwannomas do not express CD34; SMA-positive expression is the typical characteristic of leiomyoma, and S-100-positive expression is the typical characteristic of schwannomas^[32]. The positive expression rates of CD34, CD117, Dog-1, S-100 and SMA in the 69 patients were 81.2% (56/69), 82.6% (57/69), 82.6% (57/69), 0% and 17.4% (12/69), respectively, which demonstrated that, among the 69 patients, there were 12 leiomyomas, which express SMA, and the other 57 patients had gastric stro-

mal tumors confirmed with immunohistochemistry. Currently, the main treatment method for GISTs is surgical operation; the majority of benign GISTs in this study had a good prognosis after resection, and there was no recurrence in a short time. GISTs are not sensitive to radiotherapy or chemotherapy. For malignant GISTs that could not be completely resected or with recurrence, patients could take imatinib for therapy. Imatinib is an inhibitor of tyrosine kinase; it has significant therapeutic effects on malignant GISTs that express CD117^[33-37].

With the maturation of EFR, more and more gastric stromal tumors originating from the muscularis propria would avoid surgical operations, sharing the benefits provided by therapeutic endoscopy techniques.

COMMENTS

Background

The traditional method for the treatment of gastric stromal tumor originating from the muscularis propria was laparoscopic or surgical operation; in recent years, with the development of endoscopic techniques and instruments, endoscopic therapy for gastric stromal tumors originating from the muscularis propria has been made possible.

Research frontiers

Gastric stromal tumor is the most common mesenchymal tissue-originated tumor in the digestive tract; based on the tissue of origin, it can be defined as originating from the muscularis mucosae or the muscularis propria. As the location of the tumor originating from the muscularis mucosae was superficial, endoscopic resection or ligation was commonly performed. In contrast, the location of a tumor originating from the muscularis propria was deep, especially when the tumor grew outside gastric wall, perforation often occurred during endoscopic therapy, and the tumor could not be resected easily and thoroughly. Therefore, this tissue of origin was often considered to be a contraindication for endoscopic therapy, and the traditional treatment for such tumors was surgical or laparoscopic operation. In recent years, on the basis of sufficient clinical practice, endoscopic treatment for gastric stromal tumors originating from the muscularis propria has become possible.

Innovations and breakthroughs

Depending on the size of gastric stromal tumors originating from the muscularis propria, three types of endoscopic therapy could be used to treat the tumor. These techniques were endoscopic ligation and resection, endoscopic submucosal excavation and endoscopic full-thickness resection. The wound surface and the perforation of the gastric wall were closed with metal clips. These results showed that some gastric stromal tumor originated from muscularis propria can be treated successfully with endoscopic techniques, which could replace some surgical operations.

Applications

The study results suggest that some gastric stromal tumors originating from the muscularis propria could be treated successfully with endoscopic techniques, which could replace some surgical operations.

Terminology

Gastrointestinal stromal tumor (GIST): This term indicates a particular type of low-intermedius-originating tumor in the gastrointestinal tract; usually CD117 was positively expressed. Histologically, this tumor type is often composed of spindle cells and epithelioid cells. With respect to immunophenotype, it expresses the *c-kit* protein, which is driven by mutated *c-kit* or platelet-derived growth factor receptors. It is recognized that GIST originate from the intestinal cells of Cajal in gastrointestinal tract, which are in the shape of a network structure distributed in the muscular layer of gastrointestinal tract; this network is the pacemaker of slow-wave activity in the gastrointestinal tract and participates in the mechanism of exertional disease and tumor formation in the gastrointestinal tract.

Peer review

This is a good paper on endoscopic resection of gastric stromal tumors and leiomyomas. The figures are excellent.

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Recurrent ischemic strokes in a young celiac woman with *MTHFR* gene mutation

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Abstract

Celiac disease (CD) is frequently associated with neurological disorders, but very few reports concern the association with ischemic stroke. A 26-year-old woman affected by CD with secondary amenorrhea, carrier of a homozygous 5,10-methylenetetrahydrofolate reductase mutation with hyperhomocysteinemia, was affected by two occipital ischemic strokes within a period of 5 mo. At the time of the second stroke, while she was being treated with folic acid, acetylsalicylic acid and a gluten-free diet, she had left hemianopsia, left hemiparesis, and gait imbalance. Brain magnetic

resonance imaging showed a subacute right occipital ischemic lesion, which was extended to the dorsal region of the right thalamus and the ipsilateral thalamo-capsular junction. Antitransglutaminase and deamidated gliadin peptide antibodies were no longer present, while antinuclear antibodies, antineuronal antibodies and immune circulating complexes were only slightly elevated. Since the patient was taking folic acid, her homocysteine levels were almost normal and apparently not sufficient alone to explain the clinical event. A conventional cerebral angiography showed no signs of vasculitis. Finally, rare causes of occipital stroke in young patients, such as Fabry's disease and mitochondrial myopathy, encephalomyopathy, lactic acidosis and stroke-like symptoms, were also excluded by appropriate tests. Thus, the most probable cause for the recurrent strokes in this young woman remained CD, although the mechanisms involved are still unknown. The two main hypotheses concern malabsorption (with consequent deficiency of vitamins known to exert neurotrophic and neuroprotective effects) and immune-mediated mechanisms. CD should be kept in mind in the differential diagnosis of ischemic stroke in young patients.

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Key words: Celiac disease; Female; Methylenetetrahydrofolate reductase; Stroke; Vasculitis; Young

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INTRODUCTION

Celiac disease (CD) is an immune-mediated enteropathy, resulting from a permanent intolerance to the gliadin component in dietary gluten. Its clinical presentation may include signs and symptoms of malabsorption, frequently associated with extra-intestinal manifestations. Several neurological disorders like peripheral neuropathy, cerebellar ataxia and dementia were found to be associated with the disease; epilepsy, bilateral occipital calcifications and rarely stroke^[1-7] have also been reported in CD. This article illustrates the case of a young woman with asymptomatic CD, and carrier of the homozygous mutation of the gene encoding 5,10-methylenetetrahydrofolate reductase (*MTHFR*), who was affected by two occipital strokes within a period of 5 mo.

CASE REPORT

A 26-year-old woman with history of migraine without aura presented acute left hemiparesthesias (prevailing at lips and upper limb), mild weakness in her left arm, gait imbalance, unsteadiness and asthenia, while she was working hard in an archaeological site during a hot day. Since symptoms persisted, on the following day she was transported to the emergency department of our hospital and then admitted to the Stroke Unit.

The patient had a documented positivity of antitransglutaminase and antigliadin antibodies, which she had tested 7 years before because her sister was affected by CD. The patient had no gastrointestinal symptoms, but presented a secondary amenorrhea of at least two years duration. Duodenal biopsy showed a subtotal villous atrophy with an increased number of intraepithelial lymphocytes, so confirming the CD diagnosis. Her treatment consisted of a gluten-free diet, plus an estrogen-progestin therapy that she received for a period of 6 mo. The hormone therapy had been stopped 5 mo earlier when she was hospitalized elsewhere because of a left occipital ischemic stroke (transient right lateral homonymous hemianopsia). On that occasion, laboratory and genetic testing revealed the presence of a homozygous mutation of the *MTHFR* gene with hyperhomocysteinemia (31.5 mmol/L, normal value < 15 mmol/L). The patient was prescribed folic acid and acetylsalicylic acid.

On admission to our hospital, the patient appeared alert, oriented, cooperative and afebrile. Her neck was supple. She had left lateral homonymous hemianopsia, left hemiparesthesias and mild weakness in her left leg.

Investigations

A brain CT scan showed, in addition to the previous

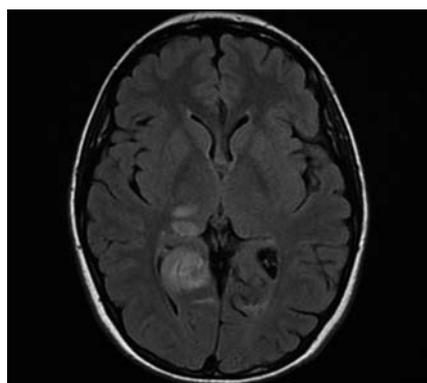


Figure 1 Magnetic resonance imaging showing a subacute right occipital lesion, extended to the dorsal region of the right thalamus and the ipsilateral thalamo-capsular junction.

left occipital infarction, a right occipital low-density area (maximum diameter 40 mm) suggesting an acute ischemic stroke involving the territory of the right posterior cerebral artery. There was no contrast enhancement of these lesions. Brain magnetic resonance imaging (MRI) confirmed the subacute right occipital lesion, which was extended to the dorsal region of the right thalamus and the ipsilateral thalamo-capsular junction (Figure 1). A conventional angiography of the aortic arch, supraortic branches and intracranial arteries showed a substantially normal picture, with poor visualization of the P3 segment and distal branches of the right posterior cerebral artery. In particular, no signs of vasculitis were found. Chest X-ray, abdominal ultrasound, electrocardiogram, electrocardiogram Holter monitoring, transthoracic echocardiogram, supraortic ultrasound and transcranial echo-Doppler with microbubbles to test the presence of patent foramen ovale, were all normal. Moreover, a bicipital muscle biopsy and an MRI spectroscopy of the gastrocnemius muscles before, during and after exercise were performed to test the hypothesis of mitochondrial disease and, in particular, of mitochondrial myopathy, encephalomyopathy, lactic acidosis and stroke-like symptoms (MELAS). Biochemical and molecular studies were performed to assess Fabry's disease. The results of all these investigations were negative.

Laboratory analyses, including complete blood count, C-reactive protein, erythrocyte sedimentation rate, renal function, cobalamin and folate levels, were normal. Serological tests for Human Immunodeficiency Virus, markers of hepatitis B and C, antigranulocyte antibodies (p-ANCA and c-ANCA), cryoglobulins, antiphospholipid antibodies and lupus anticoagulants phenomenon were also normal. The serum markers of CD, antitransglutaminase and deamidated gliadin peptide antibodies were no longer present. However, a control duodenal biopsy was not performed and antinuclear antibodies, anti-neuron antibodies and immune circulating complexes were slightly increased. Factor V Leiden and prothrombin mutation were absent. Similarly, other markers of a possible hypercoagulable status (antithrombin, protein C and

protein S) were normal. A mild hyperhomocysteinemia (16.1 mmol/L) still persisted, despite treatment with folic acid. Other laboratory tests documented secondary hypogonadism and hypothyroidism (increased FSH, LH, TSH and reduced FT4).

During hospitalization, there was a gradual improvement of neurological symptoms. The previous treatment with acetylsalicylic acid (100 mg/d) was replaced with clopidogrel (75 mg/d), and L-thyroxine (50 mcg/d) was started. Folic acid (5 mg/d), in addition to a gluten-free diet, was confirmed. At the time of discharge, the left lateral homonymous hemianopsia was still present. Five months after admission, the neurological picture was unchanged, but the patient was able to return to her job. There were no new acute cerebrovascular events.

DISCUSSION

Ischemic strokes occurring in patients younger than 30 years are a relatively rare event. The most important causes of cerebral ischemia in the young are cardioembolism, dissection of extracranial arteries, migraine, drugs and hypercoagulable states. Furthermore, premature atherosclerosis can also play an important etiological role. Uncommon causes include vasculitis, metabolic disorders and inherited diseases. However, the undetermined etiology is the most frequent reported cause of ischemic stroke in young patients^[8]. In this case report, we have considered in the differential diagnosis two rare causes of occipital stroke in young adults: MELAS syndrome and Fabry's Disease. MELAS^[9] is a progressive disease characterized by acute neurological events resembling cerebral ischemia, associated with mitochondrial myopathy and hyperlactataemia. Ten different mutations of mitochondrial DNA have been identified, but most cases (about 80%) are due to the A3243G mutation encoding the transfer RNA (tRNA) of leucine. CT and MRI often show lesions located in the occipital lobes, which are sometimes associated with brain atrophy and intracranial calcifications. Increased levels of lactate are often present in the blood, and almost constant in the cerebrospinal fluid. Muscle biopsy is abnormal in 85% of patients, showing atypical mitochondrial proliferation with presence of "ragged red fibers" at the Gomori's stain. The diagnosis is based on mitochondrial DNA analysis. Fabry's disease^[10] is a progressive multisystem inherited disease of glycosphingolipid metabolism. It is caused by mutations in the gene of α galactosidase A (chromosome Xq22). Its classic form, which mainly affects hemizygous males, is characterized by angiokeratomas, renal insufficiency with proteinuria, cardiomyopathy, and cochlear, vestibular and cerebrovascular symptoms. The final diagnosis for hemizygous males is based on the demonstration of α -galactosidase A deficiency, while in heterozygous females enzyme analysis is often inconclusive and genetic analysis is needed. In the present case, the diagnostic investigations performed for both MELAS and Fabry's disease were negative.

Our patient presented mild hyperhomocysteinemia, which is recognized as an independent risk factor for cardiovascular diseases^[11], acting through several possible prothrombotic mechanisms including the toxic action on vascular endothelium resulting in endothelial dysfunction, enhancement of both platelet adhesion and aggregation, endothelial factor V activation, protein C inhibition, and reduction of antithrombin III levels. Hyperhomocysteinemia may result from reduced elimination due to renal insufficiency, from inadequate intake or malabsorption of folic acid and B vitamins, or from a mutation of genes coding for one of the three enzymes involved in homocysteine metabolism: cystathionine β synthetase (CBS), MTHFR and methionine synthetase^[12]. The *MTHFR* gene mutation was carried by our patient. This is a fairly common condition in the Caucasian population, with a prevalence of 8%-15% for the homozygous genotype and 65% for the heterozygous genotype. It does not seem to be more prevalent among patients with CD^[13,14]. The *MTHFR* gene is located in the short arm of chromosome 1. Its mutation may be a cardiovascular risk factor only in subjects with a low intake of folic acid. At the time of the second stroke the patient was already taking folic acid. Thus, her homocysteine levels were only slightly elevated and apparently not sufficient alone to explain the clinical event.

Since no other cerebrovascular risk factors were found, we concluded that CD could be involved in the pathogenesis of recurrent strokes in this patient. CD is an immune-mediated disorder of the small bowel, resulting from a permanent gluten intolerance. Its prevalence in the general population is about 1% and its presentation may be at any age: childhood, adolescence and also adulthood. The clinical picture of CD varies greatly, ranging from asymptomatic forms, to the "classic symptomatic" form with mainly intestinal symptoms, to "atypical" forms dominated by extra-intestinal symptoms^[15]. The systemic involvement of CD may include skin, thyroid, pancreas, heart, joints, muscles, bones, reproductive system, liver, central and peripheral nervous system^[16]. Approximately 10% of CD patients show neurological symptoms, including idiopathic cerebellar ataxia, peripheral neuropathy, various forms of epilepsy, myoclonus, migraine and dementia^[17-20].

There are two main possible hypotheses concerning the pathogenesis of neurological manifestations in CD. Firstly, malabsorption with consequent deficiency of vitamins known to exert neurotrophic and neuroprotective effects (especially folic acid, vitamin B12, vitamin E) has long been thought to contribute to the neurodegenerative changes observed in patients with CD-related neurological manifestations. However, even plenty of vitamin supplementation does not improve the clinical course in these patients. Secondly, an emerging hypothesis is the possible participation of immune-mediated mechanisms. This hypothesis is strengthened by the evidence of lymphocytic infiltration of the central and peripheral nervous system^[21-22] and the presence of serum antineuronal

antibodies^[23] in CD patients with neurological complications. In this regard, neurological manifestations might result from a combination of sub-threshold neurotoxic effects caused by gluten-related immune markers (e.g., antigliadin antibodies, deamidated gliadin peptide antibodies and antibodies to one or more tissue transglutaminase isoenzymes) in combination with antibodies directed against nervous system epitopes, including anti-Purkinje cell and antiganglioside antibodies^[24]. However, the role of these antibodies in the pathogenesis of neurological dysfunction is not yet understood, even if there are elements supporting a mitochondrial-dependent neuronal apoptosis^[25].

Although the association between CD and ischemic stroke has been described by some authors, a causal link between these two conditions has never been demonstrated^[1-7]. Rush *et al*^[1] described the case of a 51-year-old white man affected by CD who had an ischemic stroke with tonic-clonic seizures, resulting from a vasculitis of the central nervous system that was documented by brain biopsy. Also, Ozge *et al*^[2] hypothesized that a vasculitis of the central nervous system could be a possible explanation for the association between CD and stroke. Moreover, Goodwin *et al*^[4] described the case of a child with CD and recurrent episodes of transient hemiplegia, suggesting an autoimmune vascular disease as the underlying mechanism. In fact, tissue transglutaminase, which is the major autoantigen of CD^[26], is also present in the central and peripheral nervous system, and it is thought to maintain vascular endothelial integrity. Pratesi *et al*^[27] found that anti-endomysium IgA antibodies, which were shown to bind to transglutaminase^[28], reacted with human brain vessel structures. This suggests an autoimmune mechanism for the association between CD and ischemic stroke. This hypothesis has also been proposed by Dogan *et al*^[7], who presented a case of stroke and dilated cardiomyopathy associated with CD.

As far as the present case is concerned, the cause of recurrent ischemic strokes remains uncertain. The negativity of indices of inflammation and autoantibodies would exclude systemic vasculitis. The hypothesis of a primary vasculitis of the central nervous system still remains. In fact, this condition cannot be ruled out even by the negativity of cerebral angiography^[29], and also cerebral biopsy is of limited value for the diagnosis due to its low sensitivity^[29]. However, in addition to the lack of small vessel involvement on neuroimaging, the benign course of the disease in our patient in the absence of immunosuppressive treatment makes this hypothesis unlikely.

In conclusion, although the involved mechanisms are still unclear, CD should be considered as a potential etiology of stroke of unknown cause, particularly in young patients, even in the absence of gastrointestinal manifestations.

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Endoscopic diagnosis of Barrett's esophagus

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Abstract

The Prague C and M Criteria have been developed for the objective endoscopic diagnosis of Barrett's esophagus (BE). BE arises between the squamocolumnar junction and the gastroesophageal junction at the proximal margin of the gastric folds. In this study, we reported that 43.0% of the subjects examined were diagnosed with BE based on the Prague C and M Criteria. Previous criticism by John Dent proposed that our data should be considered invalid because the prevalence of BE reported in our study was extraordinarily high and discordant with previous studies. Dent predicted that the position of the gastroesophageal junction in our study was judged to be lower than the actual position due to the effacement of the proximal ends of the gastric folds because of the routine use of a high degree of air distension during typical Japanese

endoscopic examinations. The endoscopic evaluation of the superior gastric folds is certainly influenced by the degree of air distension of the esophagus. However, in our study, the proximal limit of the gastric mucosal folds was prospectively imaged while the esophagus was minimally insufflated. Then, under a high level of air distension, the distal ends of the palisade-shaped longitudinal vessels were imaged because they are more easily observed when distended. In the majority of patients, the distal ends of the palisade-shaped longitudinal vessels correspond to the proximal limit of the gastric mucosal folds. Our endoscopic evaluation was appropriately performed according to the Prague C and M Criteria. We suspect that the high prevalence of BE in our study may be due to the inclusion of ultra-short-segment BE, which defines BE with an affected mucosal length under 5 mm, in our positive results.

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Key words: Barrett's esophagus; Gastroesophageal junction; Squamocolumnar junction; Digital endoscopic images; Endoscopy

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TO THE EDITOR

In our previously published retrospective study^[1], we reported that a total of 43.0% [short-segment Barrett's esophagus (SSBE, 42.6%); long-segment Barrett's esophagus (LSBE, 0.5%)] of the patients examined were diagnosed with Barrett's esophagus (BE) based on the Prague

C and M Criteria^[2]. The study population included 869 patients, of whom 463 were men and 406 were women. The median age of the patients was 66 years and the patient age ranged from 29 to 91 years. The study population consisted of patients who underwent upper gastrointestinal endoscopic examination as part of a routine health check at the Yokohama City University Hospital. During endoscopic examination and imaging of the esophageal mucosa, the gastroesophageal junction (GEJ) was prospectively photographed prior to air distension. All of the endoscopic images were digitalised and were independently and retrospectively reviewed by two endoscopists trained to diagnose BE based on the Prague C and M Criteria^[2]. Any inconsistencies in the assessment of the endoscopic images were resolved by a joint review of the questionable endoscopic images by the two endoscopists. The Prague C and M Criteria for the endoscopic diagnosis of BE is based on the presence of columnar-appearing mucosa between the squamocolumnar junction and the GEJ, which composes the proximal margin of the gastric folds.

The findings of our study are consistent with a previous Japanese study on the occurrence of BE diagnosed based on the Prague C and M Criteria^[3]. The reported frequency of BE could be affected by differences in the interpretation of what constitutes BE, especially with regard to whether histological confirmation of specialised intestinal metaplasia of the esophagus is required. Many physicians from Western countries believe that confirmation of intestinal metaplasia by an esophageal biopsy is necessary to correctly identify BE^[4] because this condition is considered a risk factor for esophageal adenocarcinoma^[5]. However, in our study, BE was diagnosed endoscopically based on the Prague C and M Criteria without histological confirmation; therefore, positive results were referred to as endoscopic BE.

In a recent review article, Dent^[6] opined that our study appeared to be fatally flawed to the extent that our data should be considered invalid. His rationale was that the reported prevalence of BE in our study was extraordinarily high and discordant with previous studies. Dent predicted that the position of the GEJ was judged to be lower than its actual position due to the effacement of the tops of the gastric folds by the routine use of high levels of air distension during our endoscopic explorations.

The endoscopic evaluation of the proximal limit of the gastric folds is certainly influenced by the degree of air distension of the oesophagus. Furthermore, the American Gastroenterological Association workshop in Chicago has concluded that the proximal limit of the gastric mucosal folds is best visualised when the esophagus is minimally distended, although further work

may be required to define minimal insufflation^[2]. When endoscopically examining and imaging the GEJ, our protocol includes the prospective imaging of the proximal limit of the gastric mucosal folds while the esophagus is minimally insufflated. Then, we image the extreme distal ends of the palisade-shaped longitudinal vessels, which are considered topographical landmarks for the GEJ, under high levels of esophageal air distension^[7]. The distal ends of the palisade-shaped longitudinal vessels are much more easily observed under high levels of oesophageal air distension than when the oesophagus is minimally insufflated. In most study populations, the most distal ends of the palisade-shaped longitudinal vessels correspond to the proximal limit of the gastric mucosal folds.

Therefore, our endoscopic evaluation of the position of the GEJ was appropriately performed according to the Prague C and M Criteria without histopathological examination. We suspect that the relatively high prevalence of BE reported in our study may be due to the inclusion of ultrashort-segment BE, which defines BE with an affected mucosal length under 5 mm, in our positive results.

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 The International Liver Congress
 2012
 Barcelona, Spain

April 19-21, 2012
 Internal Medicine 2012
 New Orleans, LA 70166,
 United States

April 20-22, 2012
 Diffuse Small Bowel and Liver
 Diseases
 Melbourne, Australia

April 22-24, 2012
 EUROSON 2012 EFSUMB Annual

Meeting
 Madrid, Spain

April 28, 2012
 Issues in Pediatric Oncology
 Kiev, Ukraine

May 3-5, 2012
 9th Congress of The Jordanian
 Society of Gastroenterology
 Amman, Jordan

May 7-10, 2012
 Digestive Diseases Week
 Chicago, IL 60601, United States

May 17-21, 2012
 2012 ASCRS Annual Meeting-
 American Society of Colon and
 Rectal Surgeons
 Hollywood, FL 1300, United States

May 18-19, 2012
 Pancreas Club Meeting
 San Diego, CA 92101, United States

May 18-23, 2012
 SGNA: Society of Gastroenterology
 Nurses and Associates Annual
 Course
 Phoenix, AZ 85001, United States

May 19-22, 2012
 2012-Digestive Disease Week
 San Diego, CA 92121, United States

June 2-6, 2012
 American Society of Colon and
 Rectal Surgeons Annual Meeting
 San Antonio, TX 78249,
 United States

June 18-21, 2012
 Pancreatic Cancer: Progress and
 Challenges
 Lake Tahoe, NV 89101, United States

July 25-26, 2012
 PancreasFest 2012
 Pittsburgh, PA 15260, United States

September 1-4, 2012
 OESO 11th World Conference
 Como, Italy

September 6-8, 2012
 2012 Joint International

Neurogastroenterology and Motility
 Meeting
 Bologna, Italy

September 7-9, 2012
 The Viral Hepatitis Congress
 Frankfurt, Germany

September 8-9, 2012
 New Advances in Inflammatory
 Bowel Disease
 La Jolla, CA 92093, United States

September 8-9, 2012
 Florida Gastroenterologic Society
 2012 Annual Meeting
 Boca Raton, FL 33498, United States

September 15-16, 2012
 Current Problems of
 Gastroenterology and Abdominal
 Surgery
 Kiev, Ukraine

September 20-22, 2012
 1st World Congress on Controversies
 in the Management of Viral Hepatitis
 Prague, Czech

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

November 3-4, 2012
 Modern Technologies in
 Diagnosis and Treatment of
 Gastroenterological Patients
 Dnepropetrovsk, Ukraine

November 4-8, 2012
 The Liver Meeting
 San Francisco, CA 94101,
 United States

November 9-13, 2012
 American Association for the Study
 of Liver Diseases
 Boston, MA 02298, United States

December 1-4, 2012
 Advances in Inflammatory Bowel
 Diseases
 Hollywood, FL 33028, United States

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1352 experts in gastroenterology and hepatology from 64 countries.

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The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systematically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensus and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

Name of journal

World Journal of Gastroenterology

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Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

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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. Immunolog-

ic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

In press

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

Organization as author

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

Both personal authors and an organization as author

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

No author given

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

Volume with supplement

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

Issue with no volume

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

No volume or issue

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

Books

Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary 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. Wiecezorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

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

Conference paper

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

Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious dis-

eases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

Patent (list all authors)

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

Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

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

Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose) 6.4 \pm 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 24.5 μ g/L; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

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

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Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kbo I*, *Kpn I*, etc.

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Evolution of continent ileostomy

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Abstract

Continent ileostomy can be defined as a surgical procedure that facilitates planned intermittent evacuation of a bowel reservoir through an ileostomy. It was devised by Nils Kock in 1969. Subsequently, continent ileostomy (or Kock pouch) became a viable alternative in the management of patients who had traditionally required an end ileostomy. Kock pouch appeared to provide substantial physical and psychosocial benefits over a conventional ileostomy. The procedure became popular until ileal pouch anal anastomosis (IPAA) was introduced in 1980. Despite its benefits, continent ileostomy had many short term complications including intubation problems, ileus, anastomotic leaks, peritonitis and valve problems. Operative mortalities have also been reported in the literature. Most of these problems have been eliminated with increasing experience; however, valve-related problems remain as an "Achilles' heel" of the technique. Many modifications have been introduced to prevent this problem. Some patients have had their pouch removed because of complications mainly related to valve dysfunction. Although revision rates can be high, most of the patients who retain their reservoirs are satisfied with regard to their health status and quality of life. Today, this procedure is still appropriate for selected patients for whom pouch surgery is not possible or for patients who have failed IPAA. Both the patient and their physician must be highly motivated to

accept the risk of failure and the subsequent need for revisional operations.

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Key words: Continent ileostomy; Kock pouch; Ileal reservoir; Surgical technique

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INTRODUCTION

Continent ileostomy (CI) allows planned evacuation of the small bowel through a reservoir equipped with a nipple valve. The operation was first reported by Kock in 1969^[1]. Subsequently, CI (or Kock pouch) became a viable alternative to conventional end ileostomy. This new procedure was met with initial skepticism since most patients did well with a Brooke type end ileostomy^[2]. However, some complained that an external appliance was inconvenient, some experienced complications from the conventional ileostomy, while others sought alternative procedures because of sexual and social reasons. The procedure became popular until ileal pouch anal anastomosis (IPAA) was introduced in 1980^[3]. Today, the Kock pouch is still appropriate for selected patients with ulcerative colitis (UC) and familial polyposis who are not candidates for IPAA or for those patients who have failed IPAA^[4-6].

THE EVOLVING TECHNIQUE OF CONTINENT RESERVOIR

Kock's original pouch design did not have a continence

valve mechanism (Figure 1). A segment of ileum is opened longitudinally and sutured together to create U-shaped limbs. The pouch is formed with vertical folding. A corner of the reservoir is sutured to the skin of the abdominal wall. Next, an efferent limb is added to the reservoir. His initial intent was to use the rectus abdominis muscle to serve as a constricting force around the efferent loop of bowel and thus provide continence; some of the patients achieved continence. Later, a nipple valve was provided by retrograde intussusception of the efferent limb into the pouch (Figure 2). This configuration achieved complete continence. Many surgeons have adopted this technique^[7,8]. All have expressed major concern in regard to the incidence of valve slippage. Dessusception of the valve (slippage) has been the most frequent complication in the long-term follow-up studies^[9-14]. Total valve slippage renders the pouch incontinent; partial slippage makes intubation difficult or impossible.

In the following years, many modifications of operative technique were proposed to overcome this problem. Steichen introduced the use of staples for valve stabilization^[15]. Fazio also described a stapled technique for nipple valve fixation^[16]. These modifications have led to a decrease in the incidence of valve slippage (Figure 3). Later on, synthetic materials such as polypropylene (Marlex) or polyester (Mersilene) were also used to buttress the valve mechanism. The valve slippage problem was largely controlled but the use of these materials could lead to fistula formation^[17,18].

Barnett introduced an isoperistaltic valve to avoid the slippage problem^[19]. In his technique, an afferent limb of bowel is used to construct a nipple valve with isoperistaltic intussusception. He initially reported that none of his sixteen isoperistaltic CI patients needed reoperation for valve slippage during three years of follow-up. Barnett's later works^[20] and a Cleveland Clinic study^[21] revealed that valve slippage occurs whether it is constructed in an isoperistaltic or anisoperistaltic fashion. The weakest point of the valve is on the mesenteric side where intussusception produces a large bulky mass that leads to slippage (Figure 4). Barnett was convinced that a support was absolutely necessary to avoid the problem and sought a suitable material that would not erode the valve. In 1986, he added an intestinal segment around the exit conduit to control valve slippage. This "living intestinal collar" buttressed the mesenteric side of the valve where slippage is initiated (Figure 5). The Barnett continent intestinal reservoir, including an intestinal collar and an isoperistaltic valve, has reduced the incidence of valve slippage and fistula formation^[22]. Because of the complications related to valve intussusception, Stein developed a new design pouch incorporating a serosal lined ileal antireflux mechanism for urinary diversion after cystectomy in 1998^[23]. Preliminary results of this innovative flap valve mechanism reservoir (I-pouch) indicated complete continence of the pouch but long-term results have not been reported^[24].

A novel CI has been reported by the authors^[25]. The porcine model was used to create a valveless reservoir

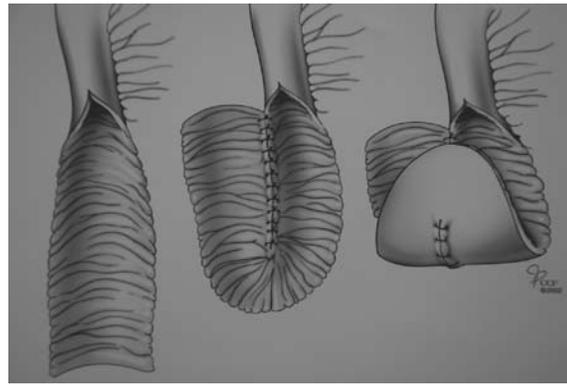


Figure 1 Original Kock pouch design without valve mechanism.

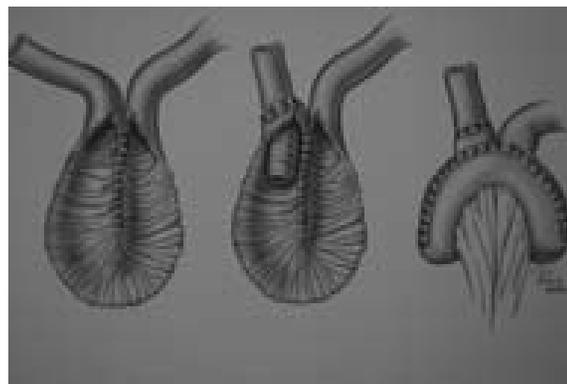


Figure 2 Continent Kock pouch.

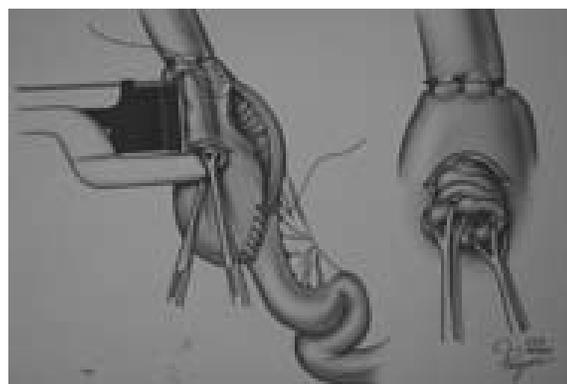


Figure 3 Stapled technique for nipple valve fixation.

and its integrity was tested. Although promising results have been achieved with this simple technique, the method has not been tested in humans.

LONG-TERM RESULTS AND QUALITY OF LIFE

Berglund *et al*^[26] and Ojerskog *et al*^[27] published their long-term experiences of CI with or without valve mechanism. A total number of 435 patients had been provided with a CI; of these 50% had their pouch constructed at the time of proctocolectomy (one-stage operation). These

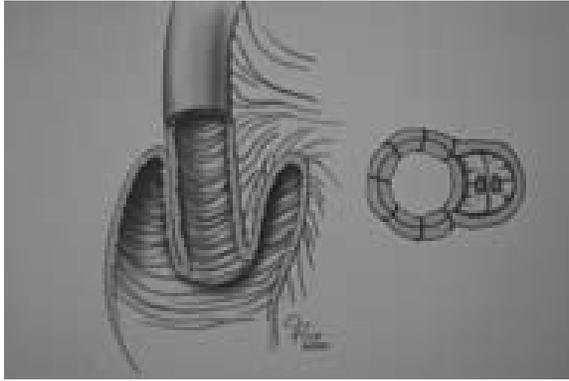


Figure 4 Valve intussusception produces a large bulky mass leading to slippage.

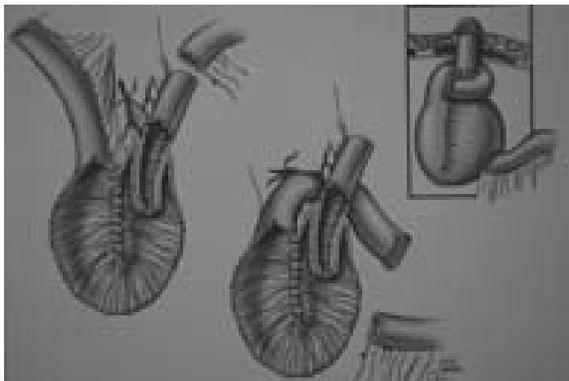


Figure 5 Barnett continent intestinal reservoir.

pioneers of CI reported a high complication rate (23%) and operative mortality (4.3%) in their early series but these figures dropped to a complication rate of 8% with no mortality in the later period. The long-term complications of CI were mainly related to the valve mechanism, including sliding of the nipple valve, fistula formation, partial or total prolapse of the valve, necrosis of the valve and/or the outlet, stricture of the stoma and inflammatory changes in the reservoir (pouchitis). Valve revisions were performed within the first postoperative year in most of the patients. They concluded that patients with CI have perfect continence unless they develop complication in the valve mechanism.

The long-term results of CI were evaluated in 129 consecutive patients who had this procedure performed by one surgeon at the University of California, San Francisco, between 1975 and 1995^[28]. The primary disease was UC in 119 (92%) patients, familial adenomatous polyposis (FAP) in 7 (5%), and multiple neoplasms in one (1%). Patient satisfaction, pouch durability and the details of the operation were evaluated using a modified 36-item short form health survey, phone calls, chart review and the personal records of the surgeon. Mean follow-up was 11.4 years (range: 1-21 years) and the late outcome was available for 85 patients (66%). Thirty-three of the patients (39%) had undergone an elective one-stage operation with total proctocolectomy and CI, whereas the

remainder had undergone emergent and elective procedures prior to CI creation. Of these patients, 54 (64%) had a functional pouch, whereas 31 patients (36%) had their pouch excised because of complications mainly related to valve dysfunction. The important feature of this study was the overall pouch failure rate which was significantly higher for those of the patients who had their CI as a secondary procedure. Despite many revisions, 97% of the survey respondents who retained their pouch were very satisfied and considered their quality of life to be good or excellent.

The Cleveland Clinic study described 330 patients who underwent Kock pouch surgery between 1977 and 2001^[21]. The mean patient age at the time of surgery was 34.9 years (range: 14-65 years) with a male to female ratio of 1:1.3. Indications for surgery included UC in 251 patients (76%); Crohn's disease in 42 (12.7%); FAP in 23 (7%); indeterminate colitis in five (1.5%); and other diagnoses including colonic inertia, Hirschsprung disease, rectal villous adenoma, and imperforate anus in nine (2.8%). Patients were followed for a median of 11 years (range: 1-27 years); during this period, 51 (16.6%) patients had their Kock pouch excised. At 10 years, 87% of all patients maintained their pouches. At 20 years, 77% of all patients still had their pouches. The 10-year and 20-year continent pouch survival for UC and FAP disease patients were 92.5% and 83.3%, respectively. Patients with Crohn's disease or indeterminate colitis had 10-year and 20-year pouch survival rates of 58.4% and 39.1%, respectively. The median complication-free pouch interval was 7 mo; the median revision-free pouch interval was 14 mo. Valve slippage was the most frequent complication leading to pouch revision (29.7%). The second most common complication was fistula formation (25.2%). The median time to valve slippage was two years. Parastomal hernia occurred in 15.5% of patients. Complete valve prolapse and stoma stricture were other complications. Patients had an average of 3.7 complications (range: 1-28 complications) and 2.9 pouch revisions (range: 1-27 pouch revisions) during their follow-up period. Patients undergoing staged procedures (such as prior restorative proctocolectomy, proctocolectomy with end ileostomy or ileorectal anastomosis) were 1.4 times more likely to undergo pouch revision compared with patients undergoing a single stage operation.

Quality of life for Kock pouch patients was evaluated in the Cleveland Clinic study. Patients with an end ileostomy were more than twice as likely to report social, work, and sexual restrictions compared with CI patients. End ileostomy patients reported higher antidiarrheal medication and fiber intake. A higher percentage of patients with CI reported having a better appetite. In addition, a higher proportion of end ileostomy patients reported having pouch-related complications. Finally, patients with CI were more likely to have the same procedure again and would recommend the procedure to someone else in comparison to the end ileostomy group of patients.

Wasmuth *et al.*^[29] evaluated 50 patients who underwent Kock CI between 1983 and 2002. The reoperation and

pouch excision rates were 44% and 8%, respectively. Re-operation rate was higher among the patients having CI as a second procedure. The Mount Sinai group evaluated 31 patients who had revisions after more than 10 years of normal Kock pouch function^[30]. Delayed pouch failure reasons were mostly related to the valve, and the pouch salvage rate was 93% with good functional outcome. Another study from Holland compared CI patients with a historical group of IPAA patients and end ileostomy patients^[31]. They found that the quality of life of CI patients was not significantly better or worse than that of the patients with either a conventional ileostomy or an IPAA.

FUTURE OF THE CONTINENT ILEOSTOMY

CI studies have revealed that this procedure has many short and long-term complications, mainly related to the valve mechanism. Despite many revisions, patients who retained their pouch were very satisfied and considered their quality of life to be good or excellent. There is a need to develop a safe and reliable continent abdominal reservoir because there are still indications for the CI. These procedures have to be performed in specialized centers.

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Natural history of Barrett's esophagus

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Abstract

The natural history of Barrett's esophagus (BE) is difficult to quantify because, by definition, it should describe the course of the condition if left untreated. Pragmatically, we assume that patients with BE will receive symptomatic treatment with acid suppression, usually a proton pump inhibitor, to treat their heartburn. This paper describes the development of complications of stricture, ulcer, dysplasia and adenocarcinoma from this standpoint. Controversies over the definition of BE and its implications in clinical practice are presented. The presence of intestinal metaplasia and its relevance to cancer risk is discussed, and the need to measure the extent of the Barrett's epithelium (long and short segments) using the Prague guidelines is emphasized. Guidelines and international consensus over the diagnosis and management of BE are being regularly updated. The need for expert consensus is important due to the lack of randomized trials in this area. After searching the literature, we have tried to collate the important studies regarding progression of Barrett's to dysplasia and adenocarcinoma. No therapeutic studies yet reported show a clear reduction in the development of cancer in BE. The effect of pharmacological and surgical intervention on the natural history of Barrett's is a subject of ongoing research,

including the Barrett's Oesophagus Surveillance Study and the aspirin and esomeprazole cancer chemoprevention trial with interesting results. The geographical variation and the wide range of outcomes highlight the difficulty of providing an individualized risk profile to patients with BE. Future studies on the interaction of genome wide abnormalities in Barrett's and their interaction with environmental factors may allow individualization of the risk of cancer developing in BE.

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Key words: Barrett's esophagus; Columnar lined esophagus; Dysplasia; Adenocarcinoma; Gastroesophageal reflux; Surgery

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INTRODUCTION

Barrett's esophagus (BE) is commonly defined as the replacement of esophageal squamous epithelium with metaplastic columnar epithelium, from the gastroesophageal junction proximally, that has been visualized endoscopically and confirmed histologically^[1-3]. The importance of the diagnosis of BE lies in the fact that it is known to increase the future risk of developing adenocarcinoma^[4]. The presence of BE is also associated with patients who have a more severe degree of acid and bile reflux compared to patients with gastro-esophageal reflux

disease without Barrett's columnar lining in their esophagus^[5,6]. This has implications in their clinical management.

There is debate about the degree to which intestinal metaplasia (IM) of the columnar-lined esophagus increases the risk of esophageal adenocarcinoma, and some observers have included the presence of IM in the definition of BE^[3,7]. Some authorities, however, do not specify IM as they believe its absence is only a reflection of sampling error and that it will invariably be present if meticulously searched for^[8-10]. It is clear that the presence of IM is common in patients who have no other diagnostic criteria of BE^[11,11], and without a consistent endoscopic abnormality the diagnosis of IM on biopsy only may have no clinical relevance. Patients with BE have a range of histological abnormalities including gastric metaplasia (fundic and/or body) and non specialized IM, often with a mosaic of different cell types spread across the epithelium.

SYMPTOMS

Patients with BE usually present with symptoms of gastroesophageal reflux disease (GERD) or its complications^[12]. Amongst a cohort of 309 BE patients described by Rudolph *et al*^[13], 98.6% reported a history of heartburn or acid regurgitation spanning at least a decade or more. Lieberman *et al*^[14] further confirmed the correlation between a long history of GERD and the presence of BE. Also, patients with uncomplicated BE seem to have less symptoms than those who have esophagitis without BE^[6]. Patients with BE have a greater frequency and severity of defective anti-reflux mechanisms^[15].

Symptom correlation with onset or progression of BE is very poor. This could be due to the observation that patients with BE have an alteration in their pain perception and thus repeated reflux events and associated tissue injury remain asymptomatic to the patient^[16]. In a prospective non randomized study of 35 patients with low grade dysplasia (LGD) in BE, only 63% had typical symptoms of GERD and 15% had no predominant symptom^[17]. Also, up to 40% of patients with BE-associated esophageal adenocarcinoma do not have reflux symptoms^[18]. Only a minority of patients with reflux symptoms develop BE. In a large prospective study of GERD patients followed up for several years, BE was found in 11% of the studied population of 6250 patients^[19], and in the recent LOTUS trial BE was diagnosed in 10.8% of a population of 554 patients with chronic reflux symptoms^[20].

Screening studies for BE in asymptomatic subjects^[21,22] use the definition of BE which relies on the endoscopic appearance of salmon pink mucosa plus the microscopic diagnosis of IM. In the absence of a defined endoscopic abnormality, they use the term specialized IM of the esophagogastric junction. The introduction of the presence of IM and the circular reasoning of assuming that any IM defines BE has created a degree of confusion in the clinical epidemiology of this condition. The abnormality of BE which has a clinical relevance has been

the endoscopic diagnosis of columnarization. When this columnarization is > 3 cm long, the likelihood that this is a hiatus hernia diminishes. When IM is found, the risk of cancer is considered greater, and, any patient with > 3 cm length of BE is likely to eventually show IM on surveillance endoscopy even if the first series of biopsies are negative. Thus an overemphasis on the presence of IM is very unhelpful. Therefore, the endoscopic finding should be the primary recorded abnormality, supported by histology.

Endoscopic features should be reported according to the Prague C and M criteria described by Sharma *et al*^[23] with precise definitions of endoscopic abnormalities, including the tongues, the circumferential extent and the position and extent of the associated hiatus hernia. The criteria include assessment of the circumferential (C) and maximum (M) extent of the endoscopically visualized BE segment, as well as endoscopic landmarks. This is very useful for long-term follow-up of individual patients and for standardizing results in clinical trials. The data in almost all of the studies reported in this review have not been recorded within the standards of the Prague classification, but future studies should uphold this current standard.

Onset of diagnosis and observation on natural history of BE over time

BE develops in the distal esophagus following tissue injury due to GERD. It is believed to be an acquired condition because of its association with more severe forms of GERD, its prevalence in older patients^[24] and the evidence from animal models^[25]. Two theories have been proposed for the evolution of BE.

Progressive theory: Amongst the proposed theories for the evolution of BE, the progressive theory is the most supported. Microscopic changes first start in the squamocolumnar junction in the form of a change from neutral to acid mucin production and formation of goblet cells. This is then subsequently visible as a columnar lined esophagus of varied length depending on the duration and severity of reflux^[26]. Thereafter, the segment length and the degree of differentiation of cells progresses according to the stimulus to which it is exposed. BE may occur after resection of the lower esophagus as observed by Hamilton *et al*^[27] and also in the upper esophagus as seen in patients who have survived cancer resections for BE-induced adenocarcinoma^[28].

Instantaneous field change theory: Most patients with BE do not demonstrate a significant increase in the length of the affected segment with time^[24,29]. This observation and the lack of good evidence for the progression of BE led to an alternative hypothesis suggested by Cameron *et al*^[24]. This instantaneous field change theory proposed that in response to a specific reflux injury, there is immediate change in the lining of the esophagus of a certain length which then remains constant. However, there is in-

creasing evidence to suggest that there is progression of BE (with regard to segment length and de-differentiation) with time^[30-32], and that long segment BE has more severe acid exposure than short segment BE^[33].

Male Caucasians (non-Hispanic whites) in the age range 60-70 years have consistently been shown to have a higher incidence of BE^[34,35]. Not surprisingly, the prevalence of BE, particularly the long segment type, is low in East Asians^[36]. Also, although BE is considered to affect the elderly, this trend seems to be changing. In a retrospective analysis of 7220 patients with BE, the mean age of diagnosis of BE had decreased between the years 1990 and 2005, with an increase in newly diagnosed BE patients below the age of 50 years^[37]. Guardino *et al.*^[38] also found that 25% of BE patients from their 837 patients registry were younger than 50 years of age. These differences in the demographics of patients with BE has not been explained by any study yet although it has implications in surveillance programs. Future studies need to address the influence of increased availability of endoscopy, lower threshold for health-seeking behavior and increasing obesity in Western countries with the increased prevalence/incidence of BE in these countries.

The influence of the extent of BE on its natural history is controversial. Patients with short segment BE were not considered to be predisposed to esophageal adenocarcinoma, and hence were often excluded from earlier studies of the natural history of BE^[39,44]. Rudolph *et al.*^[13], however, did not observe an association between segment length of BE and the risk of carcinoma in their cohort of 309 patients with BE. In their study, 32 patients with high grade dysplasia (HGD) progressed to cancer and 8 patients developed adenocarcinoma directly from benign BE, giving an overall incidence of 3.4/100 patient years (1184 years of follow-up). The length of BE, also did not influence the symptomatology of their patients significantly^[13]. More recent studies have observed a strong relationship between length of BE and development of adenocarcinoma and dysplasia^[32,45]. The demographic data for both short and long segment BE are similar, indicating that these are a continuum of the same process^[46]. The site of malignant degeneration also seems to depend on the anatomical configuration of the esophagogastric junction because cancer tends to occur in the right lateral quadrant of the esophagus in patients with BE^[47]. This is supported by Prasad *et al.*^[48] who have comprehensively studied the current evidence of various predictors that may be useful in determining the progression of BE, including clinical and demographic factors, endoscopic factors, pathologic factors and molecular biomarkers.

DEVELOPMENT OF COMPLICATIONS

Esophagitis

Erosive esophagitis occurs along with BE in a similar frequency to those symptomatic GERD patients without BE. Zaninotto *et al.*^[45] demonstrated esophagitis in 19%

of BE patients. BE can be present in nearly 27% of patients with erosive esophagitis, and its diagnosis may be missed due to the presence of inflammation. Therefore, repeat evaluation should be considered after complete healing of esophagitis^[49].

The degree and extent of inflammation is variable. Fitzgerald *et al.*^[50] showed that most patients without macroscopic evidence of esophagitis had microscopic evidence of inflammation with T cell, neutrophil and eosinophil infiltration. They also showed a higher degree of inflammation and interleukin-8 cytokine expression in proximal compared with distal BE^[51]. This proximal part of the columnar lined esophageal segment is known to be the area with the greatest risk of inflammatory complications such as stricture formation.

Stricture

In early retrospective series, strictures were present in up to 100% of cases^[52] but in prospective series, stricture rates of 15%-40% are found. They occur within the distal esophagus most frequently near the squamocolumnar junction^[53].

Ulceration

The development of ulceration within the columnar lined segment can occur in up to 60% of cases. They may be found incidentally or may present with complications such as bleeding^[16] in up to 50%, or more rarely with perforation into the mediastinum^[54], or fistula formation. Fistulation due to erosion through the esophageal wall into adjacent structures has been reported into the aorta^[55], pericardium^[56] and respiratory tree^[57].

Dysplasia

During the development of adenocarcinoma there is a gradual increase in dysplastic features of the epithelium through LGD and HGD culminating in invasive cancer^[58]. The reported incidence of dysplasia varies with different publications and is generally around 2%-5%^[43,59-63]. Studies on the natural history of patients with dysplasia in BE are summarized in Table 1.

LGD: In prospective studies, LGD is more commonly seen than HGD^[13,45]. This can progress to HGD/cancer, regress or remain static for several years^[43,58-60]. HGD is frequently found in specimens containing adenocarcinoma indicating that adenocarcinoma develops from HGD^[44].

The time it takes to progress from dysplasia to adenocarcinoma is highly variable with some rapidly developing adenocarcinoma, some having LGD for long periods^[68,69] and some progressing from LGD to HGD^[43,57-59]. Regression from HGD to LGD and HGD/LGD to absence of dysplasia is also variable. In the majority of patients, LGD is relatively stable and does not tend to progress to invasive adenocarcinoma when observed in the short term^[43,59]. However, when compared to patients with no dysplasia, those with LGD have a significantly higher risk of progressing to cancer/HGD^[61-67]. For patients with a

Table 1 Barrett's esophagus: Development of dysplasia

Author	Patients with BE	Dysplasia at diagnosis	Patient years follow up	New LGD	New HGD	New dysplasia incidence (%)
Miros <i>et al</i> ^[43]	81	13	290	10	1	7.5
Katz <i>et al</i> ^[59]	102	5	563	19	4	4.1
O'Connor <i>et al</i> ^[60]	136	Excluded	570	24	4	4.9
Basu <i>et al</i> ^[61]	138	3	405	7	0	1.7
Alcedo <i>et al</i> ^[63]	155	Excluded	3875	83	12	2.7
Ferraris <i>et al</i> ^[64]	187	5	562	5	2	2.1
Weston <i>et al</i> ^[65]	108	Excluded	362	-	5	1.4
Oberg <i>et al</i> ^[66]	140	Excluded	946	44	4	5.0
Sharma <i>et al</i> ^[67]	618	-	2546	156	22	7.14

LGD: Low grade dysplasia; HGD: High grade dysplasia; BE: Barrett's esophagus.

Table 2 Barrett's esophagus: Outcome of series exclusively following up high grade dysplasia

Author and year	Patients	Follow-up (yr)	HGD to cancer	Cancer/patient years
Schnell <i>et al</i> ^[73] , 2001	79	7.3	12	2
Rastogi <i>et al</i> ^[75] , 2008	236	-	69	6.5
Weston <i>et al</i> ^[76] , 2000	15	3	6	13

HGD: High grade dysplasia.

diagnosis of LGD, the cumulative risk of progressing to HGD or carcinoma was reported as 85.0% in 109.1 mo compared with 4.6% in 107.4 mo for patients with non-dysplastic BE ($P < 0.0001$). The incidence of HGD or cancer was 13.4% per patient per year for patients with LGD^[70].

In patients with BE, dysphagia/odynophagia and nausea/vomiting were associated with a higher risk of development of dysplasia^[71]. We do not know the risk factors in patients with LGD which predispose to development of cancer, and are unable to individualize the interval for surveillance endoscopy.

HGD: HGD, similarly, has a variable course with both regression and rapid progression well documented^[58,72-76] (Table 2). Most will have HGD for several years before progressing to adenocarcinoma^[73,74]. Also, an intensive surveillance program can still miss adenocarcinoma in HGD patients^[74].

The presence of nodularity in HGD increases the likelihood that there will be submucosal invasion, and the recommendation is that all such lesions should, at a minimum, be removed by endoscopic resection, if not by esophagectomy^[75-80]. The work of Manner *et al*^[78] shows that most HGD lesions with nodularity can still be resected completely by endoscopic resection, but if the depth of submucosal invasion is beyond the upper third (into the SM3 level), lymph node involvement becomes a possibility. Hence the endoscopic appearance of nodularity alone raises the risk of an underlying cancer being present. The natural history of cancer evolution is vari-

able and patients need to be managed by individualized assessment.

Resected specimens of esophagectomy for HGD variably confirm the presence of invasive adenocarcinoma^[8,81,82], but this often quoted statistic fails to give a useful overview of the likelihood of cancer. In follow-up studies, cancer frequency in HGD patients ranged from 2%-13%. Table 2 describes the range of incidence of cancer in patients with HGD. It is important to remember while reviewing these studies that dysplastic/neoplastic changes are frequently localized within the segment, and are not a field change^[44]. Therefore areas of HGD or cancer may be missed on initial biopsy, and are only detected on follow-up biopsy, leading to apparent rapid progression.

Series in specialized centers which attract tertiary referrals are very selective. Such is the Seattle group of Levine *et al*^[83] who studied 70 patients undergoing prospective surveillance. Twelve patients were found to have invasive cancer on early follow-up (mean 2 mo). Fifteen progressed to cancer over a mean of 27 mo, while 43 remained stable or regressed during a mean of 30 mo follow-up. Such tertiary referral centers do not provide a useful guide to the management of the full range of community observed dysplastic BE and may skew practice.

Adenocarcinoma

Adenocarcinoma of the esophagus and gastroesophageal junction is amongst the fastest growing cancers in the Western world^[84,85], and is thought to be due to the increased incidence of GERD and its complications in this population. It may be important to note the recent epidemiological study of Pohl *et al*^[86] who studied the Surveillance Epidemiology and End Results database of the National Cancer Institute of USA 1973-2006. They found the incidence of esophageal adenocarcinoma to have plateaued. Whether this is true in Europe has not been reported.

The estimated risk of developing adenocarcinoma in BE varies widely (Table 3). The reasons cited for this observation are the surveillance program in place, the biopsy protocol and sampling error, publication bias and geographical variation^[87-100].

Some commentators raised the possibility that determination of cancer incidence in BE suffered particularly from reporting bias where positive studies were more likely to be published and smaller population groups tended to have a higher cancer incidence. This may be true of American series but Jankowski *et al*^[101] contended that this was not the case for European studies where there is a more normal (Gaussian) distribution of cancer incidence and population study size.

The recent very large prospective study by de Jonge *et al*^[100] however, seems to show a lower rate of progression to cancer than the previous smaller European studies (Table 3).

There may be a sex difference in cancer risk in patients with BE^[100,102]. Falk *et al*^[102] have shown that Barrett's segment length was greater in men than in women

Table 3 Barrett's esophagus: Development of adenocarcinoma

Author and year	Patients	Years follow-up	Cancers	% cancer/patient years
American series				
Spechler <i>et al</i> ^[87] , 1984	105	3	2	0.6
Sprung <i>et al</i> ^[88] , 1984	84	4	4	1.2
Cameron <i>et al</i> ^[89] , 1985	104	8	2	0.23
Achkar <i>et al</i> ^[90] , 1988	62	3	1	0.6
Williamson <i>et al</i> ^[39] , 1991	176	3	5	1
Drewitz <i>et al</i> ^[91] , 1997	170	5	4	0.48
Streitz <i>et al</i> ^[92] , 1998	149	3	7	1.37
Katz <i>et al</i> ^[59] , 1998	102	5	4	0.71
Weston <i>et al</i> ^[65] , 1999	108	3.3	5	1.39
Rudolph <i>et al</i> ^[31] , 2000	309	3.8	40	3.4
Sharma <i>et al</i> ^[67] , 2006	618	4.12	12	0.47
European and others series				
Robertson <i>et al</i> ^[93] , 1988	56	3	3	1.79
Van der Veen <i>et al</i> ^[94] , 1989	155	4	4	0.59
Hameeteman <i>et al</i> ^[40] , 1989	50	5	5	2
Miros <i>et al</i> ^[43] , 1991	81	3.6	3	1
Iftikar <i>et al</i> ^[41] , 1992	102	4	4	1
Sánchez <i>et al</i> ^[95] , 1995	46	3.6	2	0.96
Wright <i>et al</i> ^[96] , 1996	166	3	6	1.2
Ferraris <i>et al</i> ^[64] , 1997	88	3	3	1.25
Bujanda Fernández de Piérola <i>et al</i> ^[97] , 1999	46	3.5	2	1.2
Basu <i>et al</i> ^[61] , 2004	138	2.9	2	0.5
Oberg <i>et al</i> ^[66] , 2005	140	5.8	3	0.74
Aldulaimi <i>et al</i> ^[98] , 2005	506	9	13	4
Lim <i>et al</i> ^[62] , 2007	356	11	25	0.62
Zaninotto <i>et al</i> ^[43] , 2007	397	1.5	3	0.5
Gatenby <i>et al</i> ^[99] , 2009	217	17	17	2.68
Alcedo <i>et al</i> ^[63] , 2009	386	4.2	19	0.5
de Jonge <i>et al</i> ^[100] , 2010	42 207	15	2709	0.43

(mean, 5.06 ± 4.2 cm *vs* 4.05 ± 3.27 cm, $P = 0.003$). Of 839 patients with BE, there were 114 cases of HGD or cancer (96 men, 18 women). Women were less likely to have HGD or cancer than men (odds ratio, 0.52; 95% confidence interval, 0.31-0.88; $P = 0.015$). There were 13 new cases of HGD or cancer (11 men, 2 women) during a mean follow-up of 4.72 years, with an incidence of 1 in 179 patient-years of follow-up for women and 1 in 91 patient-years of follow-up for men.

Accurate risk estimation is critically important to the economics of surveillance and other interventions to prevent carcinoma in BE patients, and thus to the specification of optimal clinical management policies.

Although many observers believe that the presence of IM in BE raises the cancer risk, it is now clear that cancer can occur without IM being detected^[8,103,104]. Comparison of rates of malignant degeneration are made more complex because some authors (such as Oberg *et al*^[66]) exclude all dysplastic patients at the start of the observation period while many others include all patients and document the subsequent cancer rate.

Overall health outcomes in BE patients

A very important issue for patients with BE is to understand that the natural history of BE is for the patient to suffer chronic GERD symptoms usually for a lifetime and to need a lifelong strategy of symptomatic care. This

may be by medication or by antireflux surgery. They may also be subject to regular planned endoscopic surveillance. The need for surveillance is not the subject of this article *per se*. The true value of surveillance must be assessed in each geographical and economic environment. Such a study is the BE surveillance study in the United Kingdom (BOSS trial)^[105] which will address this for the United Kingdom population and will also highlight the patients' own perceptions of the necessity for repeated endoscopic examination.

Studies of the true natural history of BE without therapy are not reported because every case series is given some form of therapy. Most patients are offered lifelong proton pump inhibitors (PPI) which are effective in symptom control. However, their ability to prevent complications, either benign or malignant has not been studied in controlled clinical trials.

Most reported studies on the outcome of reflux control by drugs or antireflux surgery are relatively short-term. Studies of open antireflux surgery have previously been compared with H2 receptor antagonist treatment and suggested better control of symptoms and prevention of complications of BE in patients who underwent antireflux surgery^[106]. However, acid suppression with PPI is more effective than H2 receptor antagonists, and the recent studies in the LOTUS trial have compared laparoscopic antireflux surgery with dose adjusted esomeprazole (often given 20 mg *bid*). Three-year results from the LOTUS trial^[20] have suggested that antireflux surgery is as efficacious in symptomatic control of BE as medical management, without significant operative or postoperative complications.

Interfering with the natural history of BE especially where treatments are directed at cancer prevention also is not the subject of this paper *per se*. It requires well controlled studies which are sparse. The aspirin and esomeprazole cancer chemoprevention trial in the United Kingdom^[107-110] is looking at the issue of dose-response of esomeprazole with or without aspirin on overall survival (both cancer and cardiovascular related).

Some authors have suggested that there might be a benefit from antireflux surgery in prevention of cancer, but there are no controlled studies on this topic. Case series and cohort studies have been reviewed by Chang *et al*^[108]. They found 25 publications with original data reports and their analysis supports this hypothesis but no conclusions can be drawn. Oberg *et al*^[66] looked specifically at 140 patients in a surveillance program, of whom 46 had undergone antireflux surgery and none developed adenocarcinoma or HGD. In patients treated with antireflux surgery, the risk of developing LGD was reduced 2.3-fold compared with patients receiving conventional acid suppression therapy^[66]. Whether a competent antireflux repair can indeed reduce the rate of malignant progression in patients with BE is still unclear, and further studies are needed to clarify this issue. Rossi *et al*^[7] reported regression from LGD to BE in 63% patients with PPI and in 94% of those who had antireflux surgery ($P = 0.03$).

Other forms of cancer prevention now proposed are ablation of dysplastic BE which again will require long-term prospectively controlled studies. Early ablation studies used Argon beam plasma coagulation which indicated potential efficacy in controlling the progression of HGD to cancer; 86% of patients studied responded to this treatment with a follow-up evaluation over 7 years^[109]. Recently, radiofrequency ablation has been introduced, which is a balloon-based technology that provides more easily standardized tissue destruction. This, when combined with endoscopic mucosal resection may dramatically alter the natural history of dysplastic BE^[110,111].

Some authors contend that survival rates of patients with BE are virtually identical to those of age- and sex-matched control populations^[87], and it is important to appreciate that, notwithstanding the increased risk of developing esophageal adenocarcinoma, the absolute risk of death from this tumor is small. In a cohort of 166 BE patients in the Netherlands with 1440 patient-years of follow-up, 79 patients died but only 2 of the deaths were due to esophageal carcinoma^[42]. Most patients with BE die from causes unrelated to their esophageal disease^[112,113], and reducing the risk of adenocarcinoma can produce no more than a small effect on overall life expectancy. Long term studies^[105,107] are eagerly awaited to guide future understanding of BE.

Understanding the natural history of BE in an individual patient requires an estimate of risk based on the geographical variations of disease progression, and an individualized assessment of patient characteristics, race, obesity, *etc.* Presenting such a risk assessment in context is important for patients so that they have a balanced perspective of risk.

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Multicausality in fatty liver disease: Is there a rationale to distinguish between alcoholic and non-alcoholic origin?

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Abstract

Apart from alcohol, there are other factors that may induce complications, which resemble alcohol-related liver disorders. In particular, obesity has been brought into focus as a risk factor for fatty liver disease. The term "non-alcoholic" fatty liver disease is commonly used to distinguish between obesity-related and alcohol-related hepatic steatosis. This review uses the epidemiological perspective to critically assess whether it is necessary and useful to differentiate between alcoholic and "non-alcoholic" fatty liver disease. The MEDLINE database was searched using the PubMed search engine, and a review of reference lists from original research and review articles was conducted. The concept to distinguish between alcoholic and "non-alcoholic" fatty liver disease is mainly based on specific pathomechanisms. This concept has, however, several limitations including the common overlap between alcohol misuse and obesity-related metabolic disorders and the non-consideration of additional causal factors. Both entities share similar histopathological patterns. Studies demonstrating differences in clinical presentation and outcome are often biased by selection. Risk factor reduction is the main principle of prevention and treatment of both disease forms. In conclusion, alcoholic and "non-alcoholic" fatty liver diseases are one and the same disease caused by

different risk factors. A shift from artificial categories to a more general approach to fatty liver disease as a multicausal disorder may optimize preventive strategies and help clinicians more effectively treat patients at the individual level.

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Key words: Fatty liver disease; Hepatic steatosis; Risk factors; Clinical epidemiology

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INTRODUCTION

There is longstanding clinical knowledge that chronic alcohol misuse may cause severe liver damage. Hepatic steatosis is regarded as the early stage of alcohol-induced liver damage, and steatohepatitis may follow if alcohol misuse is continued. Liver cirrhosis as an end-stage of liver damage is accompanied by potentially life-threatening conditions including esophageal bleeding, hepatic encephalopathy, increased susceptibility to infections and impaired hemostasis.

It has been recognized for many decades that, apart from alcohol, other factors may induce complications,

which resemble alcohol-related liver disorders in many ways^[1]. In particular, obesity and metabolic syndrome, both with an increasing prevalence in developed communities, have been brought into scientific and clinical focus as risk factors for hepatic steatosis. Ludwig *et al*^[2] described the potential role of obesity and metabolic syndrome in hepatic steatosis in their paper on nonalcoholic steatohepatitis as a hitherto unnamed disease, which was published in 1980. From that work, the term “non-alcoholic” fatty liver disease has been established and is now commonly used to distinguish between obesity-related and alcohol-related hepatic steatosis.

Fatty liver disease is common in populations and has potential consequences for individual health. In Southern Italy, it was estimated from a population-based study^[3] that alcohol misuse accounted for 46.5% of all cases diagnosed with impaired liver function, and a further 24.0% of cases were attributed to “non-alcoholic” fatty liver disease. In Northeast Germany, where alcohol misuse, obesity and metabolic syndrome are highly prevalent^[4-6], 29.9% of adults aged between 20 and 79 years had a hypercholesterolemic pattern in their liver ultrasound. Given the expected increase in the global burden of overweight and obesity^[7], the prevalence of “non-alcoholic” fatty liver disease will continue to rise over the next years. The potential impact of fatty liver disease for societies is reflected by the fact that, over the following five years, subjects with current hepatic steatosis will cause 26% higher health care costs compared to subjects without hepatic steatosis^[8].

Currently, a large amount of research is being performed to explore risk factors, histopathological features, pathogenesis, clinical symptoms and options for the prevention and treatment of “non-alcoholic” fatty liver disease. At this moment in time, it is common in research and clinical practice to distinguish between alcoholic and “non-alcoholic” fatty liver disease. The rationale for this distinction, however, has not yet been the issue of thorough analyses.

This review uses the clinical-epidemiological perspective to critically assess whether it is necessary and useful to differentiate between alcoholic and “non-alcoholic” fatty liver disease. The general aim of this review is to summarize the evidence that alcoholic and “non-alcoholic” fatty liver diseases represent one and the same disorder with an underlying multicausal origin.

MATERIALS

Two search strategies were used to find appropriate articles for the research question. The MEDLINE database (from January 1, 1966 to December 17, 2011) was searched using the PubMed search engine to find articles on alcoholic and “non-alcoholic” fatty liver disease. The keywords used were “alcoholic” or “non-alcoholic” and “fatty liver” or “hepatic steatosis” or “steatohepatitis” or “cirrhosis”. Hereafter, a review of reference lists from original research and review articles was conducted. Articles in English and German were considered on the basis of their relevance to this review’s topic.

To illustrate some major points, data from the large-scale population-based Study of Health in Pomerania (SHIP) are used^[9]. For this study, a sample from the general population aged 20 to 79 years was drawn from population registries. Baseline examinations were performed in 4308 men and women. The longitudinal data collection included a five-year examination, morbidity and mortality follow-ups^[10]. SHIP is conducted in Northeast Germany, where both metabolic syndrome and alcohol misuse are highly prevalent and, consequently, hepatic steatosis is also commonly found on liver ultrasound^[11].

RESULTS

Definitions and risk factors

The term alcoholic fatty liver disease refers to hepatic steatosis and its liver-related sequelae, which are related to alcohol misuse, whereas the term “non-alcoholic” fatty liver disease comprises the same liver disorders attributed to obesity and metabolic syndrome^[12]. At a first glance, these definitions sound clear and intuitive, but there are at least five drawbacks for applying these definitions in clinical practice and research. These drawbacks are the main reason why the term “non-alcoholic” is put in quotation marks throughout this review.

Firstly, the term “non-alcoholic” fatty liver disease is imprecise. It implies that all risk factors for hepatic steatosis are summarized under this name. This, however, is not the case, because the term rather refers to the obesity-related causes that underlie the fatty liver disease. In addition to obesity and metabolic syndrome, other causes of fatty liver disease exist including other metabolic and hormonal disorders, acute starvation and abdominal surgery, as well as pharmacotherapeutic, toxic and genetic factors, which actually would have been also summarized under the term “non-alcoholic” fatty liver disease (Figure 1).

Secondly, with regard to hepatic steatosis, there is no consistent definition of alcohol misuse. For example, some scientists^[13,14] consider alcoholic fatty liver disease when men consume at least 80 g alcohol and define “non-alcoholic” liver disease by excluding patients who report a daily alcohol consumption of less than 20 g^[15,16]. Unfortunately, subjects with alcohol consumption of between 20 g and < 80 g are not assigned to a specific group. The resulting practical problem is illustrated by SHIP data. In fact, 26.7% of all male participants aged 20 to 79 years had reported a daily alcohol consumption in the range of 20 g to < 80 g during the past 7 d.

Thirdly, the term “non-alcoholic” implies that alcohol plays no role in the development of fatty liver disease in affected patients. Certain amounts of alcohol consumption, however, are usually tolerated in the definition of “non-alcoholic” fatty liver disease. Contrary to this, chronic daily alcohol consumption of e.g., 20 g may well increase the risk of liver damage in a considerable proportion of susceptible individuals. Thus, female sex, dietary habits, alcohol dehydrogenase deficiency and other genetic factors are major predictors of such increased susceptibility^[17,18].

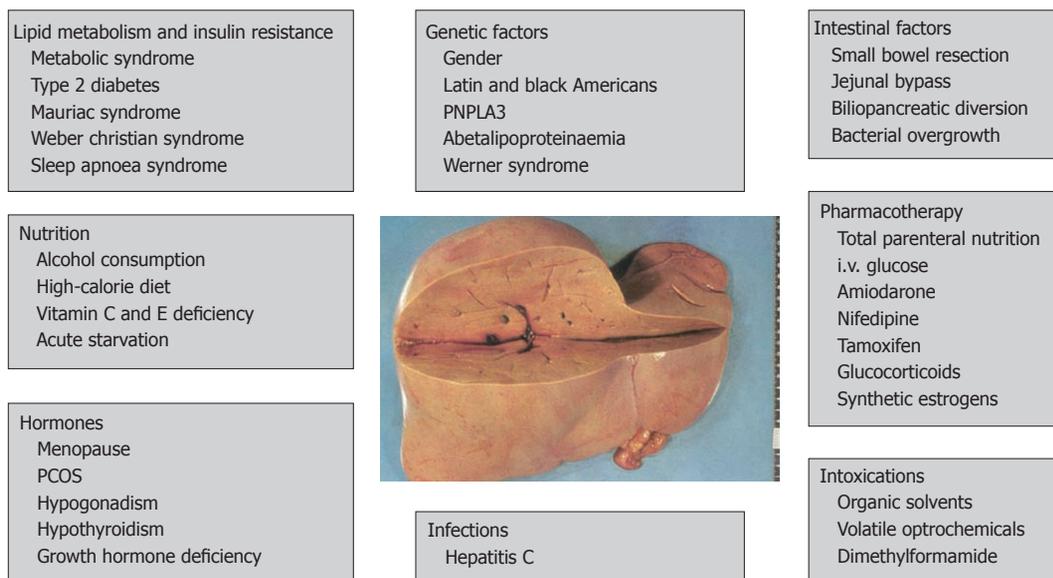


Figure 1 Risk factors for fatty liver disease^[17,32,72,82-87]. PNPLA3: Patatin-like phospholipase domain-containing protein 3; PCOS: Polycystic ovary syndrome.

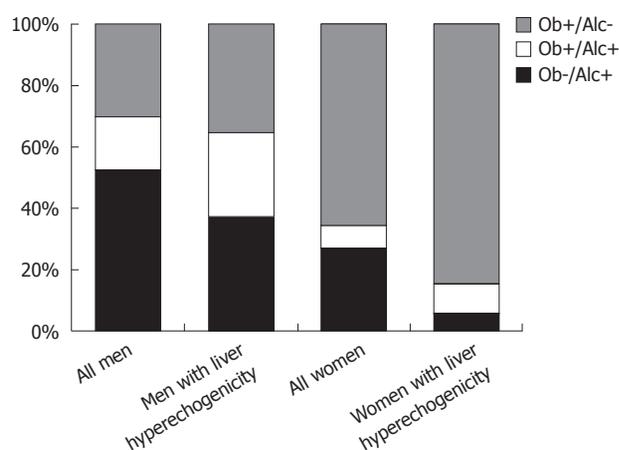


Figure 2 Obesity and alcohol consumption in the general population of Northeast Germany. Data are taken from the population-based Study of Health in Pomerania. The columns indicate the proportions of obesity (Ob; body mass index > 30 kg/m²), harmful alcohol consumption (Alc; daily alcohol consumption > 20 g in women and > 30 g in men), the combined presence of both risk factors in all subjects (1122 men, 781 women) and subjects with a hyperechogenic pattern on liver ultrasound (535 men, 276 women), in whom at least one of both risk factors was present.

Fourthly, many cases cannot be clearly assigned to either the alcoholic or the “non-alcoholic” category, because an overlap between alcohol consumption and metabolic disorders exists within many individuals. SHIP data exemplify this issue (Figure 2). In the general adult population of Northeast Germany, obesity and harmful alcohol consumption are not mutually exclusive characteristics. Rather, a broad overlap between both characteristics exists, particularly in men. Among men who are either obese or report a daily alcohol consumption of > 30 g, 17.5% fulfill both criteria. In men with hyperechogenicity on liver ultrasound, this proportion is even as high as 27.3%. In women, for whom harmful alcohol consumption of > 20 g per day is much less prevalent than in men, the overlap

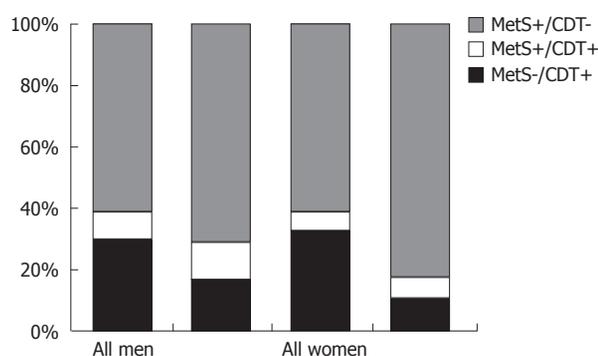


Figure 3 Metabolic syndrome and increased serum carbohydrate-deficient transferrin in the general population of Northeast Germany. Data are taken from the population-based Study of Health in Pomerania. The columns indicate the proportions of metabolic syndrome (MetS), increased serum carbohydrate-deficient transferrin (CDT > 6%), the combined presence of both risk factors in all subjects (970 men, 685 women) and subjects with a hyperechogenic pattern on liver ultrasound (486 men, 288 women), in whom at least one of both risk factors was present.

is smaller and reaches a proportion of 7.3% in the whole female population and 9.4% in women with hyperechogenic findings on liver ultrasound. After applying stricter definitions for risk factors, metabolic syndrome^[19] and increased serum carbohydrate-deficient transferrin levels are co-existent in 9.0% of men with at least one of both risk factors in the whole study population and in 11.7% in men with risk factors and liver hyperechogenicity, respectively (Figure 3). In women, these proportions are 5.8% and 7.3%. Similar findings were found in Finnish adults^[20], where subjects with alcoholic fatty liver disease were as often obese as subjects with “non-alcoholic” fatty liver disease, and the metabolic syndrome was even more common in alcoholic than in “non-alcoholic” fatty liver disease.

Current definitions of alcoholic and “non-alcoholic” fatty liver disease disregard the common presence of

risk factors for hepatic steatosis. What is the correct diagnosis for obese patients with hepatic steatosis who consume too much alcohol? Do they have alcoholic fatty liver disease, “non-alcoholic” fatty liver disease or both? And how do we name fatty liver disease if one or more additional risk factors listed in Figure 1 are present? Furthermore, it has been convincingly demonstrated that metabolic factors contribute to the risk of pure steatosis in alcoholic patients. Apolipoprotein A1 levels, body mass index, waist circumference and blood pressure are closely associated with the risk of fatty liver in these patients^[21]. On the other hand, especially in obese women, low amounts of alcohol may provoke the risk of “non-alcoholic” fatty liver disease^[22]. These data suggest that interactions among alcohol use, metabolic characteristics and other factors (Figure 1) do exist, and that the complexity of risk factor interplay is much greater than the simple distinction between alcoholic and “non-alcoholic” fatty liver disease indicates.

Finally, misclassification due to information bias represents a serious problem in correctly distinguishing between alcoholic and “non-alcoholic” forms of fatty liver disease. Given the potential stigmatization through alcohol use among patients and study participants, the under-reporting of alcohol consumption is a common problem in clinical practice and research. Thus, it can be expected that a significant alcoholic component contributes to the development of “non-alcoholic” fatty liver disease in a certain proportion of patients. Misclassification also arises from the fact that a history of alcohol consumption is usually evaluated in the present or for the very recent past. The Dionysos study^[25,24] demonstrated, however, that life-time history of alcohol consumption is more valid to define a threshold for liver cirrhosis than is the current information. Studies performed in patients with “non-alcoholic” fatty liver disease confirmed that misclassification was present in up to 10% of all cases^[22].

Misclassification of alcohol consumption may also have biased studies^[25-28], which suggested that low-to-moderate alcohol consumption is inversely associated with the risk of fatty liver diseases. The challenge in such studies is to correctly define the reference group. If this definition is only based on current self-reported denial of alcohol consumption, the reference group might not only include lifelong teetotallers, but also sick quitters with high alcohol-related morbidity^[29]. This phenomenon may also have led to oversimplified interpretation of J-shaped associations between alcohol consumption and cardiovascular mortality^[30,31] and should be considered in future studies on associations between alcohol consumption and fatty liver disease.

Taken together, the definitions of alcoholic and “non-alcoholic” fatty liver disease are not very practical for clinical applications. The current concept of “non-alcoholic” fatty liver disease does not sufficiently take into account risk factors for fatty liver disease other than obesity and metabolic syndrome.

Histopathology

Liver biopsy is the gold standard for the diagnosis of

fatty liver disease. The general stages of both alcoholic and “non-alcoholic” fatty liver disease are as follows: (1) **simple steatosis**; (2) **steatohepatitis**; and (3) **cirrhosis**. Macrovesicular, microvesicular or mixed patterns of simple steatosis are the first step of both alcoholic and “non-alcoholic” fatty liver disease^[32,33]. The diagnostic criteria for steatohepatitis are steatosis accompanied by liver cell injury, inflammatory changes and fibrosis^[32,34]. Common histopathological features of liver cell injury are ballooning of hepatocytes, vacuolated nuclei, Mallory bodies and megamitochondria. Inflammatory changes usually follow a lobular pattern. Perisinusoidal fibrosis typically occurs in acinar zone 3^[32,34]. Cirrhosis, as an end stage of multiple liver disorders, is characterized by progressive perivenular fibrosis, which may form septa between terminal hepatic venules. Regenerative nodules or diffuse pericellular fibrosis throughout the acini may develop if risk factors persist^[32].

Several studies^[34-36] have investigated the differences in the histopathological picture between alcoholic and “non-alcoholic” fatty liver disease. In one study^[35], patients with alcoholic fatty liver disease had a more diminished regional blood flow and hepatic oxygen consumption relative to patients with “non-alcoholic” fatty liver disease, suggesting that a more impaired hepatic circulation exists in the former than in the latter. Although in this small study^[35] both of the patient groups had an otherwise similar picture of hepatic steatosis, major differences existed in the extent of risk factors. Whereas the six patients with alcoholic fatty liver disease consumed a heavy amount of at least 180 g alcohol daily, the relatively moderate inclusion criterion for the “non-alcoholic” fatty liver disease group was to be “at least 30% overweight in terms of ideal standards for height”^[35]. Unfortunately, no further details on the distribution of metabolic risk factors were given in that study^[35].

Regarding steatohepatitis, a previous review^[34] has summarized current evidence by stating that the “non-alcoholic” form of steatohepatitis has greater amounts of steatosis and nuclear vacuolization, but less necroinflammatory activity, canalicular cholestasis, Mallory hyaline and periportal fibrosis than the alcoholic form. Although these differences probably exist, the limited comparability between patients with and without alcoholic steatohepatitis may limit the conclusions of many studies.

One example illustrates this notion. To investigate the histopathological disparities between patients with and without alcoholic hepatitis, Pinto *et al*^[13] compared the histopathological characteristics of patients with “non-alcoholic” and alcoholic steatohepatitis, whereby the latter group was divided into ambulatory and hospitalized patients. In relation to hospitalized alcoholic patients, those with “non-alcoholic” steatohepatitis had less severe histopathological signs of steatohepatitis, whereas ambulatory patients with alcoholic steatohepatitis displayed an intermediate histopathological picture.

Without a doubt, the study of Pinto *et al*^[13] confirmed the expectation that the clinical presentation of patients

with alcoholic steatohepatitis is associated with the histopathological severity of the disease. It still remains to be determined, however, whether it is also possible to conclude that “non-alcoholic” steatohepatitis has a less severe histopathological pattern than the alcoholic form, because additional information on the clinical status of the patients would be necessary. Unfortunately, it is unclear from that study^[13], whether the “non-alcoholic” steatohepatitis patients were ambulatory or hospitalized. It has also not been stated what the indication of liver biopsy was in the “non-alcoholic” patients. Thus, it does seem likely that, for example, asymptomatic hepatomegaly in patients with known heavy alcohol consumption may be tolerated, since this finding is well explained by alcoholism; whereas the same constellation in patients who deny alcohol abuse gives rise to greater clinical efforts to find reasons for hepatomegaly. Hence, it is unclear whether the less severe clinical status in “non-alcoholic” steatohepatitis patients and the earlier liver biopsy account for the lower severity of histopathological findings compared to patients with alcoholic steatohepatitis.

Furthermore, although Pinto *et al*^[13] provide data on medication taken by the study patients, they leave uncertainty whether liver-related side effects of these drugs were suspected by treating physicians. Hence, it is not clear whether patients were correctly assigned to “non-alcoholic” steatohepatitis as a metabolic liver disorder.

Taken together, although histopathological differences between alcoholic and “non-alcoholic” fatty liver disease exist, the general pattern of findings is very similar. Therefore, pathologists are not able to distinguish between both entities by themselves without information on risk factors provided by clinicians^[32,37,38].

Pathogenesis

The pathogenesis of alcoholic and “non-alcoholic” fatty liver disease has specific as well as common components. The relatively specific components of alcoholic fatty liver disease include the toxic effects of acetaldehyde and an increase in NADH^[18,39] leading to acidosis, hypoglycemia and, as important factors for the development of hepatic steatosis, an increased activity of lipogenic pathways and a reduced export of triglycerides from the liver^[18]. In “non-alcoholic” fatty liver disease, insulin resistance activates the breakdown of peripheral adipose tissue with the consequence of increased hepatic absorption of free fatty acids, *de novo* synthesis of fatty acids and accumulation of triglycerides in the liver^[18]. Furthermore, high serum insulin levels stimulate fatty acid synthesis and inhibit the conversion of triglycerides to very low density lipoproteins^[18].

At least five common mechanisms exist that are important for the development and progression of both alcoholic and “non-alcoholic” fatty liver disease. Firstly, inadequately high energy uptake may not only induce key mechanisms in “non-alcoholic” fatty liver disease, but may also contribute to the alcoholic form of the disease. Alcoholic beverages are calorically dense, and in

the absence of severe malnutrition in affected patients, this may result in an impaired energy balance in chronic alcoholism^[36]. Secondly, triglycerides are synthesized from fatty acids in both forms of fatty liver disease. Fatty acids are mainly derived from lipolysis of adipose tissue, but may also be generated by *de novo* lipogenesis^[36]. Thirdly, oxidative stress is highly relevant to the progression from hepatic steatosis to steatohepatitis and cirrhosis in both forms of fatty liver disease. In alcoholic fatty liver disease, ethanol generates free radicals, and the activation of CYP2E1 and mitochondrial activities release reactive oxygen species^[18,40]. Free fatty acids and mitochondrial dysfunction seem to be key mediators for the inflammatory processes induced in “non-alcoholic” fatty liver disease^[41,42]. Mitochondrial dysfunction and oxidative stress are the major contributors to the progression from pure steatosis to steatohepatitis in both alcoholic and “non-alcoholic” fatty liver disease^[43-45]. Also, in both disease forms, inflammatory cytokines reduce insulin sensitivity and thereby increase the risk of fatty liver disease^[45-47]. Fourthly, endotoxin, a toxic lipopolysaccharide located in the cell wall of Gram-negative bacteria, enhances complications of fatty liver disease by stimulating inflammatory processes. In alcoholic and “non-alcoholic” fatty liver disease, the release of endotoxin is triggered by increased gut permeability^[18,48,49]. Finally, intestinal bacteria may add an alcoholic component to the pathogenesis of “non-alcoholic” fatty liver disease. It has been demonstrated in mice studies that intestinal bacteria produce alcohol, and that the amount of alcohol production is higher in obese than in lean animals^[50]. In line with these findings from animals, hepatocytes from young human patients with “non-alcoholic” steatohepatitis and without any history alcohol consumption demonstrated expression of genes encoding all known pathways of alcohol degradation, which was much stronger than in hepatocytes from age-matched controls^[51]. These findings support the notion that alcohol may play a central role in the development of “non-alcoholic” steatohepatitis.

Taken together, there are, particularly in the early stages of fatty liver disease, some pathways that are specific for the underlying cause of the disease. In other important aspects, pathomechanisms leading to alcoholic as well as “non-alcoholic” fatty liver disease share many similarities.

Clinical presentation

Upon investigation of the clinical status of patients with “non-alcoholic” compared to alcoholic fatty liver disease, studies usually find that the former patients are less symptomatic with nausea, abdominal pain, jaundice and gastrointestinal bleeding, than are the latter^[37,52]. To interpret these findings correctly, the time of recruitment of patients for the studies during the course of the disease has to be taken into account. These studies^[37,52] are commonly histology-based. Thus, the time of recruitment is the day the biopsy was performed. As already suggested above, invasive diagnostic procedures may be performed

much later in the time course of the disease in patients with clear alcohol misuse than in those with no or less alcohol consumption. Thus, the longer exposure and, consequently, larger cumulative dose of risk factors may have significantly influenced the outcomes of studies comparing the clinical features between patients with and without alcoholic fatty liver disease.

Taken together, current research suggests that “non-alcoholic” fatty liver disease is accompanied by relatively mild symptoms compared to alcoholic fatty liver disease, but studies generally lack a well-balanced standardization with respect to the extent of underlying risk factors.

Outcome

Hepatic complications: Pure hepatic steatosis is commonly regarded as a benign disorder. However, in both alcoholic and “non-alcoholic” fatty liver disease, hepatic steatosis may progress to steatohepatitis. Fibrosis is regarded to be the result of wound healing following inflammatory changes. The final stage of alcoholic and “non-alcoholic” fatty liver disease is liver cirrhosis. Hepatic steatosis and steatohepatitis are also associated with an increased risk of hepatocellular carcinoma, and alcohol misuse and obesity are the most common risk factors for this malignant tumor in developed countries^[53,54].

One study^[37] has compared the histopathological patterns between consecutive patients with alcoholic and “non-alcoholic” hepatic steatosis, demonstrating an increased risk of steatohepatitis and fibrosis in patients with alcoholic relative to “non-alcoholic” fatty liver disease. The conclusions from that study^[37] are, however, hampered by the limited comparability between the exposure groups with respect to the extent of risk factors. The prevalence of liver fibrosis in patients who admittedly consumed at least 80 g alcohol daily was compared to that in “non-alcoholic” fatty liver disease patients, of whom 77% had a body weight of > 10% above their ideal body weight. The higher grades of lobular inflammation, fibrosis and cirrhosis might well be explained by unbalanced risk factors in that study^[37]. Another study^[55] supports this notion. Histopathological findings were investigated in 160 patients with morbid obesity who underwent gastric bypass or gastric banding surgery. The proportion of “non-alcoholic” steatohepatitis was 33.8% and thus reached higher percentages than have been previously described in heavy alcoholic drinkers^[23].

Similar to the risk of pure steatosis, there is also a possible overlap of risk factors for steatosis-related sequelae. After investigating risk factors for steatohepatitis and liver cirrhosis in patients with alcoholic fatty liver disease, it was demonstrated that the co-existence of alcohol misuse with obesity and metabolic syndrome increases the risk of complications of alcoholic fatty liver disease^[56]. Conversely, in patients with “non-alcoholic” fatty liver disease, moderate and, particularly, heavy episodic alcohol consumption increases the risk of hepatic fibrosis^[57].

A Danish register study^[58] demonstrated that, after excluding patients with liver cirrhosis, both alcoholic and

“non-alcoholic” fatty liver disease was associated with an increased risk of primary liver cancer, and that this risk was higher in the alcoholic (standardized incidence ratio 9.5; 95% CI: 5.7-14.8) than in the “non-alcoholic” group (standardized incidence ratio 4.4; 95% CI: 1.2-11.8). Unfortunately, as is commonly inherent to register studies, only a limited amount of baseline data was available. Hence, uncertainty exists as to whether “non-alcoholic” fatty liver disease was mainly due to obesity and metabolic syndrome or whether other causes were also present.

Taken together, given individual susceptibility, which cannot be fully defined by the current knowledge, hepatic steatosis may progress to steatohepatitis, liver cirrhosis and hepatocellular carcinoma. The major determinant for this progression is the persistence of risk factors, which has led to hepatic steatosis.

Extrahepatic complications: The association between hepatic steatosis and extrahepatic sequelae has become an important issue of current research. Since “non-alcoholic” hepatic steatosis is regarded to be closely related to metabolic syndrome, multiple studies have investigated its associations with atherosclerotic diseases^[59-61] and diabetes mellitus^[62-65]. Most of these studies^[59,61-63,65], however, used the relatively unspecific serum transaminase levels to define the exposure variables and commonly did not compare the risks of the outcomes between subjects with “non-alcoholic” and alcohol-related fatty liver disease.

In SHIP, we extensively studied the associations between metabolic disorders and hepatic steatosis as defined by the combined presence of ultrasound and laboratory findings. We identified strong inverse relations of hepatic steatosis with the anabolic hormones testosterone in men^[66] and insulin-like growth factor-1 in both genders^[67]. All of these relations were independent of alcohol consumption. Moreover, stratified analyses in subjects who consume more or less alcohol did not reveal any significant difference between both groups. A recent case-control study confirmed that carotid intima-media thickness was higher in all patients with fatty liver disease compared to healthy controls, but there was no difference in intima-media thickness between patients with alcoholic or “non-alcoholic” fatty liver disease^[68].

Using the longitudinal data, we demonstrated that subjects with hyperechogenicity on liver ultrasound and increased serum alanine aminotransferase levels at baseline more commonly used health care services over the following five years^[8]. Consequently, hepatic steatosis was also related to increased health care costs. Another analysis showed that increased serum γ -glutamyl transpeptidase levels were predictive of mortality if a hyperechogenic liver echo pattern was also present^[69]. All of these associations of hepatic steatosis with mortality as well as future health care utilization and costs were independent of alcohol consumption. Also, stratification of the study population according to more or less alcohol consumption did not reveal any difference in the subgroups with respect to the outcomes.

In good agreement with the hypothesis that not only the risk of fatty liver disease itself but also its outcome is determined by multiple risk factors, the NHANES III study demonstrated that the components of the metabolic syndrome are associated with overall mortality in both “non-alcoholic” and alcoholic fatty liver disease^[70]. In line with our findings, an extension of the aforementioned study by Cortez-Pinto *et al*^[13,71] demonstrated that the clinical status of patients, but not the major cause of fatty liver disease, is predictive of the outcome. Patients with “non-alcoholic” steatohepatitis as well as ambulatory and hospitalized patients with alcoholic hepatitis were followed up over a mean time of six years. Survival was similar between patients with alcoholic and “non-alcoholic” steatohepatitis. Only the subgroup of hospitalized alcoholics had a shorter survival than patients with “non-alcoholic” steatohepatitis^[71]. As mentioned earlier, this finding might be explained by the more advanced disease stage of hospitalized alcoholics, when compared to patients with “non-alcoholic” steatohepatitis at the time of recruitment.

Taken together, it is still not clear whether extrahepatic complications of “non-alcoholic” fatty liver disease are specific for the metabolic origin. Rather, it seems likely that fatty liver disease confers this risk, independent of its causes.

Prevention and therapy

The question arises as to whether specific strategies for primary and secondary prevention and treatment represent the major difference between alcoholic and “non-alcoholic” fatty liver disease. In the former, abstinence from alcohol is the major goal, while in the latter the major challenge is improvement of insulin resistance. The most efficient risk factor reduction can be achieved by lifestyle changes. In patients with alcoholic fatty liver disease the major treatment target is total abstinence from alcohol, while in patients with “non-alcoholic” fatty liver disease the central goal is weight reduction by calorie reduction and optimized food quality^[18]. Treatment can be pharmacologically supported by drugs, which improve the insulin resistance^[72].

From a more general perspective, risk factor reduction is the major principle of prevention and treatment in both forms of fatty liver disease. This more general perspective is particularly necessary, given the overlap between risk factors and their potential interactions.

Risk factor reduction is not only important to prevent the natural course of the disease from benign hepatic steatosis to steatohepatitis and cirrhosis, but it is also required to avoid recurrent fatty liver disease in allografts following liver transplantation^[72]. This general statement is appropriate for both alcoholic and “non-alcoholic” fatty liver disease and also holds true for alcoholic fatty liver disease with a “non-alcoholic” component and *vice versa*.

Beyond risk factor reduction and liver transplantation in end stage liver failure, no specific therapies for alcoholic fatty liver disease are currently accepted in clinical

medicine, and none have proven to be exclusively sufficient in patients with “non-alcoholic” or alcoholic fatty liver disease. Thus, several therapeutic strategies that aim at, for instance, supporting the antioxidative system or reducing inflammation have been tested only in specific forms of fatty liver disease^[18,72,73].

Taken together, risk factor reduction is the major goal of prevention and the basic principle of treating fatty liver disease.

CONCLUSION

This review followed the hypothesis that fatty liver disease is a multifactorial disease with alcohol consumption and metabolic factors being the most common risk factors. For many aspects, studies were found that directly confirmed the hypothesis. Other studies, which apparently did not support this notion, were critically assessed from the epidemiological perspective. Following the line of evidence presented herein, the hypothesis underlying the strict separation of alcoholic from “non-alcoholic” fatty liver disease may be less justified than taking fatty liver disease as it probably is—a multifactorial disorder.

The current concept to distinguish between alcoholic and “non-alcoholic” fatty liver disease is mainly based on the presence of alcohol misuse and obesity-related metabolic disorders as common risk factors and, at least evident for the early phase of the disease, specific pathogenetic mechanisms. This concept has, however, several limitations. These limitations include the clear distinction between both entities in the common presence of alcohol misuse and obesity-related metabolic disorders and the non-consideration of other causal factors listed in Figure 1. Both entities share similar histopathological patterns and pathways. Studies demonstrating differences in clinical presentation and outcome are sometimes biased by selection. Risk factor reduction is the main principle of prevention and treatment of both forms of the disease.

Fatty liver disease parallels other multicausal diseases in many aspects. For example, various risk factors for atherosclerosis have been established including tobacco consumption, obesity and metabolic syndrome. For all of these risk factors, specific pathways have been described^[74,75]; the outcome following acute coronary syndrome or stroke differs depending on the type and amount of risk factors accumulated^[76-78] and, beyond basic therapeutic principles, the choice of pharmaceutical drugs depends on the pattern of individual risk factor profiles. However, the presence of underlying risk factors is not mutually exclusive, the histopathological findings are not risk factor specific, and risk factor reduction is the main goal of primary and secondary prevention. In cardiovascular medicine, however, there is no concept to distinguish between tobacco and non-tobacco-related atherosclerosis.

The distinction between alcoholic and “non-alcoholic” fatty liver disease is arbitrary and artificial. It should be replaced by a concept which regards fatty liver disease as

a multicausal disorder. Such a concept opens up multiple questions, which are potentially of high clinical relevance. Examples of these questions are: Are there cumulative thresholds for the effect of risk factors for fatty liver disease and related complications, and how are those hypothetical thresholds decreased by the additional presence of other risk factors? As expected, the association between risk factors and alcoholic fatty liver disease follows a dose-response pattern^[23]. Similar findings have been documented for “non-alcoholic” fatty liver disease. In the Dionysos study^[79], the prevalence of hepatic steatosis was estimated to be 91% in obese, 67% in overweight and 24% in normal weight individuals. However, it still remains to be determined whether these risk factors interact with other factors listed in Figure 1. The multicausal approach could also be applied to investigation of the factors defining individual susceptibility for this multicausal disorder and its complications. From the clinical perspective, it would be highly valuable to explore risk scores similar to those scores established for coronary artery disease^[80] or diabetes mellitus^[81], for instance. Finally, complex therapeutic strategies, in addition to risk factor reduction, could be found.

In conclusion, alcoholic and “non-alcoholic” fatty liver diseases are one and the same disease caused by different risk factors. A shift from artificial categories to a more general approach to fatty liver disease as a multicausal disorder may optimize preventive strategies and help clinicians more effectively treat patients at the individual level.

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Significance of regenerating islet-derived type IV gene expression in gastroenterological cancers

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Abstract

The regenerating islet-derived members (*Reg*), a group of small secretory proteins, which are involved in cell proliferation or differentiation in digestive organs, are upregulated in several gastrointestinal cancers, functioning as trophic or antiapoptotic factors. Regenerating islet-derived type IV (*RegIV*), a member of the *Reg* gene family, has been reported to be overexpressed in gastroenterological cancers. *RegIV* overexpression in tumor cells has been associated with carcinogenesis, cell growth, survival and resistance to apoptosis. Cancer tissue expressing *RegIV* is generally associated with more malignant characteristics than that without such expression, and *RegIV* is considered a novel prognostic factor as well as diagnostic marker in some gastroenterological cancers. We previously investigated the expression levels of *RegIV* mRNA of 202 surgical colorectal cancer specimens with quantitative real-time reverse-transcriptase polymerase chain reaction and reported that a higher level of *RegIV* gene expression was a significant independent predictor of colorectal cancer. The biologic functions of *RegIV* protein in cancer tissue, associated with carcinogenesis, anti-apoptosis and invasiveness, are being elucidated by

molecular investigations using transfection techniques or neutralizing antibodies of *RegIV*, and the feasibility of antibody therapy targeting *RegIV* is being assessed. These studies may lead to novel therapeutic strategies for gastroenterological cancers expressing *RegIV*. This review article summarizes the current information related to biological functions as well as clinical importance of *RegIV* gene to clarify the significance of *RegIV* expression in gastroenterological cancers.

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Key words: Regenerating islet-derived type IV protein; Gastrointestinal neoplasms; Prognosis; Epidermal growth factor receptor/protein kinase B

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INTRODUCTION

It is generally accepted that cancer develops as a result of multiple genetic alterations. A better understanding of the changes in gene expression that occur during carcinogenesis may lead to improvements in diagnosis, treatment and prevention. Identification of novel biomarkers for cancer diagnosis and novel targets for treatment is a major goal^[1]. Genes encoding transmembrane/secretory proteins strongly expressed in cancer may be ideal biomarkers for cancer diagnosis^[2]. In addition, if the function of the gene product is involved in the neoplastic process, such genes may constitute a therapeutic target.

The regenerating islet-derived members (*Reg*) are a

family of genes belonging to the calcium-dependent lectin (C-type lectin) gene superfamily^[3-6]. Reg represents a group of small secretory proteins that are essential for cell regeneration and proliferation and form an immune system^[7,8]. Reg plays a wide range of roles in human physiology as well as in disease^[9-15] (Table 1). Among the human Reg family, special attention has been paid to Regenerating islet-derived type IV (RegIV), the most recently discovered member. RegIV was originally isolated from a cDNA library of ulcerative colitis tissues by Hartupée *et al.*^[3]. It is expressed not only in various normal tissues such as stomach, colon, small intestine and pancreas^[16,17], but also in various malignant diseases, including colorectal^[18], gastric^[19], pancreatic^[20] and gallbladder cancer^[21]. The biological functions of RegIV in cancers are not fully understood; however, several possible functions have been proposed.

MOLECULAR CHARACTERISTICS OF REGIV AND OTHER REG GENES

Using sequence analysis, Hartupée *et al.*^[3] mapped the RegIV gene to chromosome 1 and determined that the RegIV gene contains 6 exons, the structure of which is preserved among members of the Reg gene family. Zhang *et al.*^[22] also showed that most members of the Reg family have similar organization with respect to exon number and chromosome location.

Previous studies have revealed that the Reg family shares strong similarities with C-type lectin^[3], which is distinguished from other lectins by sharing a calcium-dependent carbohydrate recognition domain (CRD). This domain of lectin might account for the complex events induced by RegIV and other Reg genes^[23-25]. Unlike RegIV, however, other Reg genes are present at low or undetectable levels in most tumors^[11]. Analysis of the unique structural similarities and differences between RegIV and other members of this gene family are expected to provide a basis for investigations of structure-function relations in this gene family.

As for binding of RegIV to other molecules or putative receptors, detailed interactions have not been elucidated. Ho *et al.*^[26] recently provided evidence that human RegIV binds to polysaccharides and mannan in the absence of calcium, unlike other C-type lectins. Utilizing nuclear magnetic resonance to elucidate the structural basis for carbohydrate recognition of RegIV, they found that RegIV has two calcium-independent mannan-binding sites serving as CRDs, suggesting a potential role in specific carbohydrate recognition^[26]. These findings might provide clues to understanding the sugar-binding role of RegIV proteins, as well as molecular interactions with currently unknown receptors.

REGIV OVEREXPRESSION IN GASTROENTEROLOGICAL CANCER

Although various normal tissues express RegIV, expres-

sion levels are much lower in normal tissues than in cancerous tissues^[27]. Violette *et al.*^[11] showed that the RegIV is more strongly expressed in colorectal tumors (particularly in mucinous carcinomas) and normal small intestine than in normal colorectal tissue. They also demonstrated that RegIV-positive tumor cells display different phenotypes: mucus-secreting, enterocyte-like, or undifferentiated.

Several studies have demonstrated overexpression of RegIV in gastric cancer. Oue *et al.*^[16] reported that RegIV expression was significantly higher in gastric carcinoma than in normal tissue on quantitative real-time polymerase chain reaction (PCR). Moreover, RegIV expression was associated with both intestinal mucin phenotype and neuroendocrine differentiation.

In pancreatic ductal adenocarcinoma^[20] as well as in gallbladder carcinoma^[21] amplification of RegIV in normal tissues was not apparent on quantitative real-time PCR. In contrast, high levels of RegIV were found in cancer tissues. These data suggest that overexpression of RegIV is associated with generating and maintaining cancer tissue.

IMPORTANT ROLES OF REGIV IN CANCER TISSUES

Overexpression of RegIV is an early event in carcinogenesis

Carcinogenesis is a multistep process involving somatic mutations or epigenetic changes affecting tumor suppressor genes and oncogenes. Several studies have indicated that RegIV may participate in early carcinogenesis in certain cancers.

Most colorectal carcinomas are thought to develop through the “adenoma-to carcinoma sequence” model^[28], in which adenomas are recognized as precursor lesions of the vast majority of colorectal cancers. Elucidation of the adenoma-carcinoma sequence along with its corresponding molecular genetic alterations will significantly enhance our understanding of the pathogenesis of colorectal carcinoma. However, since research on genetic mutations of adenomas is scant, the mechanism of the adenoma-adenocarcinoma sequence remains elusive.

Zhang *et al.*^[9,14] generated a large collection of candidate, differentially expressed genes in primary colorectal adenomas and found that RegIV was one of the differentially expressed genes in colorectal adenoma and adenocarcinoma. They proposed that overexpression of RegIV may be an early event in colorectal carcinogenesis.

In gastric cancer, one of the important precancerous changes is intestinal metaplasia caused by chronic inflammation^[29]. Oue *et al.*^[27] performed serial analysis of gene expression of gastric carcinoma and identified several genes potentially involved in invasion, metastasis and carcinogenesis, and reported that RegIV is a candidate gene for cancer-specific expression. They also performed immunohistochemical analysis of RegIV in gastric tissues and showed that RegIV protein was immunohistochemically expressed in the goblet cells of intestinal metaplasia of the stomach and gastric carcinoma, suggesting an association of RegIV protein with intestinal differentiation of the

Table 1 Human Reg family

Superfamily member	Length of amino acid	Chromosome localization	Main function
Reg I A	166	2p12	Islet cell regeneration, diabetogenesis and pancreatic lithogenesis
Reg I B	166	2p12	Islet cell regeneration, diabetogenesis and pancreatic lithogenesis
Reg III A	175	2p12	Cell proliferation and differentiation in pancreatic inflammation and liver carcinogenesis
Reg III G	175	2p12	Lectin-mediated innate immunity by binding intestinal bacteria, forming symbiotic host-microbial relationships
RegIV	158	1p12	Cell proliferation and cell regeneration. Carcinogenesis and anti-apoptosis in gastroenterological cancers

Reg: Regenerating gene; RegIV: Regenerating islet-derived type IV.

stomach and the pathogenesis of intestinal-type gastric carcinoma^[16].

Gallbladder carcinoma is also thought to arise from epithelial dysplasia, and dysplasia appears to arise from metaplasia^[50]. Tamura *et al.*^[21] reported that RegIV participates in gallbladder carcinogenesis *via* intestinal metaplasia because RegIV expression was found not only in the cancer cells but also in the intestinal metaplastic epithelium of patients with adenomyomatosis. In contrast, RegIV expression was never apparent in the normal epithelium of the gallbladder.

Intraductal papillary mucinous neoplasms (IPMN) of the pancreas show a wide spectrum of histological differentiation from hyperplasia and adenoma, and the existence of an adenoma-carcinoma sequence has been documented^[31-33]. Adsay *et al.*^[34-36] suggested that the intestinal-type IPMN to colloid carcinoma sequence is a distinct pathway of carcinogenesis involving intestinal-related genes caudal-type homeobox transcription factor 2 (*CDX2*) and Mucin 2 intestinal (*MUC2*). They described this pathway as the “intestinal” pathway of carcinogenesis. *CDX2* is a transcriptional factor that is important for the maintenance of intestinal identity^[57], and *MUC2* is a major mucin detected in intestinal epithelium^[58]. Nakata *et al.*^[58] analyzed RegIV and *CDX2* expressions in patients with IPMNs using immunohistochemical staining and microdissection-based quantitative real-time reverse transcription PCR. The positive rates of both RegIV protein and mRNA expression were significantly higher in intestinal-type IPMN than in the other types of IPMN. A significant correlation between *RegIV* and *CDX2* mRNA levels was also demonstrated. They concluded that RegIV plays an important role in differentiation of the “intestinal” pathway of IPMN and may be regulated by *CDX2*.

Carcinogenesis is a complex, multistep process involving somatic mutations or epigenetic changes affecting tumor suppressor genes and oncogenes^[39,40]. RegIV may contribute a part of these process, however further investigation is essential for comprehensive elucidation.

RegIV as an antiapoptotic factor

Advanced malignancies are often associated with poor responses to chemotherapy or radiation. Additional generic alterations in tumorigenesis create a permissive environment for clonal expansion of cells that are resistant to apoptosis^[41]. So far, considerable attention has been given to the B-cell lymphoma 2 (*Bcl-2*) family of genes as pos-

sible regulators of intrinsic tumor resistance to therapy^[42]. Repressors of programmed cell death, such as *Bcl-2* and B-cell lymphoma-extra large (*Bcl-xL*), are known to decrease radiation- and chemotherapy-induced apoptotic cell death in cell cultures^[43] *via* the Akt signaling pathway, which is an important determinant of the response to anticancer therapy^[44]; overexpression of these genes suggests a poor prognosis in colon cancer^[45]. However, key cellular factors that regulate expression of anti-apoptotic genes in tumors are not fully clarified. Defining dominant pathways responsible for regulating apoptosis could broaden current strategies for therapeutic intervention.

Bishunpuri *et al.*^[46] investigated possible roles of RegIV in colon cancer cells, using an *in vitro* radiation-survival colony assay. Colon cancer cells were cultured with or without recombinant human RegIV (rhR4) and exposed to 4 Gy of irradiation. After irradiation, colony counts increased significantly in rhR4-treated cell lines, but decreased in untreated cells. In the absence of irradiation, rhR4 treatment did not alter the numbers of colonies in treated cells. These data indicate that RegIV promotes tumor cell survival following a potent apoptotic stimulus. Furthermore, to establish a causative association between *RegIV* and the anti-apoptotic genes *Bcl-2* and *Bcl-xL*, *rhR4* was added to cultures of colon carcinoma cell lines, and *Bcl-2* and *Bcl-xL* mRNA expression levels were analyzed. Both *Bcl-2* and *Bcl-xL* expression levels increased significantly after rhR4-treatment in colon cancer cell lines, indicating that exogenous RegIV regulates expression of the *Bcl-2* and *Bcl-xL*.

Mitani *et al.*^[19] also transfected gastric cancer cells with vector expressing RegIV to investigate the biologic significance of RegIV. To evaluate the effects of RegIV on the response to 5-fluorouracil (5-FU) treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays were performed. Overexpression of RegIV in gastric cancer cells was confirmed to significantly inhibit 5-FU-induced apoptosis compared with cells transfected with empty vector on both MTT assay and measurement of DNA fragments. They also examined the expression of dihydropyrimidine dehydrogenase (DPD), because DPD has important roles in the pharmacokinetics and toxicity of 5-FU^[47,48]. Induction of DPD expression was demonstrated in cells overexpressing RegIV compared with a negative control on Western blotting. They next examined the relevance of RegIV expression to the response of gastric cancer to low-dose 5-FU and cisplatin in patients with

Table 2 Articles reporting regenerating islet-derived type IV as anti-apoptotic factor

Author	Year	Type of cancer	Result
Bishnupuri <i>et al</i> ^[46]	2006	Colorectal	RegIV induces cell survival against radiation, and regulates Bcl-2 and Bcl-xL
Mitani <i>et al</i> ^[19]	2007	Gastric	RegIV inhibited 5-fluorouracil-induced apoptosis, and induced DPD expression
Eguchi <i>et al</i> ^[44]	2009	Pancreatic	RegIV decreased the sensitivity to radiation and gemcitabine

RegIV: Reporting regenerating islet-derived type IV; Bcl-2: B-cell lymphoma 2; Bcl-xL: B-cell lymphoma-extra large; DPD: Dihydropyrimidine dehydrogenase.

recurrent gastric cancer. Among 36 patients who received this regimen, the response was no change or progressive disease in all 14 patients with RegIV expression, whereas partial responses were obtained in 8 (36.4%) of the 22 patients without RegIV expression ($P = 0.013$).

To investigate the relevance of RegIV to resistance to chemotherapy or radiotherapy, Eguchi *et al*^[44] established stable RegIV-expressing cells by transfection of plasmid into RegIV-negative pancreatic cancer cells. Fluorescence-activated cell scanning analysis of radiated cells showed that the apoptotic population was 28.7% among radiated control cells, compared with only 10.7% among RegIV-expressing cells ($P < 0.001$). Similarly, the 50% inhibitory concentration (IC₅₀) of gemcitabine was 100 nmol/L against RegIV-expressing cells, but only 30 nmol/L against control cells. These *in vitro* findings suggest that RegIV has anti-apoptotic properties in pancreatic cancer cells exposed to radiation and chemotherapy.

These studies revealed that RegIV not only promotes the expression of certain factors known as anti-apoptotic proteins, but also contributes to increasing resistance to apoptotic death during treatment *in vivo* as well as *in vitro* (Table 2).

RegIV as a proinvasive factor

Rafa *et al*^[49] investigated the potential function of RegIV as a proinvasive factor in colorectal cancer cells. Colon cancer cells secreting RegIV or not were used to analyze the autocrine and paracrine effects of RegIV. They evaluated the invasive properties of cancer cells by performing collagen type I invasion assays and calculating invasion index, which is useful for judging the invasion ability^[50,51]. They demonstrated that cell lines which secreted RegIV were spontaneously invasive, whereas cells which did not secrete RegIV were non-invasive compared with positive control cells. They also added the RegIV protein to non-RegIV-secreting cell lines, and confirmed a dose-dependent increase in the invasive index. Addition of an anti-RegIV antibody to assays with the invasive cell lines significantly limited their invasive properties. These results suggest that RegIV promotes *in vitro* invasion of colon cancer cell lines in both an autocrine and a paracrine manner.

REGIV EXPRESSION AND CLINICOPATHOLOGICAL FEATURES

Several studies have contrasted clinicopathological features with expression levels of RegIV. Among 36 patients with

colorectal cancer, Oue *et al*^[16] demonstrated that lymph node metastasis was positive in 69.2% of the RegIV-positive group, but only 30.4% of RegIV-negative group, suggesting that RegIV contributes to lymph node metastasis in colorectal cancer. A relation between overexpression of RegIV and liver metastasis has also been demonstrated in patients with colorectal cancer^[18,52]. One study of 30 patients with colorectal cancer showed that metastatic recurrence in the liver during follow-up was more frequently associated with the presence than the absence of RegIV staining (63% *vs* 9%, $P = 0.010$)^[18]. Bishnupuri *et al*^[53] suggested that liver metastasis might result from RegIV inducing matrix metalloproteinase-7, which promotes liver metastasis^[54].

We studied surgical specimens of cancer tissue and adjacent normal mucosa obtained from 202 patients with colorectal cancer. We examined the relations between the expression levels of RegIV and clinicopathological features. High expression levels of RegIV were significantly related to well-differentiated histological type, deeper invasion, lymphatic invasion, liver metastasis, and advanced stage (stage IV). In contrast, RegIV expression levels were unrelated to age, gender, tumor size, tumor location, lymph node metastasis, and venous invasion^[55].

In gastric cancer, positive RegIV expression has been associated with poorly differentiated tumors, although there was no clear correlation between RegIV expression and tumor depth or lymph node metastasis^[16]. The results of an *in vitro* study by Kuniyasu *et al*^[56] suggest that RegIV might accelerate peritoneal metastasis in gastric cancer.

These studies generally discussed the unfavorable impact of RegIV expression on clinicopathological features. Cell proliferation and anti-apoptotic effect induced by RegIV may accelerate progression of cancer.

RegIV as a prognostic factor

RegIV has recently been recognized to be associated with poor prognoses in specific gastroenterological cancers. Macadam *et al*^[13] reported a significant increase in colorectal cancer-related deaths among patients with non-metastatic early stage disease whose tumors expressed Reg genes. This subgroup of patients was also at high risk for recurrent colorectal cancer after curative surgery, and such patients may benefit from adjuvant therapy^[13].

We also demonstrated that RegIV is significantly associated with outcomes in colorectal cancer. In our study, overall survival rates were significantly higher in RegIV low patients (83.5%) than in RegIV high patients (55.1%, $P = 0.002$). On multivariate Cox regression analysis, tu-

Table 3 Articles reporting regenerating islet-derived type IV as biomarker

Author	Year	Type of cancer	Result
Oue <i>et al</i> ^[18]	2007	Colorectal	Serum RegIV concentration was significantly elevated in patients with liver metastases than those without
Mitani <i>et al</i> ^[19]	2007	Gastric	Presurgical serum RegIV was significantly elevated compared with healthy individuals
Kobayashi <i>et al</i> ^[59]	2010	Gastric	Presurgical serum RegIV was significantly different in early and advanced cancer, compared with healthy individuals

RegIV: Reporting regenerating islet-derived type IV.

mor size, liver metastasis, and a higher level of *RegIV* gene expression ($P = 0.029$) were significant independent predictors of overall survival in colorectal cancer^[55].

Miyagawa *et al*^[57] estimated RegIV mRNA levels in the peritoneal washes of 95 patients with gastric cancer by real-time reverse transcription-PCR. The RegIV mRNA level was correlated with the extent of wall penetration and peritoneal metastases. They also found that the outcomes of RegIV-positive patients were significantly worse than those of RegIV-negative patients. Multivariate analysis suggested that RegIV is an independent prognostic factor^[57].

Tao *et al*^[58] measured RegIV mRNA levels by immunohistochemical staining of tissue, and enzyme-linked immunosorbent assay of serum. Their results confirmed that the mean survival time was significantly shorter in patients with RegIV-positive gastric cancer than in those with RegIV-negative gastric cancer ($P = 0.013$)^[58].

In gallbladder cancer, Tamura *et al*^[21] reported that high expression of RegIV correlated with a well-differentiated phenotype accompanied by better outcomes, where lower expression correlated with a poorly differentiated phenotype accompanied by worse survival, suggesting that loss of RegIV expression might be associated with more malignant characteristics.

According to these data, RegIV expression is generally associated with poor outcomes in colorectal cancer and gastric cancer. Further studies will hopefully clarify differences in clinical outcomes according to the type of cancer.

RegIV as a diagnostic biomarker

Serum biomarkers for the detection of cancer are needed in order to find a larger number of candidates for suspected cancer. Several studies have assessed the feasibility of using RegIV as a serum diagnostic marker (Table 3). Oue *et al*^[18] measured serum RegIV levels in patients with colorectal cancer by enzyme-linked immunosorbent assay to investigate the diagnostic potential of RegIV. Increased preoperative levels of RegIV were found in a low numbers of serum samples from patients with stage 0-III colorectal cancer, indicating that serum RegIV is unsuitable for the detection of early colorectal cancer. In contrast, in patients with stage IV disease, the serum RegIV concentration was significantly higher in the presence than in the absence of liver metastasis, suggesting that RegIV is a good marker for metastatic recurrence in the liver after resection of colorectal cancer.

Mitani *et al*^[19] demonstrated that the serum RegIV concentration in presurgical patients with gastric cancer was significantly higher than that in healthy individuals ($1.96 \pm 0.17 \mu\text{g/L}$ vs $0.52 \pm 0.05 \mu\text{g/L}$, $P < 0.001$). The diagnostic sensitivity of serum RegIV (36.1%) was superior to that of serum carcinoembryonic antigen (CEA, 11.5%) or carbohydrate antigen 19-9 (CA19-9, 13.1%).

Kobayashi *et al*^[59] also evaluated the usefulness of serum RegIV levels as a diagnostic marker for gastric cancer. They collected pretreatment serum samples from patients with gastric cancer and healthy control subjects without cancer. RegIV levels were significantly higher in patients with early gastric cancer (median $8.42 \mu\text{g/L}$) than in the control subjects (median $5.01 \mu\text{g/L}$) ($P < 0.001$). RegIV levels were also higher in patients with advanced gastric cancer (median $13.12 \mu\text{g/L}$) than in those with early gastric cancer ($P < 0.02$). The sensitivity for gastric cancer was 73.0%, the specificity was 70.8%, and the accuracy was 71.8%, which is superior to the respective values for CEA and CA19-9. Serum RegIV levels were thus suggested to be potentially useful as a screening marker for gastric cancer, including early disease.

In pancreatic cancer, Takehara *et al*^[20] detected significantly elevated serum RegIV levels, measured with the use of an enzyme-linked immunosorbent assay system, in patients with early-stage pancreatic ductal adenocarcinomas. Their findings suggested that RegIV may become a new serological marker of pancreatic ductal adenocarcinoma.

Serum RegIV level can be a useful indicator to distinguish between patients with cancer and healthy subjects. RegIV has the potential to be used as a screening serum marker for certain cancers, including cancers in the early stages.

SIGNALING PATHWAY OF REGIV, AND REGIV-TARGETED THERAPY

Monoclonal antibody therapy has become an important option for the management of gastroenterological cancer. Bevacizumab, a humanized monoclonal antibody to vascular endothelial growth factor, is currently approved in combination with intravenous 5-FU-based regimens for first-line treatment of metastatic colorectal cancer. Besides the anti-angiogenesis factor antibody, antibodies against circulating ligands, such as hepatocyte growth factor^[60] and interleukin-6^[61], are under review as anticancer drugs.

The signaling pathway activated by *RegIV* is poorly understood; however, Bishunpuri *et al*^[53] recently demonstrat-

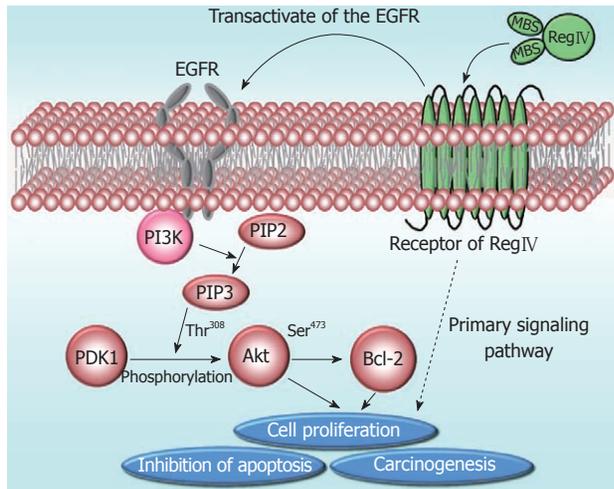


Figure 1 Schematic representation of the regenerating islet-derived type IV signaling pathway. Regenerating islet-derived type IV (RegIV) contributes to cell proliferation, inhibition of apoptosis, and carcinogenesis *via* protein kinase B (Akt) signaling pathway by transactivating the epidermal growth factor receptor (EGFR). The primary signaling pathway is now being investigated. MBS: Mannan-binding sites; PI3K: Phosphoinositide 3-kinase; PIP2: Phosphatidylinositol 4,5-bisphosphate; PIP3: Phosphatidylinositol 4,5-triphosphate; PDK1: Phosphoinositide-dependent kinase-1; Bcl-2: B-cell lymphoma 2.

ed that *RegIV* is likely to function as an anti-apoptotic factor in colon cancers through the phosphorylation of Akt and epidermal growth factor receptor (EGFR). EGFR expression and activation are common in adenocarcinomas and are associated with poor prognoses^[62-68]. The importance of EGFR in colorectal cancer has been underpinned by the clinical use of the cetuximab, a therapeutic monoclonal antibody that binds to the external domain of EGFR and blocks ligand-mediated dimerization and activation^[68].

Akt is another important downstream target of EGFR because EGFR activates Akt *via* a signaling pathway involving phosphoinositide 3-kinase (PI3K) and phosphoinositide-dependent kinase-1^[69,70]. Akt overexpression and activation by phosphorylation at Thr³⁰⁸ and Ser⁴⁷³ are well-established early events in sporadic colon carcinogenesis and are detectable in the neoplastic epithelium, but are generally absent in normal colonic epithelium^[71].

Bishunpuri *et al.*^[53] examined the effects of purified rhR4 on colon adenocarcinoma cells to determine the signaling pathways responsive to RegIV. They reported that rhR4 treatment resulted in rapid phosphorylation of EGFR at Tyr⁹⁹² and Tyr¹⁰⁶⁸ and Akt at Thr³⁰⁸ and Ser⁴⁷³. They concluded that RegIV is a potent transactivator of the EGFR/PI3K/Akt signaling pathway *via* an unknown receptor and proposed that disruption of PI3K signaling may have utility as a novel therapeutic intervention for colorectal cancer.

To examine the role of RegIV overexpression in pancreatic cancer cells, Takehara *et al.*^[20] constructed several expression vectors designed to express siRNA specific to RegIV and transfected them into a pancreatic cancer cell line that endogenously expressed RegIV at a high level. The cell line had a knockdown effect on endogenous RegIV transcription, resulting in a significant reduction in

the number of viable cells as measured by MTT assay as well as by colony formation assay compared with negative controls. RegIV was thus suggested to have a critical role in pancreatic cancer cell survival and growth. They also generated recombinant human RegIV and incubated pancreatic cancer cells in its presence to examine whether RegIV activates the Akt signaling pathway. Recombinant human RegIV treatment was confirmed to significantly increase phosphorylated Akt, suggesting that RegIV stimulates cell growth *via* the Akt signaling pathway in pancreatic cancer cells^[20]. Furthermore, by using monoclonal antibodies specific to RegIV, they succeeded in neutralizing secreted RegIV in the culture medium *in vitro* and found that treatment with these neutralizing antibodies significantly suppressed pancreatic cancer cell growth by blocking Akt phosphorylation.

Legoffic *et al.*^[72] similarly examined the effect of RegIV antibody by Western blotting analysis in mice with pancreatic cancer. They confirmed that the injection of RegIV antibody significantly reduced the intra-tumor level of proteins associated with apoptosis (Akt, Bcl-2 and Bcl-xL) after treatment compared with a control group, resulting in significant reduction of tumor.

The results of these studies suggest that RegIV may contribute to cell proliferation and anti-apoptosis by transactivating EGFR *via* an unknown receptor (Figure 1). Although a cell surface receptor for RegIV has not been identified, there are numerous examples where EGFR is transactivated by signaling molecules that do not bind directly to receptor. Bishunpuri *et al.*^[53] speculated that the RegIV receptor might be a G protein-coupled receptor, because ligands capable of transactivating EGFR usually act on such receptors. They are now focusing on identifying the RegIV binding receptor and further delineating primary intracellular signaling events^[53].

The findings outlined above suggest the feasibility of antibody therapy targeting RegIV. Neutralizing antibody therapy targeting RegIV may lead to novel therapeutic strategies for gastroenterological cancers expressing RegIV (Table 4).

CONCLUSION

This is the first systematic review of the *RegIV* gene as related to gastroenterological cancer, focusing on its role in cancer tissue and its impact on clinical outcomes. RegIV is generally upregulated in gastroenterological cancers, including those of the stomach, colorectum, and pancreas, as well as in benign diseases such as ulcerative colitis. Available evidence suggests that the basic biological effects of RegIV seem to be induction of cellular proliferation, invasion and inhibition of apoptosis, resulting in relatively worse clinicopathological features, or worse survival in patients with high-RegIV expression than those without.

Recent studies revealed that the serum RegIV level can be a novel biomarker to detect patients with colorectal, gastric, and pancreatic cancer. These studies suggested RegIV has the potential to be used as a screening serum

Table 4 Articles reporting regenerating islet-derived type IV-targetted therapy

Author	Year	Method/type of cancer	Result
Bishunpuri <i>et al</i> ^[53]	2006	Transfection of rhR4/ colorectal	Increase in pEGFR and pAkt
Takehara <i>et al</i> ^[20]	2006	Transfection of rhR4 and siRNA/pancreatic	Significant reduction in viable cells and increase in pAkt
Legoffic <i>et al</i> ^[72]	2009	RegIV antibody injection/pancreatic	Significant reductions in Akt, Bcl-2 and Bcl-xL

rhR4: Recombinant human regenerating islet-derived type IV; RegIV: Human regenerating islet-derived type IV; siRNA: Small interfering RNA; pEGFR: Phosphorylated epidermal growth factor receptor; pAkt: Phosphorylated protein kinase B; Akt: Protein kinase B; Bcl-2: B-cell lymphoma 2; Bcl-xL: B-cell lymphoma-extra large.

marker. In addition, our multivariate analysis suggested that overexpression of the *RegIV* gene is a useful prognostic biomarker in patients with colorectal cancer, which corresponds to other reports.

The signaling pathway activated by RegIV is not fully understood. However, recent studies demonstrated that RegIV is a potent activator of the EGFR/Akt signaling cascade, which is associated with cell survival and proliferation. Further investigation of RegIV, particularly its cell surface receptors and signaling pathways, will further our understanding of the basic mechanisms of this gene. Strategies designed to reduce endogenous RegIV expression or to block downstream signaling warrant additional investigations to delineate their potential roles in the prevention or treatment of established gastrointestinal adenocarcinomas.

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Hepato-biliary profile of potential candidate liver progenitor cells from healthy rat liver

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Abstract

AIM: To evaluate the presence of progenitor cells in healthy adult rat liver displaying the equivalent advanced hepatogenic profile as that obtained in human.

METHODS: Rat fibroblastic-like liver derived cells (rFLDC) were obtained from collagenase-isolated liver cell suspensions and characterized and their phenotype profile determined using flow cytometry, immunocytochemistry, reverse transcription polymerase chain reaction and functional assays.

RESULTS: rFLDC exhibit fibroblastoid morphology, express mesenchymal (CD73, CD90, vimentin, α -smooth muscle actin), hepatocyte (UGT1A1, CK8) and biliary (CK19) markers. Moreover, these cells are able to store glycogen, and have glucose 6 phosphatase activity, but not UGT1A1 activity. Under the hepatogenic differentiation protocol, rFLDC display an up-regulation of hepa-

toocyte markers expression (albumin, tryptophan 2,3-dioxygenase, G6Pase) correlated to a down-regulation of the expression of the biliary marker CK19.

CONCLUSION: Advanced hepatic features observed in human liver progenitor cells could not be demonstrated in rFLDC. However, we demonstrated the presence of an original rodent hepato-biliary cell type.

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Key words: Hepato biliary profile; Hepatogenic differentiation; Liver; Progenitor cell; Rat

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INTRODUCTION

Liver transplantation is considered to be the standard treatment for end-stage liver diseases. Unfortunately, clinical applications are restricted by the scarcity of organs and uncertainty about the very long-term success of the procedure.

In recent years, liver cell transplantation using hepatocytes was successfully performed in patients with inborn errors of metabolism as an alternative, or at least as a bridge to orthotopic liver transplantation^[1-5]. However, the success of such a therapeutic approach remains limited by the quality of transplanted cells. In fact, cryopreservation procedures induce significant alterations at morphological

and functional levels of the thawed hepatocytes^[6,7].

To overcome these problems, several approaches to isolate and propagate liver stem or progenitor cells have been developed. In our laboratory, Najimi *et al.*^[8] isolated adult derived human liver stem/progenitor cells (ADHLSC) with hepato-mesenchymal profile. Under specific hepatogenic conditions, these cells exhibit hepato-specific functions like glycogen storage, gluconeogenesis, urea synthesis, glucuronoconjugation, and pharmacologic properties such as activity of phase I and II enzymes^[9]. These cells are also able to specifically engraft and differentiate into mature human hepatocytes in mouse liver parenchyma^[8].

Preclinical studies using homologous animal models of human liver metabolic diseases are attractive. It is therefore a prerequisite to obtain homologous cells from syngenic animals to perform such studies. The relevance of using human progenitor cells in immunosuppressed animal models is indeed questionable.

In this context, we evaluated the presence of a liver progenitor cell in adult rat liver that would express the same specifications as the previously reported human progenitor cell, referred to as ADHLSC.

In the current study we isolated and characterized rat fibroblastic-like liver derived cells (rFLDC) from healthy adult rats. Characterization included proliferation rate, phenotype, genotype and hepatic-specific functional assays.

MATERIALS AND METHODS

Rat fibroblastic-like liver derived cells

Five male Wistar rats weighing \pm 200 g were purchased from UCL *Animalerie Centrale* (Brussels, Belgium) and treated in accordance with the internal Animal Ethic and Welfare Committees (UCL/MD/2009/003).

We isolated rat liver parenchymal cells in a two-step collagenase A (1100 units/L) (Roche, Mannheim, Germany) perfusion procedure according to the Seglen method^[10]. We then obtained a hepatocyte enriched cell fraction following low-speed centrifugation (160 r/min for 3 min).

Viable hepatocytes, 1.5 million ($>$ 75%, trypan blue exclusion), were seeded onto rat tail collagen I-coated plates (Greiner, Wemmel, Belgium) in Williams' E medium (Invitrogen, Merelbeke, Belgium) supplemented with 10% fetal bovine serum (FBS) (AE Scientific, Marcq, Belgium), 25 μ g/L human epidermal growth factor (EGF) (Peprotech, London, United Kingdom), 10 mg/L human insulin (Lilly, Brussels, Belgium), 1 μ mol/L dexamethasone (Sigma, Bronem, Belgium), and 1% penicillin/streptomycin (P/S) (Invitrogen) at 37 °C in a fully humidified atmosphere containing 5% CO₂. After 24 h we changed the medium in order to eliminate the non-adherent cells and thereafter we renewed it every 3 d. On days 7-12, hepatocytes died and small colonies of spindle-shaped fibroblastic cells emerged and proliferated. At this time, we switched the culture medium to Dulbecco's modified Eagle's medium (DMEM medium) [DMEM high glucose (Invitrogen) supplemented with 10% FBS and 1% P/S]

in order to accelerate the proliferation of emerging cells. When cell cultures reached 90% confluence, we trypsinized them with 0.05% trypsin-1 mmol EDTA solution (Invitrogen) and replated them on a collagen-coated plate at a density of 10⁴ cells/cm². The medium was refreshed every 3 d.

Population doubling (PD) was evaluated after each passage using the following equation: [log (harvested cells)/log (seeded cells)]/log 2. Cumulative population doubling (CPD) was calculated with the sum of PD at all passages.

At passages 2, 4 and 8, cells were analyzed using reverse transcription polymerase chain reaction (RT-PCR), immunocytochemistry and flow cytometry.

Bone marrow mesenchymal stem cells

We obtained bone marrow from Wistar rats by flushing the femur and tibia with ice cold phosphate-buffered saline (PBS) (Lonza, Verviers, Belgium) and isolated the cell fraction using Ficoll (GE Healthcare, Uppsala, Sweden) density gradient centrifugation at 340 r/min for 30 min.

Cells were then resuspended in α -MEM (Invitrogen) supplemented with 10% FBS (Perbio, Erembodegem, Belgium) and 1% P/S (Invitrogen) and seeded in 75 cm² culture flasks. We removed non-adherent cells after 1 d and then refreshed the medium every 3-4 d. When cultures had reached 80%-90% confluence, we harvested the cells with 0.05% trypsin-1 mmol EDTA solution and replated them at a density of 7×10^3 cells/cm². These cells were used as the internal control in mesodermal differentiation studies.

Characterization of rFLDC

Flow cytometry: Cells from the initial parenchymal fraction or after passaging were suspended at a concentration of 1000 cells/ μ L in PBS and 0.5% bovine serum albumin (BSA, Sigma) and then incubated for 25 min at room temperature with the following antibodies: CD29-PE (rabbit monoclonal, 1/70), CD44-FITC (mouse monoclonal, 1/20), CD45-FITC (mouse monoclonal, 1/20) (Abcam, Belgium), CD73-FITC (mouse monoclonal, 1/20), CD90-PE (mouse monoclonal, 1/20) (BD, Erembodegem, Belgium). Unspecific binding of antibodies, was evaluated using mouse IgG1 FITC and the PE isotypes control (BD).

We then washed and fixed them in cytofix/cytoperm (BD) until analysis with a FACSCanto II flow cytometer (BD).

Immunocytochemistry: We fixed rFLDC cultured on 24 well rat collagen type-1 coated plates with 3.5 % formaldehyde (v/v, VWR, Leuven, Belgium) for 15 min at room temperature. After rinsing in PBS, we permeabilized cells with 1% Triton \times 100 (w/v Roche) in PBS for 10 min. Before incubation with specific rat antibodies, endogenous peroxidase activity was inhibited with PBS supplemented with 3% H₂O₂ (VWR) solution for 3 min. Non-specific immunostaining was prevented by incubation with 3% BSA solution (Sigma) for 1 h. Cells were

then incubated for 1 h with 0.3% BSA containing the following antibodies: fibronectin (rabbit polyclonal, 1/50) (Dako, Heverlee, Belgium), vimentin (mouse monoclonal, 1/100), and α -smooth muscle actin (ASMA) (rabbit polyclonal, 1/100) (BD). After rinsing with PBS, cells were finally incubated for 30 min with Envision[®], a secondary antibody against mouse or rabbit (Dako). Immunostaining results were evidenced by the addition of diaminobenzidine and urea reagents (Sigma) and counterstained with Mayer hematoxylin solution.

RT-PCR analysis: We extracted total RNA from expanded or differentiated rFLDC using the TriPure isolation reagent (Roche) and carried out cDNA with the Thermoscript RT-PCR system (Invitrogen) using 1 μ g total RNA, according to the manufacturer's instructions. Rat specific primers used for gene amplification are listed in Table 1. We thereafter electrophoresed amplified cDNA on a 1% agarose gel (Invitrogen) followed by 0.01% ethidium bromide (Sigma) staining.

Plasticity assessment

Hepatogenic differentiation: rFLDC from passage four were seeded at a density of $10^4/\text{cm}^2$ into 6 wells and 24 wells rat tail type I collagen-coated plates in the presence of expansion medium. When cell cultures reached 90% confluence, we switched the medium to Iscove's modified Dulbecco's medium (IMDM, Invitrogen) supplemented with 20 $\mu\text{g}/\text{L}$ human EGF (Peprotech, London, United Kingdom) and 10 $\mu\text{g}/\text{L}$ human basic fibroblast growth factor-2 (FGF2) (Peprotech) for 2 d. Thereafter, we subjected cells to differentiation induction for 10 d with IMDM containing 20 $\mu\text{g}/\text{L}$ human hepatocyte growth factor (HGF) (Peprotech), 10 $\mu\text{g}/\text{L}$ FGF2, nicotinamide 0.61 g/L (Sigma), and 1% insulin-transferrin-selenium (ITS) (Invitrogen). An intermediate step of differentiation/maturation in IMDM containing 20 $\mu\text{g}/\text{L}$ HGF, 20 $\mu\text{g}/\text{L}$ human oncostatin M (Peprotech), nicotinamide 0.61 g/L and 1% ITS was performed over 10 d. The subsequent maturation step consisted of treatment with IMDM containing 20 $\mu\text{g}/\text{L}$ oncostatin M, 1 $\mu\text{mol}/\text{L}$ dexamethasone (Sigma), and 1% ITS premix for 10 d. For each step, we changed the medium every 3-4 d.

Mesodermal differentiation: At passages 0, 2 4 and 8, rFLDC were plated at 1.5×10^4 cells/ cm^2 on six-well rat tail collagen I-coated plates. At confluency, we performed osteogenic differentiation with complete DMEM medium containing 0.1 μM dexamethasone, 0.1 mmol/L ascorbate and 10 mmol/L β -glycerophosphate (Sigma). After 4 wk, calcium deposition was evidenced using alizarin red staining. For adipogenic differentiation, we incubated cells with expansion medium complete DMEM containing 1 μM dexamethasone, 0.5 mmol/L isobutylmethylxanthine, 0.2 mmol/L indomethacin (Sigma) and 10 $\mu\text{g}/\text{mL}$ insulin (Lilly). Medium change was carried out twice a week. After 4 wk, oil red O staining revealed the presence of lipid vesicles. As a control of mesodermal differentiation capacity, the differentiation procedure was

Table 1 Primers used for reverse transcription polymerase chain reaction

Gene	Amplicon size (bp)	Primers
<i>Vim</i>	241	F: AAGCAGGAGTCAAACGAATA R: GAGCCATCTTTACATTGAGC
<i>Fn</i>	392	F: ACCGTGGAGTATGTGGTTAG R: GGTGACACCTGAGTGAACCT
<i>ASMA</i>	385	F: ATGCTTCTGGACGTACAACCT R: GACTCCATCCAATGAAAGA
<i>CK8</i>	378	F: TGGAGAATGAGTTTGTCTC R: TGATGTTACGGTTCATCTCA
<i>CK19</i>	287	F: GCCAGTACTTCAAGACCATC R: ACTAATTTCTCTCTCGTGGT
<i>HNF4</i>	332	F: CGGATGTGTGTGAGTCTATG R: AAAGAAAGATGATGGCTTTGA
<i>TAT</i>	275	F: CTGGACAAAAACATCCTCATT R: GATCTCGTCAGCTAAGATGG
<i>TDO</i>	366	F: CTCCTGGTACAGCAGTTCTC R: CTTTTTCGCCTGAATCTTTA
<i>αFP</i>	289	F: AACGTAGCTACCATTGTCGT R: CAGTTTCTGGAAAGTGAAG
<i>Alb</i>	365	F: TTTACGAGAAGCTTGGAGAG R: TGTGCAGATATCAGAGTGGGA
<i>UGT1A1</i>	369	F: CCATGTGTCTTTTATTAGGG R: ACAAACATGACACACAGTGA
<i>G6Pase</i>	386	F: GAAGATGTTTCCCTGATGAA R: AGTCACCATTACCATTACAGG
<i>GAPDH</i>	315	F: CCACTCAGAAGACTGTGGAT R: TGTGAAAGTCACAGGAGACA
<i>CD29</i>	321	F: TTCAATGAACCTGTGTGGTCA R: AGTGACTGCAAAAAATCGTCT
<i>CD44</i>	383	F: ACGATTTCCCCAGAACTTAG R: ACAGGTCAAGATGGAAGATG
<i>CD45</i>	321	F: TGAACATACGGATTGTGAAA R: TTTGTTCGGACTGTAAGGTT
<i>CD73</i>	341	F: ATAGTCACCTCTGACGATGG R: ATTTCACTCGGGTGTCTGAG
<i>CD90</i>	380	F: AAGGAGAAAACAGGAAACCTC R: ACAGACACAGTCCAACCTCC
<i>CD105</i>	386	F: TACCTCCAAGACACAGATCC R: TCTGCATATTGTGGTTGTA

Fn: Fibronectin; Vim: Vimentin; ASMA: α -smooth muscle actin; CK: Cytokeratin; HNF4: Hepatocyte nuclear factor 4; TAT: Tyrosine aminotransferase; TDO: Tryptophan 2,3-dioxygenase; α FP: α -fetoprotein; Alb: Albumin; UGT1A1: UDP-glucuronosyl transferase 1A1; G6Pase: Glucose-6-phosphatase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

validated with rat bone marrow mesenchymal stem cells using α -MEM complete medium.

Functional hepatic tests

Glycogen storage: Undifferentiated and differentiated rFLDC fixed with 3.5% formaldehyde (Sigma) were incubated for 10 min in 1% periodic acid (Sigma). After washing with distilled water, the cells were incubated with Schiff's reagent (Sigma) for 15 min. The preparations were then washed and mounted.

Glucose-6-phosphatase activity: We investigated glucose-6-phosphatase (G6Pase) activity in undifferentiated and differentiated rFLDC. After washing with PBS, cells were incubated for 4 h at 37 $^{\circ}\text{C}$ in 1.5 mL 50 mmol/L Tris (Sigma) and 50 mmol/L maleate (Sigma) buffer (pH

6.7) solution containing 5 mmol/L glucose-6-phosphate (G6Pate, Sigma) and 0.03 g lead nitrate (Acros, Geel, Belgium). We obtained brownish precipitates of lead sulfate following incubation of cells in a solution containing 0.1% ammonium sulfide (Sigma)^[11]. Cells were then mounted and viewed by light microscopy (Leica DM IL, Groot-Bijgaarden, Belgium).

Bilirubin conjugation assay

Undifferentiated and differentiated rFLDC were incubated in William's medium and 1% FBS containing unconjugated bilirubin (Sigma) for 24 h and 48 h. Afterwards, we harvested the supernatant and added 2 µg/mL xantobilirubinic acid (use as internal standard: IS). We then submitted the product obtained in this reaction to an alkaline methanolysis followed by nitrogen evaporation as described by Muraca *et al.*^[12]. Precipitates were resuspended with 10 µL chloroform (Sigma) and 100 µL dimethyl sulfoxide (Sigma). We then injected ten microliters of this solution into the liquid chromatograph (Waters 515 HPLC pump) and eluted it with a C18 column (Macherey-Nagel, Düren, Germany). Elutriation flow started at 1 mL/min with methanol/water/tetrabutylammonium (solvent A) and ended after 11 min with methanol/ethanol/water/tetrabutylammonium (solvent B). Elution was continued for 6 min with solvent B, and the column was re-equilibrated with solvent A. The absorbance of the eluted pigments was monitored at 436 nm using a 996 photodiode array detector (Waters, Zellik, Belgium) and the area under peak was integrated electronically (Millennium software, Waters). We calculated the concentration, in micromoles per liter, of each bilirubin fraction in samples using the following equation: $(\text{Area}_{\text{pigment}}/\text{area}_{\text{IS}}) \times (\text{IS}/\text{IV}) \times \text{RF}$.

In which IS corresponds to micrograms of internal standard added to the sample, SV to the volume of sample (mL), and RF to the response factor.

Conjugation rate (CR) was evaluated using the equation: $(\text{Conjugated bilirubin concentration})/(\text{total bilirubin concentration}) \times 100$.

In which total bilirubin concentration was the sum of unconjugated and conjugated bilirubin.

RESULTS

Isolation and expansion of rFLDC

An enriched population of hepatocytes obtained after collagenase A digestion and low speed centrifugation was plated on type I collagen-coated 6-well plates.

During the first step of culture, mature hepatic cells present in the culture died due to their inability to proliferate (Figure 1). After 7 to 12 d, cells with a fibroblastic-like shape emerged and proliferated (Figure 1B and C). These cells demonstrated a high proliferative potential with a CPD of 294.55 ± 20.91 after 50 passages (Figure 2).

rFLDCs were reproducibly isolated from at least five different liver cell suspensions.

Characterization of rFLDC

All isolated rFLDC were analyzed and characterized after

passages 2, 4 and 8 using FACS analysis and RT-PCR. Furthermore, a stable expression profile was observed up to P50 (data not shown).

Representative flow cytometry data at passage 4 demonstrated that the most described mesenchymal markers, CD73 and CD90 constituted $44\% \pm 36\%$ and $71\% \pm 43\%$ of the cell population, respectively (Figure 3), whereas the expression of CD29 protein was only detected in $2.4\% \pm 1.1\%$. The hematopoietic marker, CD45 ($1.1\% \pm 0.6\%$) was almost undetectable.

To further characterize our cell population, we performed immunocytochemistry (ICC) for vimentin, fibronectin and ASMA proteins and compared the findings with rat bone marrow-derived mesenchymal stem cells (rBM-MSC) (Figure 4). The results indicated positive staining for ASMA, vimentin and fibronectin as observed with rBM-MSC.

To confirm the phenotypic profile of isolated rFLDC we performed RT-PCR analysis using specific mesodermal, hepatocyte and cholangiocyte markers at passage 4 (Figure 5).

The mesenchymal expression profile was confirmed by the detection of vimentin, fibronectin, ASMA, integrin β -1 (CD29), hyaluronic acid receptor (CD44), ecto 5'-nucleotidase (CD73), Thy-1 (CD90) and endoglin (CD105) mRNAs. RT-PCR also confirmed the absence of CD45 expression (Figure 5).

Because of their liver origin, rFLDC were also studied for their expression of hepatocyte and cholangiocyte markers. mRNA analysis revealed the expression of UDP-glucuronosyl transferase 1A1 (UGT1A1), cytokeratin 8 (CK8) and G6Pase (Figure 5). However, tyrosine aminotransferase (TAT), tryptophan 2,3-dioxygenase (TDO), albumin (Alb), α -fetoprotein (α FP) and hepatocyte nuclear factor 4 (HNF4) transcripts were not detected, all expressed by fully differentiated hepatocytes. mRNA analysis also revealed the expression of cytokeratin 19, a biliary marker.

In-vitro differentiation

First, we checked the ability of rFLDC to differentiate into adipocytes in the presence of specific media supplemented with dexamethasone, isobutyl-methylxanthine, indomethacin and insulin (Figure 6). We noticed that at early passages (P0-P2) two out of five rat fibroblastic-like liver derived cell cultures demonstrated a weak localized adipocytic differentiation. This ability was lost in further passages. Under osteogenic induction, no calcium deposit was noted (Figure 6).

In order to demonstrate their potential to differentiate into mature hepatocytes we seeded 10^4 cells/cm² from passage 4 in serum free-medium in the presence of several "hepatogenic" factors, as described in the Materials and Methods section. After 32 d, cells showed a slight morphology change and few cells adopted a polygonal shape (Figure 7). Using RT-PCR, we compared the expression of immature and mature hepatocytic/biliary mRNA on undifferentiated and differentiated rFLDC (Figure 5). Despite a variation in serum concentration (10% vs 2%) between the expansion medium and hepatogenic control medium, respectively, no differences in mRNA expression

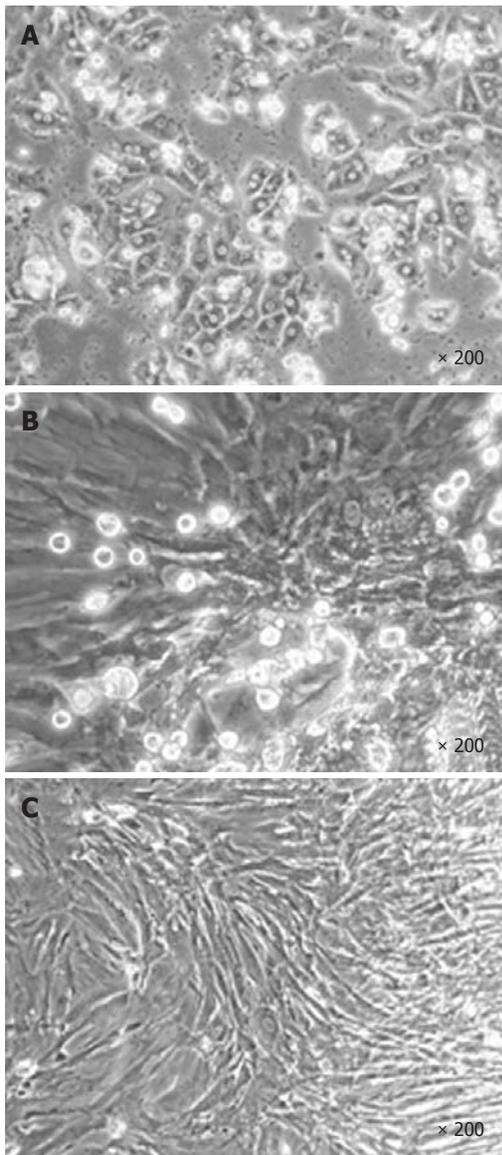


Figure 1 Rat fibroblastic-like liver derived cell obtention from adult liver parenchymal fraction. A: Cell suspension 24 h after liver parenchyma collagenase A digestion; B: 21 d culture: Rat fibroblastic-like liver derived cells (rFLDC) emergence and hepatocyte death; C: Culture purification due to rFLDC proliferation.

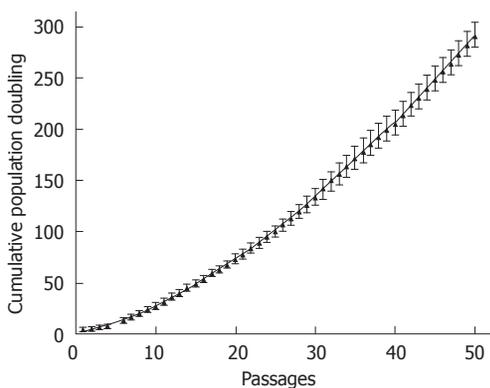


Figure 2 Proliferative capacity of rat fibroblastic-like liver derived cells. Average of cumulative population doubling of rat fibroblastic-like liver derived cells for passage 0 to passage 50.

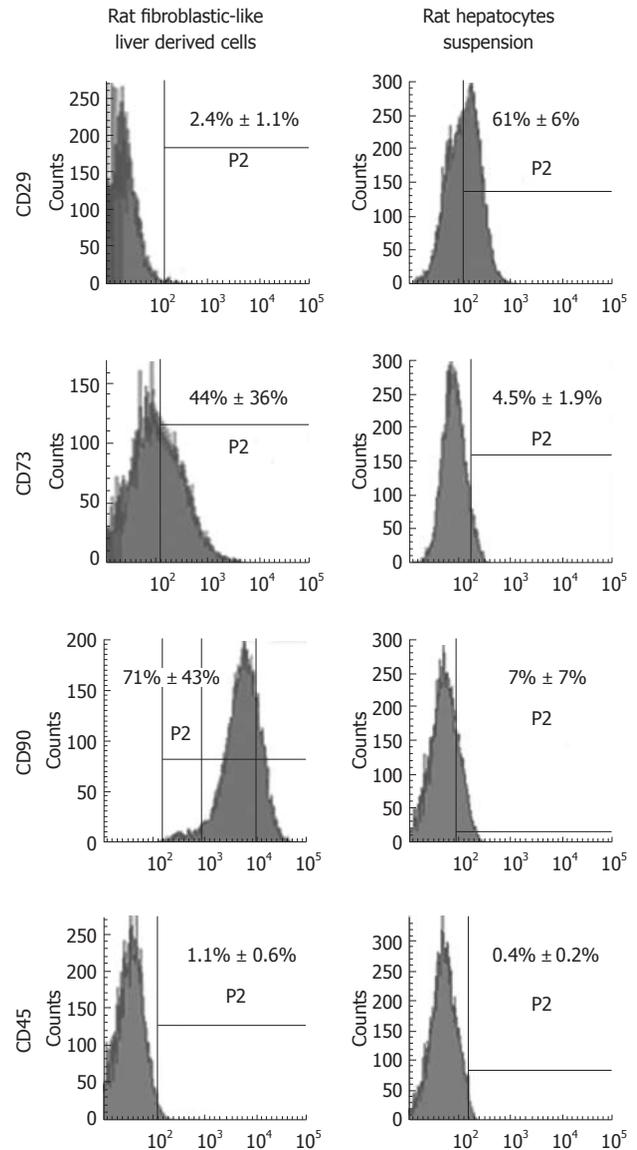


Figure 3 Representative surface markers expression analysis by flow cytometry. Results are mean \pm SE of positive cells for 5 independent experiments.

were noted (data not shown). Interestingly, differentiated rFLDC lost the expression of CK19, a biliary marker. Moreover, differentiated rFLDC acquired the expression of mature hepatocyte lineage markers including TDO and albumin.

To test their liver metabolic activity, we explored their ability to store glycogen and to perform gluconeogenesis (G6Pase activity) and their potential to conjugate bilirubin.

Glycogen storage, evidenced by periodic-acid shift staining, showed that, like rat hepatocytes (Figure 8A), undifferentiated and differentiated cells can store glycogen (Figure 8B and C).

As shown at Figure 8D-F, rat hepatocytes and rFLDC cells also revealed a basal G6Pase activity. These results were corroborated by the expression of G6Pase at the mRNA level.

In addition to glycogen storage and G6Pase activity we assessed the ability of differentiated and undifferentiated

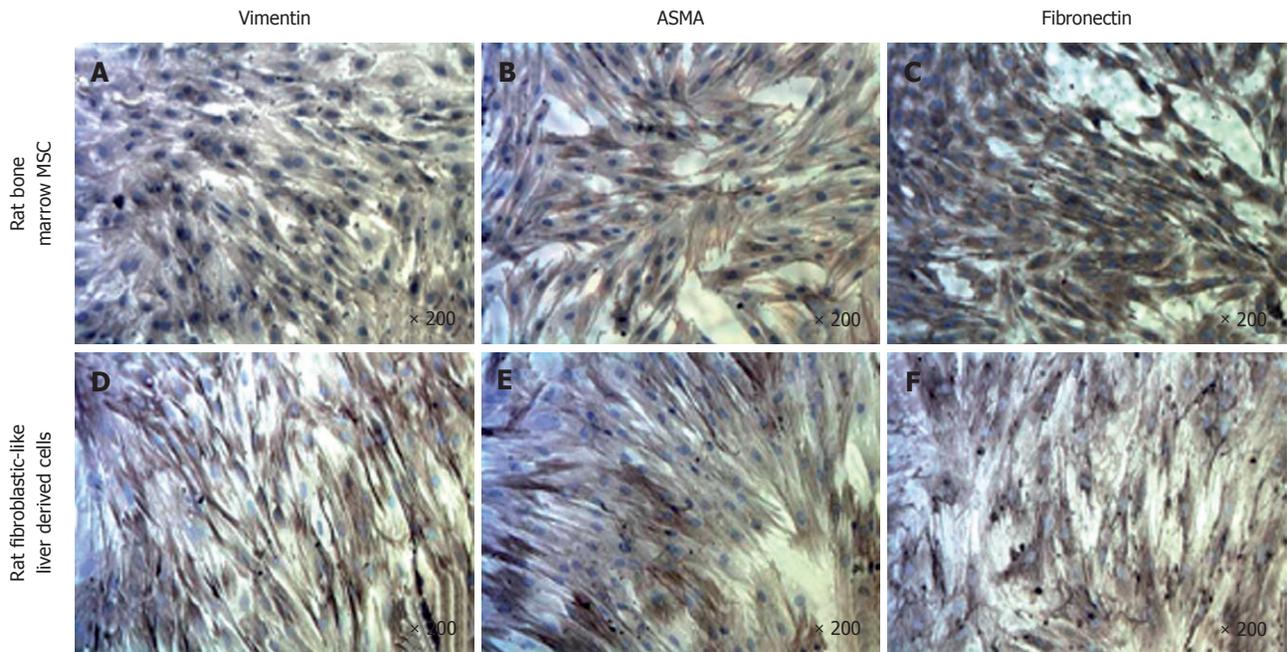


Figure 4 Rat fibroblastic-like liver derived cells mesenchymal characterization by immunocytochemistry. A-C: Rat bone marrow mesenchymal stem cells (MSC); D-F: Rat fibroblastic-like liver derived cells. ASMA: α -smooth muscle actin.

ated rFLDC to conjugate bilirubin. Therefore, we incubated 10^4 cells/cm² with William's medium-1% serum containing unconjugated bilirubin. After 24 h and 48 h, no bilirubin conjugate was observed in the culture medium in comparison with freshly isolated hepatocytes used as positive controls. For this late population the bilirubin CR reached 11% and 33% after 24 h and 48 h, respectively (Figure 9).

DISCUSSION

Because preclinical studies use animal models mimicking human diseases, we tried to isolate from rodent liver a liver progenitor cell that would display characteristics reported for ADHLSC. The use of human derived cells in animal models is considered irrelevant, as they may not engraft and function similarly in a xenogenic rodent environment.

Like human cells, rFLDC were isolated and emerged *in vitro* after culture of liver cell suspension following enzymatic-mediated disaggregation of liver. However, many differences were observed: rFLDC demonstrated a higher proliferative potential and did not reach senescence after at least 50 passages in contrast to human cells which stopped proliferating after 10-12 passages^[13]. rFLDC were able, at early passages, to differentiate into adipocytes, in contrast to ADHLSC.

Like human cells, rFLDC displayed a mesenchymal profile as evidenced by the expression of CD44, CD73, CD90 and CD105. The cell population was not contaminated by hematopoietic stem cells as evidenced by the absence of CD45 expression. These results confirmed the presence of a new enriched cell population different from the freshly isolated hepatic cells. RT-PCR also revealed expression of CK8, UGT1A1 and G6P under-

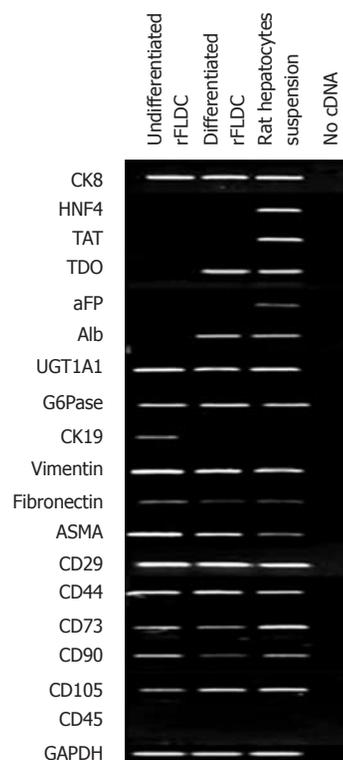


Figure 5 Representative reverse transcription-polymerase chain reaction characterization of undifferentiated and differentiated rat fibroblastic-like liver derived cells in comparison with rat hepatocyte suspension. Hepatic markers: Cytokeratin 8 (CK8), hepatic nuclear factor 4 (HNF4), tyrosine aminotransferase (TAT), tryptophan 2,3-dioxygenase (TDO), α -fetoprotein (α FP), albumin (Alb), UDP glucuronosyltransferase 1A1 (UGT1A1), glucose-6-phosphatase (G6Pase); Biliary marker: CK19; Mesenchymal markers: Vimentin (Vim), fibronectin (Fn), α -smooth muscle actin (ASMA); Cell surface markers: Integrin β -1 (CD29), hyaluronic acid receptor (CD44), tyrosine phosphatase (CD45), ecto-5'-nucleotidase (CD73), Thy-1 (CD90), endoglin (CD105); Housekeeping gene: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

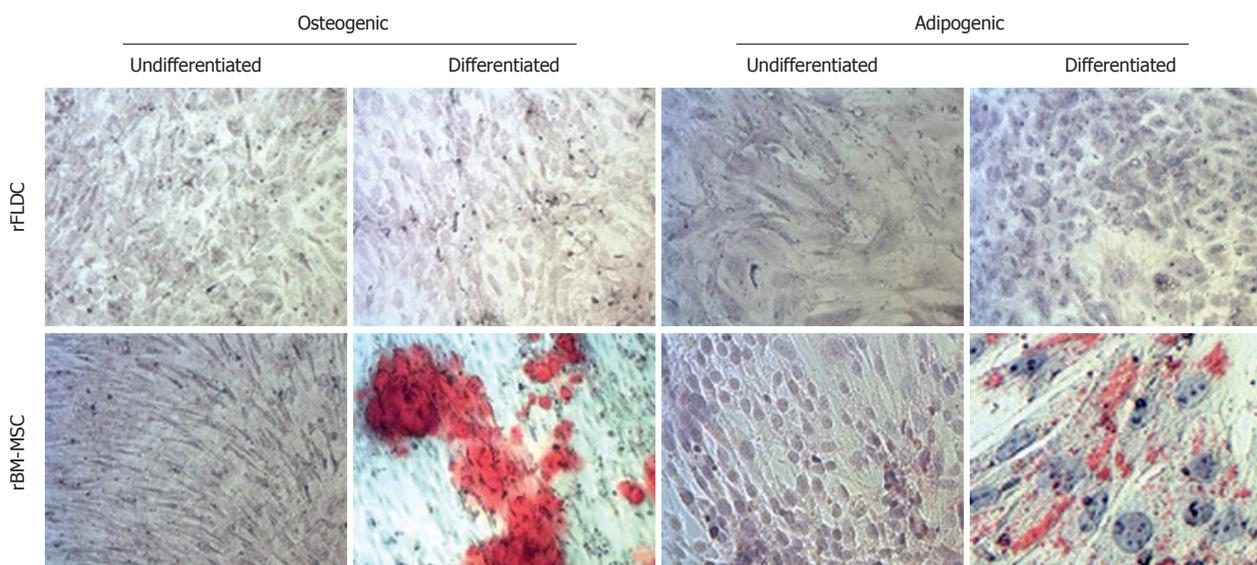


Figure 6 Evaluation of osteogenic and adipogenic differentiation potential of rat fibroblastic-like liver derived cells (alizarin red and red oil O coloration). rFLDC: Rat fibroblastic-like liver derived cells; rBM-MSC: Rat bone marrow-derived mesenchymal stromal cells.

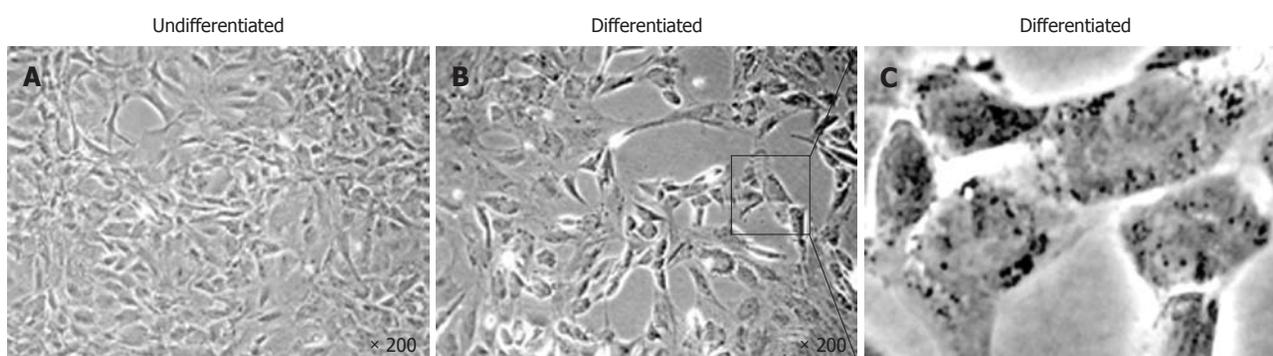


Figure 7 Rat fibroblastic-like liver derived cells hepatogenic differentiation. A: Undifferentiated; B, C: Differentiated.

lining their hepatic-like profile. Undifferentiated rFLDCs have the capacity to store glycogen and show G6Pase activity as mature hepatocytes.

In a hepatogenic differentiation medium, low numbers of rFLDC display the polygonal morphology of mature hepatocytes. Differentiated rFLDC express both albumin and TDO. However, we did not observe the expression of more specific hepatic markers such as HNF4 or TAT.

Candidate progenitor cells reported in this study were isolated from healthy rat livers, in contrast to other progenitor liver cells described elsewhere, such as oval cells, obtained after submitting animals to carcinogenic agents or toxic injuries^[14-19]. Stellate cells^[20] and rFLDC share common features like the expression of vimentin, ASMA, CK19, and CD90, although no expression of α FP was detected in rFLDC.

Differentiated rFLDC do not express CK19 or α FP, and differ therefore from small hepatocytes and epithelial cells also recovered from normal livers^[17-19].

Recently, Sahin *et al*^[19], using a 2-step collagenase protocol, reported a cell population derived from adult rat

liver and called them LDPCs (liver-derived progenitor cells). Regarding their oval morphology and expression of HNF3 β , CD45, CD34 and CD90, these cells seem to be closely related to oval cells despite the absence of CK7, CK8 and CK19 expression.

In conclusion our results showed that rodent progenitor cells homologous to ADHLSC can not easily be obtained even when the same isolation and culture protocol was applied using a rat model. However, this protocol allowed the isolation of a novel type of liver progenitor cell population with both hepatic and biliary phenotype including G6Pase activity, glycogen storage, CK8, UGT1A1 and CK19 expression.

In the presence of a hepatogenic differentiation medium, rFLDC lose the CK19 biliary marker, but do not acquire a more mature hepatic status possibly due to the use of human cytokines and growth factors, which may not be appropriate for rodent precursors, stressing again the difficulty in generating homologous models.

Further characterization and *in vitro* hepatogenic differentiation improvement are required before their relevant use in preclinical studies.

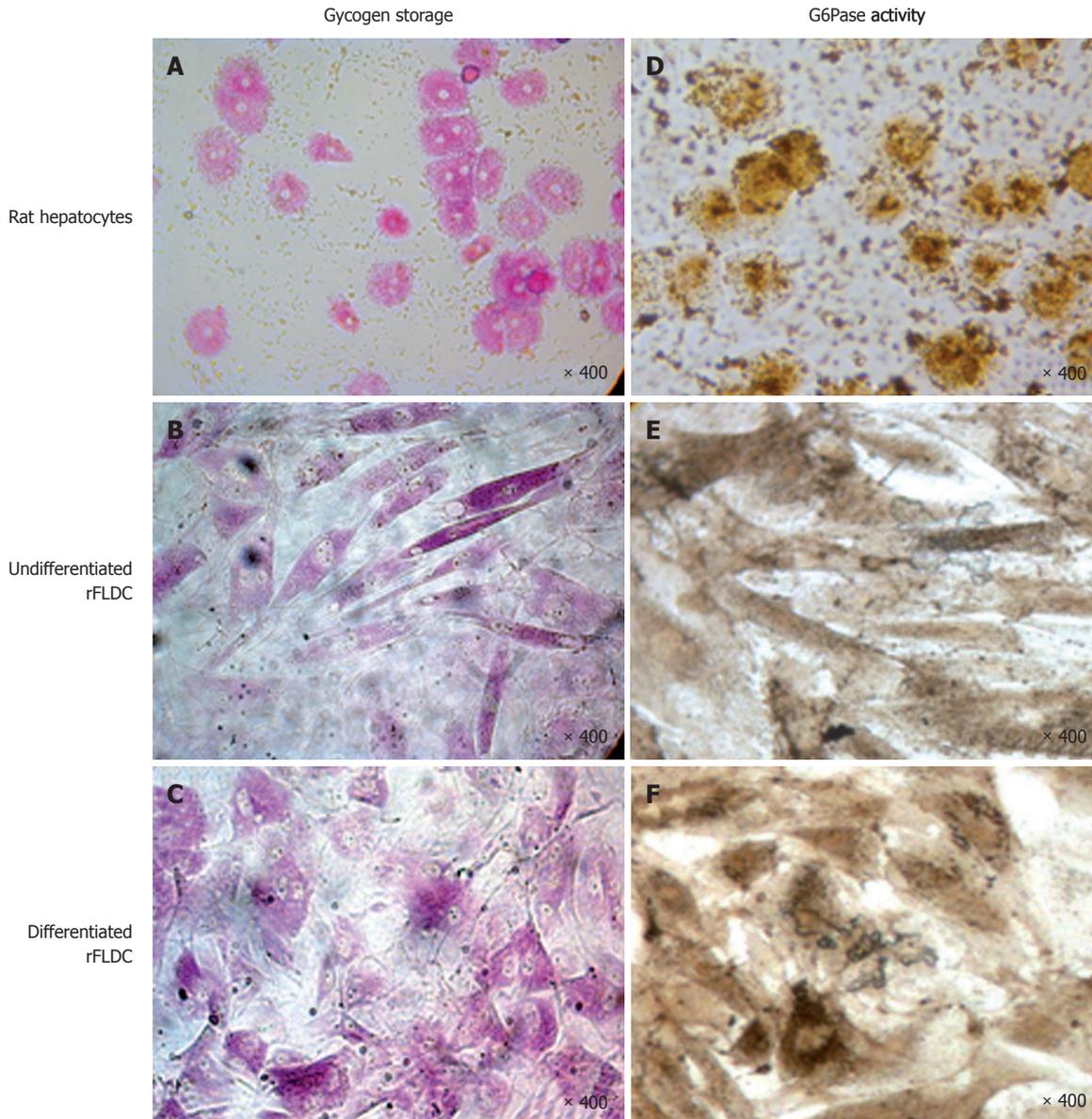


Figure 8 Hepatic functions. A-C: Glycogen storage ability assessed by periodic acid schiff reaction; D-F: Glucose-6-phosphatase (G6Pase) activity after incubation with glucose-6-phosphate and lead nitrate. rFLDC: Rat fibroblastic-like liver derived cells.

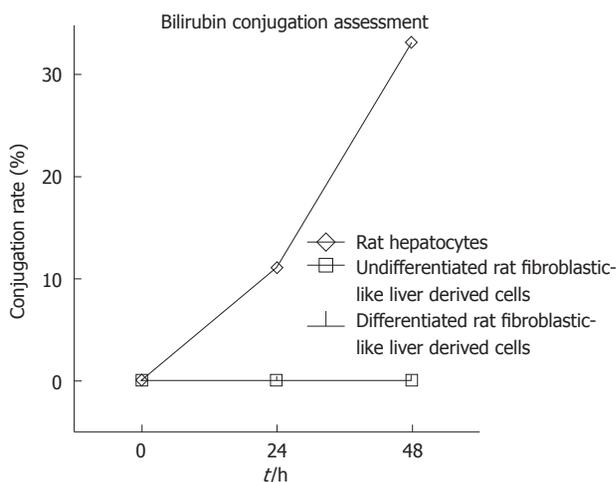


Figure 9 Bilirubin conjugation assay. Comparison between rat hepatocytes, undifferentiated and differentiated rat fibroblastic-like liver derived cells.

COMMENTS

Background

Liver cell transplantation using hepatocytes was successfully performed in patients with inborn errors of metabolism. However, the success of such a therapeutic approach remains limited by the quality of transplanted cells. To overcome these problems several approaches to isolate and propagate liver stem or progenitor cells have been developed. The capacity of those cells to restore a liver metabolic function must be demonstrated.

Research frontiers

Preclinical studies using homologous animal models of human liver metabolic diseases are attractive. It is therefore a prerequisite to isolate and propagate human homologous liver stem or progenitor cells from syngenic animals to perform such studies. In this study, the authors showed that rodent progenitor cells homologous to human adult-derived liver stem/progenitor cells can not easily be obtained even when the same protocol was applied.

Innovations and breakthroughs

In this study, the authors reported the isolation of novel potential candidate liver progenitor cells isolated from healthy rat liver called rat fibroblastic-like liver derived cells (rFLDC). These cells express both hepatic and biliary phenotype and

are able to acquire some hepatic characteristics in the presence of hepatogenic differentiation medium.

Applications

Isolation and characterization of progenitor/stem cells would be very useful to assay the *in-vivo* efficacy of liver mesenchymal progenitor cells in syngeneic animal models of liver metabolic diseases, particularly in the Gunn rat, a model of hyperbilirubinemia.

Peer review

This study shows that the advanced hepatic features of human liver progenitor cells have not been demonstrated in rFLDC. Although it strengthens the unique specificity of these human liver progenitor cells, it also shows that homologous models for cell therapy can not easily be developed even when the same isolation and culture protocols are applied. The authors should make a comparison of their cells with human established liver cells.

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Edaravone inhibits apoptosis caused by ischemia/reperfusion injury in a porcine hepatectomy model

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Abstract

AIM: To investigate the effect of E3-methyl-1-phenyl-2-pyrazolin-5-one (Edr) on hepatic ischemia-reperfusion (I/R) injury and liver regeneration in a porcine hepatectomy model.

METHODS: One hour ischemia was induced by occluding the vessels and the bile duct of the right and median lobes. A 40% left hepatectomy was performed after reperfusion. Six animals received Edr (3 mg/kg per hour) intravenously and six control animals received saline just before reperfusion. Remnant liver volume, hemodynamics, aspartate aminotransferase (AST), alanine aminotransferase, lactate dehydrogenase and lactic acid, were compared between the groups. The expression of transforming growth factor- β (TGF- β 1) and toll-like receptor (TRL) mRNA in hepatic tissues was examined using reverse transcription polymerase chain reaction. Apoptosis was demonstrated by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, respectively.

RESULTS: Serum AST ($P = 0.029$), and toll like receptor 4 level ($P = 0.043$) were significantly lower after 3 h

in animals receiving Edr. In addition, TUNEL staining in Edr-treated pigs showed significantly fewer hepatocytes undergoing apoptosis compared with control pigs. After 1 mo, all factors were non-significantly different between the two groups.

CONCLUSION: Edr is considered to reduce hepatic injury in the early stage of I/R injury in a porcine model.

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Key words: Edaravone; Ischemia-reperfusion injury; Liver resection; Transforming growth factor- β ; Toll like receptor 4

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Shimoda M, Iwasaki Y, Okada T, Kubota K. Edaravone inhibits apoptosis caused by ischemia/reperfusion injury in a porcine hepatectomy model. *World J Gastroenterol* 2012; 18(27): 3520-3526 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i27/3520.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i27.3520>

INTRODUCTION

A potent free radical scavenger, E3-methyl-1-phenyl-2-pyrazolin-5-one (Edr) has been shown to protect cardiomyocytes and brain against ischemia/reperfusion (I/R) injury^[1,2]. However, this beneficial protection after I/R injury in the liver, and the effect on liver regeneration is still unknown^[3].

Compression of the hepatoduodenal ligament is routinely used during partial hepatectomy for control of bleeding, but this can lead to postoperative I/R injury that may adversely affect outcome^[4]. Hepatic I/R damage is caused by multiple factors, including ischemia-induced

hypothermia, cytokines, coagulopathy and increased levels of cell adhesion molecules. Expression of transforming growth factor- β (TGF- β) has been demonstrated in a wide variety of diseases and in normal cells and tissues, and is known to be most abundant in platelets and bone^[5]. TGF- β 1 has inhibitory effects on cell growth, and acts in a stimulatory manner in fibrosis and certain mesenchymal cells^[6]. It is particularly germane in the liver, as TGF- β 1 is a potent inducer of apoptosis in hepatocytes, and inhibits liver cell proliferation *in vitro*^[6]. It also has a crucial role in terminating liver regeneration after partial hepatectomy in rats^[7].

Furthermore, Kupffer cells play a prominent role in I/R injury in the liver as this type of injury induces the release of high mobility group box 1 (HMGB1) from damaged liver cells, which then stimulate nonparenchymal cells, such as Kupffer cells through toll like receptor 4 (TLR4)^[8]. In addition, TLR4 triggers the secretion of tumor necrosis factor- α (TNF- α) and interleukin (IL)-6 by Kupffer cells, resulting in liver regeneration^[8].

To explore the effect of Edr in liver I/R injury and to extend the application of Edr clinically, experiments using large animal models are essential. We have attempted to define the effects of Edr after I/R injury in a porcine liver resection model.

In this study, we investigated the effect of Edr on hepatic I/R injury and liver regeneration, and found that Edr significantly inhibits apoptosis in liver.

MATERIALS AND METHODS

Experimental groups

The study was performed using male pigs, weighing 23-26 kg (SEASCO, Saitama, Japan), in accordance with the Guidelines for the Care and Use of Laboratory Animals, Dokkyo Medical University. Two groups of animals were prepared: Edr group ($n = 6$) in which Edr (supplied by Mitsubishi-Tanabe Pharma Co., Ltd., Osaka, Japan) was administered intravenously at a dose of 3 mg/kg per hour from the commencement of clamping for 30 min, and a control group ($n = 6$) which received physiological saline administered intravenously for the same period. The Edr dose was the same as that used in the treatment of human brain infarction^[9] (Figure 1).

Surgical procedure

A chevron incision was made under general anesthesia, and each branch of the portal vein, hepatic artery and bile duct were carefully isolated and taped. Hemi-hepatic (approximately 60%) liver ischemia was induced by clamping the right and middle hepatic vessels and bile duct using vessel tapes and maintained for 60 min; the left portal branch was kept patent to avoid bowel congestion, and the left lobe played a role in the bypass. After declamping, the left portal vein and artery were ligated, and a left hemi-hepatectomy was performed (approximately 40%). Liver transection was achieved by the crush-clamping method using Pean forceps. During the liver transection, the exposed Glisson's vessels were ligated

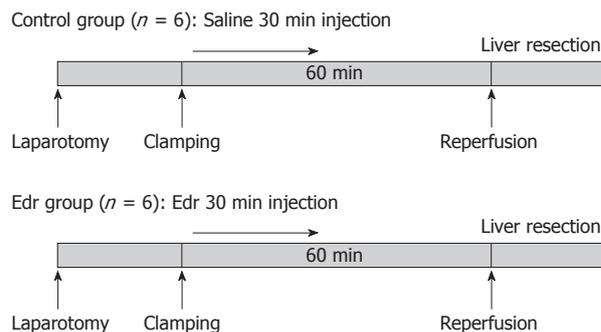


Figure 1 Two groups of animals were prepared. One group received saline and the other received E3-methyl-1-phenyl-2-pyrazolin-5-one (Edr) which was administered intravenously at a dose of 3 mg/kg per hour from commencement of clamping to completion of the liver resection.

with 2-0 or 3-0 silk. The hepatic vein was closed by continuous sutures using 4-0 proline. The influence of I/R injury was assumed to be on the right lobe only. During this procedure, hemodynamic parameters (systolic and diastolic arterial pressure) were monitored by a femoral arterial line. All pigs received Ringer's solution during the procedure. After the operation, the pigs were transferred to the Dokkyo Medical University animal center and unlimited oral intake was allowed after the first postoperative day. No antibiotics were administered either orally or intravenously after surgery.

Measurements and sampling protocol levels

Blood samples were obtained from the arterial line immediately after laparotomy, 5 and 180 min after reperfusion, and after 1 mo. The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and lactic acid (LA) were evaluated. Hepatic tissues were obtained from the right lobe at laparotomy, after reperfusion, and after 1 mo, and were subjected to terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, and TLR4 and TGF- β 1 mRNA were determined. Serum AST, ALT, LDH and LA were measured using standard clinical methods for automated analysis (Model 7170, Hitachi, Inc., Tokyo, Japan).

Experimental pigs were sacrificed 1 mo after operation and remnant liver was obtained. Total liver weight was calculated based on 30 pigs. We calculated the average percentage of total liver weight to body weight (2.64%), estimated the total liver weight from pre-operative body weight, and calculated the estimated remnant liver weight 1 mo after operation by subtracting the resected liver weight at the time of operation from the estimated total liver weight. Liver weight increasing ratio (%) was calculated as follows; estimated remnant liver weight at resection/remnant liver weight after 1 wk \times 100.

Quantitative real-time polymerase chain reaction

At laparotomy, 30 mg of surgical tissue was stored in liquid nitrogen and kept at -80°C until extraction of total RNA using a Nucleospin II kit (Macherey-Nagel, Germany). Reverse transcription reactions were performed using a SuperScript III First-strand Synthesis System for t-poly-

merase chain reaction (PCR) (Invitrogen, Carlsbad, CA, United States). Briefly, 1 µg of total RNA, oligo dT primer, and dNTPs were incubated at 65 °C for 5 min, and then 10 µL of cDNA synthesis mixture was added and incubated at 50 °C for 50 min. The reaction was terminated by adding 1 µL of RNase H and incubated at 37 °C for 20 min. Real-time PCR was performed using a ABI Prism 7700 sequence detector (Applied Biosystems, Warrington, United Kingdom). The PCR reaction was carried out in a final volume of 1 µL cDNA, 2 µL 10 × SYBR Green (Applied Biosystems), using 40 cycles at 95 °C for 30 s and 60 °C for 30 s. The specific primers were designed using Primer 3 software (http://frodo.wi.edu/cgi-bin/primer3/primer3_www.cgi) and synthesized by Sigma Genosys (Hokkaido, Japan). The sequences of each primer were as follows: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): sense 5'-CCACCCAGAAGACTGTGGAT-3', anti-sense 5'-TTCAGCTCAGGGATGACCTT-3'; TLR4 and TGF-β1: sense 5'-CCCCTGTCCATCCCTTTATT-3', anti-sense 5'-AAGCCCCAGTTCCTAATCTT-3'. For each PCR run a standard curve was constructed from serial dilutions of cDNA from the PANCI cell line. The level of expression of TLR4 and TGF-β1 were calculated using the formula: relative expression (t) = [(copy number of TLR4 + TGF-β1 number/copy number of GAPDH)] × 1000. For non-template reactions and standard cDNA dilutions from PANCI cells, liver samples were assayed in triplicate. The average and standard deviation were calculated and the *t*-value was determined from the averages.

Histological examination

Tissue samples were obtained at the time of laparotomy, the remnant liver just after hepatectomy, 3 h after declamping of the right and middle hepatic arteries and portal vein, and after 1 mo, and were fixed with 10% formalin for 24 h and embedded in paraffin. Sections 3-µm thick were stained by the *in situ* terminal TUNEL method using an apoptosis *in situ* detection kit (Wako Pure Chemical, Inc., Osaka, Japan) according to the manufacturer's instructions. The mean numbers of apoptotic cells per 10 random high-power fields were calculated and compared between the two groups.

Statistical analysis

All values are expressed as mean ± SD. Parameters were evaluated using the Student *t* test and Mann Whitney *U* test. Differences between the two groups were evaluated using analysis of variance with *P* < 0.05 considered to be significant.

RESULTS

There were no significant differences in weight, amount of intraoperative hemorrhage, liver resection time, and weight of resected liver (Table 1). There was no significant difference in liver weight increasing ratio at 1 mo after operation between the two groups (*P* = 0.228, Figure 2). There were no significant differences in systolic and diastolic blood pressures between the two groups (Figure 3).

Table 1 Weight, amount of intraoperative hemorrhage, liver resection time, and weight of resected liver in the two groups (mean ± SD)

	Control	Edr	<i>P</i> value
Body weight	24.6 ± 1.1	24.7 ± 0.5	0.99
Body weight (after 1 mo)	29.4 ± 3.3	29.4 ± 3.1	0.97
Bleeding (g)	59.5 ± 44.7	57.4 ± 46.8	0.82
Resection time (min)	24.2 ± 1.6	24.7 ± 0.4	0.49
Resected liver volume (g)	295.7 ± 25.7	271 ± 41.0	0.32
Liver volume (after 1 mo)	606.3 ± 92.7	579.3 ± 60.0	0.53

Edr: E3-methyl-1-phenyl-2-pyrazolin-5-one; RxLV: Resected liver volume.

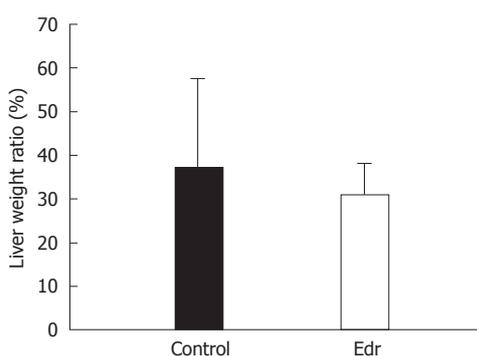


Figure 2 After 1 mo, increasing remnant liver weight ratio showed no difference between the two groups (*P* = 0.228). Liver weight increasing ratio (%) = estimated remnant liver weight at resection/remnant liver weight after 1 mo × 100. Total liver weight was calculated based on previous experimental data of 30 pigs. We calculated the average percentage of total liver weight to body weight (2.64%). Edr: E3-methyl-1-phenyl-2-pyrazolin-5-one.

Serum chemistry showed that the AST level at 3 h after reperfusion was significantly lower in the Edr-treated group than in the control group (*P* = 0.029). There were no significant differences in ALT, LDH and LA levels at any of the observation points between the two groups (Figure 4). Figure 5 shows the expression of TGF-β1-mRNA in liver tissues. Expression of TGF-β1-mRNA in the Edr-treated group tended to be lower than in the control group at 3 h (*P* = 0.285) and 1 h (*P* = 0.172) after operation. Expression of TLR4 mRNA was significantly lower in the Edr-treated group than in the control group at 3 h after operation (*P* = 0.043), however, there was no significant difference between the two groups 1 mo after operation (Figure 6). Figure 7A shows TUNEL staining. There were more TUNEL-positive cells in Control pigs than in Edr-treated pigs 5 min after hepatectomy and 3 h after declamping. Actual numbers of apoptotic cells in the Edr-treated and control groups were 22.16 ± 8.97 and 36.85 ± 1.83 at 5 min after hepatectomy (*P* = 0.015), and 24.66 ± 3.11 and 42.25 ± 3.43 at 3 h after declamping, respectively (*P* = 0.0001) (Figure 7B).

DISCUSSION

The Pringle method is usually performed to reduce the amount of hemorrhage during hepatectomy^[4]. However, this method inevitably causes I/R injury and has a risk

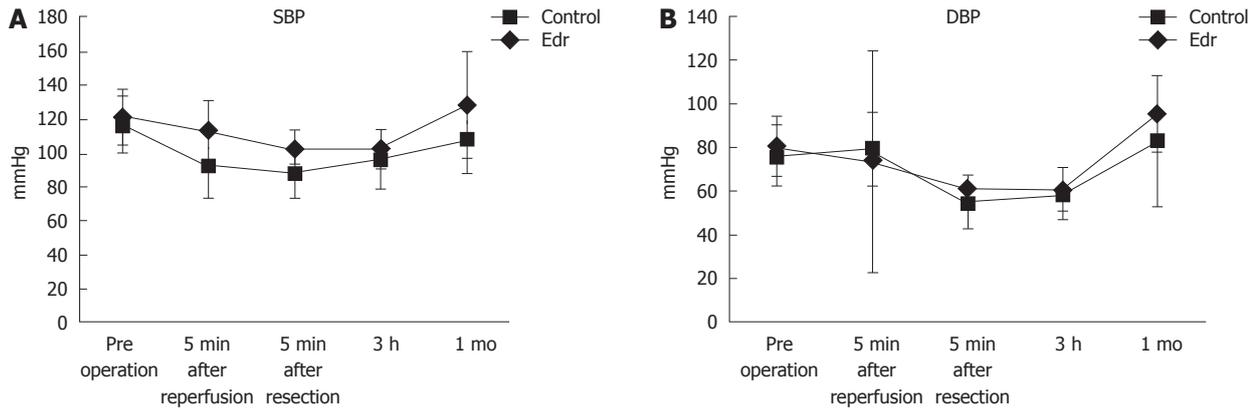


Figure 3 Systolic blood pressure and diastolic blood pressure were not different between the two groups. A: Systolic blood pressure (SBP); B: Diastolic blood pressure (DBP); Edr: E3-methyl-1-phenyl-2-pyrazolin-5-one.

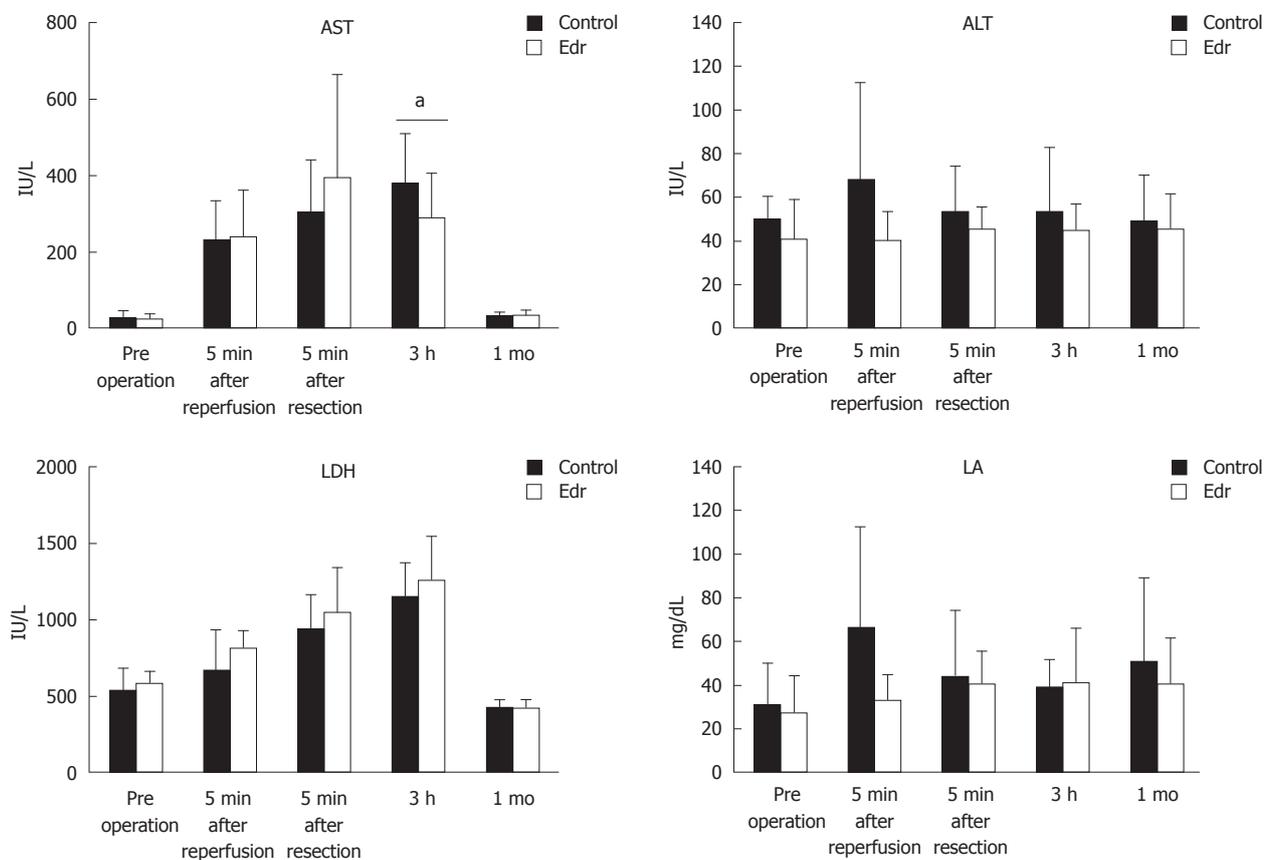


Figure 4 Aspartate aminotransferase was significantly lower 3 h after reperfusion compared to the control ($^*P = 0.029$). Alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and lactic acid (LA) were not different after reperfusion in the E3-methyl-1-phenyl-2-pyrazolin-5-one (Edr)-treated group compared to the control group. AST: Aspartate aminotransferase.

of inducing abnormally high hepatic enzyme levels, icterus, hyperammonemia, lacticidemia, and an intractable accumulation of pleural and peritoneal effusion postoperatively. I/R injury associated with the Pringle maneuver may affect outcome when the ischemic time is long, or during hepatectomy for diseased liver, such as in cases of hepatic cirrhosis and fatty liver, despite the reduction in intraoperative hemorrhage achieved using the Pringle method^[10].

I/R injury may be a causal factor in the above symp-

toms, and various countermeasures have been proposed. These include modified surgical methods, such as an intermittent Pringle method, partial hepatic pedicle clamping, and ischemic pre-conditioning, as well as drug therapy with steroids, prostaglandin E1, Sivelestat or Erythropoietin^[10-19]. However, assessment of the effectiveness of these approaches has been limited, and this may be clarified by studies in animals of similar size to humans. In general, the process of I/R injury consists of multiple steps. Hypoxia due to cessation of blood supply

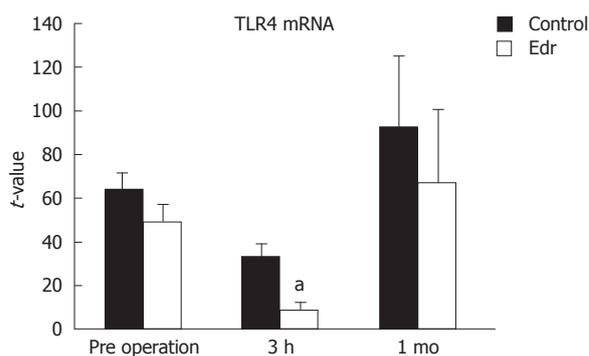
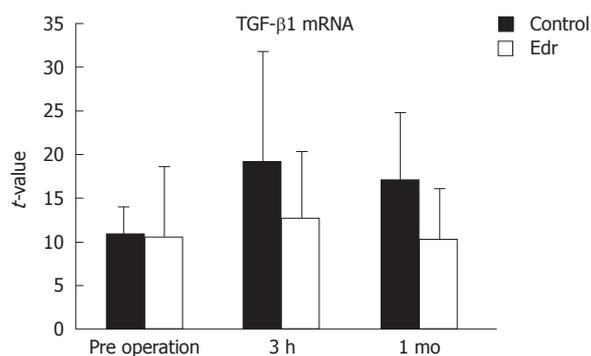


Figure 5 Transforming growth factor-β1 mRNA expression in liver tissue. Expression of transforming growth factor-β1 (TGF-β1) mRNA in the E3-methyl-1-phenyl-2-pyrazolin-5-one (Edr)-treated group tended to decrease 3 h and 1 mo after reperfusion, however, no significant difference between the two groups was observed ($P = 0.285$, $P = 0.172$).

Figure 6 Toll like receptor 4 mRNA level. Toll like receptor 4 (TLR4) mRNA level significantly decreased in the E3-methyl-1-phenyl-2-pyrazolin-5-one (Edr)-treated group at 3 h after surgery ($^aP = 0.004$). At 1 mo after operation, Toll like receptor 4 level showed no differences between the two groups.

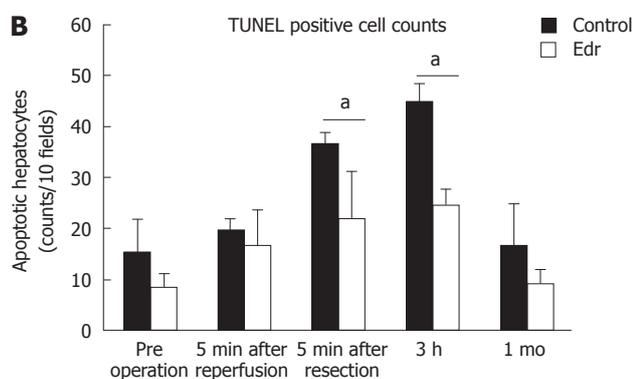
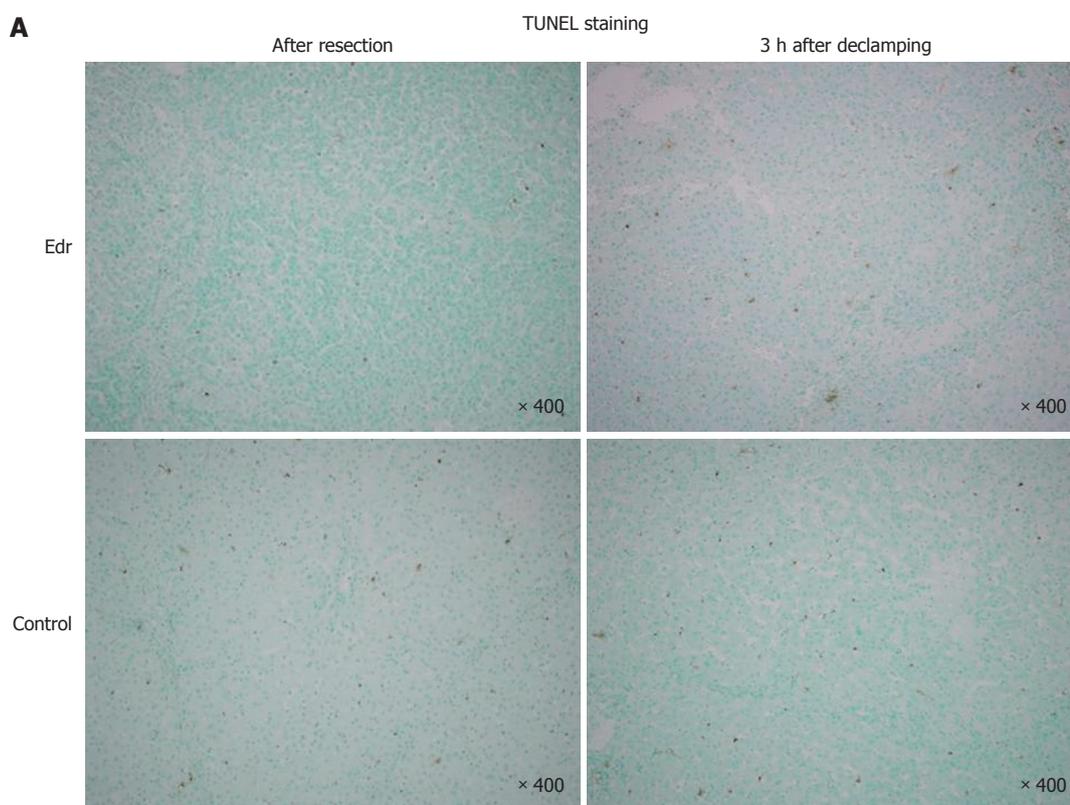


Figure 7 Morphometric evaluation of terminal deoxynucleotidyl transferase dUTP nick end labeling-positive hepatocytes in terminal deoxynucleotidyl transferase dUTP nick end labeling-stained tissue samples after resection and after 1 mo (A, B). The differences between the two groups were statistically significant 5 min after hepatectomy and 3 h after declamping (values are expressed as mean \pm SD, $n = 6$ in both groups). $^aP = 0.001$. Edr: E3-methyl-1-phenyl-2-pyrazolin-5-one; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling.

impairs oxidative phosphorylation in the mitochondria, leading to profound cellular damage^[20]. Furthermore, reperfusion exacerbates cellular damage by producing reactive oxygen species, activating pro-inflammatory cytokines such as IL-10 and TNF- α ^[21], and up-regulating cell adhesion molecules such as P-, E-, and L-selectins^[22,23], resulting in tissue injury.

Edr has potent hydroxyl radical scavenging activity^[24]. In various experimental models, Edr has been reported to protect several organs such as brain^[25], heart^[26] and liver^[27-29] from free radical-mediated injuries.

Several previous studies have investigated the effect of Edr on I/R injury in the liver. Edr improves portal flow and decreases the level of hepatic enzyme^[30]. Edr also decreases oxidative damage to mitochondria in liver^[27]. However, these findings only indicate that Edr is effective in the acute phase of I/R injury. In the present study, we investigated the effect of Edr at both very early time points and at a late point (one mo after hepatectomy). No improvements in the markers of liver function including AST, ALT, LDH and LA, except AST at 3 h after hepatectomy, were noted at any of the observation periods. However, Edr successfully reduced apoptotic cells in the liver at 5 min and 3 h after hepatectomy. The anti-apoptotic effect of Edr was not observed at one mo after hepatectomy. Thus, the results suggest that Edr has a protective effect against I/R injury in liver by inhibiting apoptosis in the early phase after injury, which was reflected in the decrease in markers. However, prevention of I/R injury, particularly in the early phase, did not affect liver regeneration.

TGF- β is a key mediator involved in the progression of liver disease. TGF- β exerts cytotoxic effects, such as induction of apoptosis and aggravation of microcirculation disorders, due to induction of the expression of cell adhesion molecules^[31]. TGF- β had antiproliferative action after partial liver resection in a rat model^[7]. When administered at the time of liver resection, TGF- β clearly delayed hepatocyte proliferation^[7]. In the present study, we observed that Edr tended to suppress TGF- β 1-mRNA expression in liver tissue at 3 h and 1 mo after hepatectomy.

TLRs consist of 13 mammalian members containing a conserved TIR Toll/IL-1 receptor domain in their intracellular domain and an individual leucine-rich repeat domain in their extracellular domain. TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface and TLR3, TLR7, TLR8 and TLR9 are expressed on the endosome-lysosome membrane. TLR4 and TLR5 are the receptors for the Gram-negative bacterial cell wall components, lipopolysaccharide, and bacterial flagellin, respectively^[32]. In the liver, TLR4 is an important key factor for liver fibrosis, I/R injury and liver regeneration. Upon I/R injury, the TLR4 ligand, HMGB1, is released from damaged hepatocytes and subsequently stimulates Kupffer cells through TLR4^[33]. Suppression of TLR4 at I/R injury, leads to the control of progress to liver regeneration by controlling liver fibrosis. Unfortunately, in this study, Edr only decreased TLR4 levels in the early phase

(less than 3 h) after liver resection. This result suggested that Edr did not have any effect on liver fibrosis and regeneration.

In conclusion, our results demonstrated that Edr has a protective effect against I/R injury in the liver by inhibiting apoptosis in early period after I/R injury. Although further studies are needed to determine the dose, timing, and duration of Edr treatment to be used in the clinical setting, we have shown, for the first time in a porcine model, that Edr may be a promising agent for ameliorating I/R injury during hepatectomy.

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COMMENTS

Background

Peritoneal adhesions can cause intestinal obstruction and other severe clinical disorders, thus, it is very important to prevent peritoneal adhesions in abdominal surgical operations. However, to date, there are still no ideal methods to prevent peritoneal adhesions in clinical practice. Chitosan is a deacetylated derivative of chitin.

Research frontiers

Chitosan is a natural biological material and has been processed into many forms for medical use. Chitosan has also been used in the prevention of peritoneal adhesions and various researchers are exploring how to modify chitosan using chemical and physical methods to improve its effectiveness in preventing adhesions, and simultaneously reduce its adverse reactions.

Innovations and breakthroughs

In previous reports on the application of chitosan gels to prevent peritoneal adhesions, it was found that the gel was much too fluid and did not remain in the target area for sufficient time, moreover, the gel degraded so fast that it could only maintain effectiveness for a short period. In order to delay degradation and decrease the fluidity of the gel, the authors processed pure chitosan into films, however, the film degraded too slowly and the residual film was encapsulated by surrounding tissue and the peritoneal adhesions worsened. In order to overcome these disadvantages, we mixed chitosan with gelatin and produced blended films. The blended film degraded much faster than the previous pure chitosan film, but it also created a foreign body reaction and formed a severe foreign body granuloma around the blended film. In the present study we chemically modified chitosan during gelatinization to develop a new type of chitosan film, and showed that the film is remarkably effective in preventing peritoneal adhesions induced by wounds, ischemia and infection except foreign body-induced adhesions.

Applications

The study results suggest that the gelatinized-chitosan film is a potential therapeutic material which could be used in preventing peritoneal adhesions induced by wounds, ischemia and infection.

Terminology

Peritoneal adhesion: Peritoneal adhesion is a type of defensive reaction to peritoneal injury mainly wounds, infection, ischemia and foreign bodies, but it can also cause intestinal obstruction and severe clinical disorders; chitosan: Chitosan is a deacetylated derivative of chitin, and chitin is the second most abundant natural biopolymer derived from the exoskeletons of crustaceans and from the cell walls of fungi and insects.

Peer review

This is a good descriptive study in which authors investigate the effect of E3-methyl-1-phenyl-2-pyrazolin-5-one (Edr) on hepatic ischemia-reperfusion (I/R) injury and liver regeneration in a porcine hepatectomy model. The results are interesting and suggest that Edr is considered to reduce hepatic injury in the early stage of I/R injury in a porcine model.

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Polo-like kinase 1, a new therapeutic target in hepatocellular carcinoma

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Abstract

AIM: To investigate the role of polo-like kinase 1 (PLK1) as a therapeutic target for hepatocellular carcinoma (HCC).

METHODS: *PLK1* gene expression was evaluated in HCC tissue and HCC cell lines. Gene knockdown with short-interfering RNA (siRNA) was used to study *PLK1* gene and protein expression using real-time reverse transcription polymerase chain reaction (RT-PCR) and Western blotting, and cell proliferation using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium (MTS) and bromodeoxyuridine (BrdU) assays. Apoptosis was evaluated using the ter-

минаl deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, and caspase-inhibition assay. Huh-7 cells were transplanted into nude mice and co-cultured with PLK1 siRNA or control siRNA, and tumor progression was compared with controls.

RESULTS: RT-PCR showed that PLK1 was overexpressed 12-fold in tumor samples compared with controls, and also was overexpressed in Huh-7 cells. siRNA against PLK1 showed a reduction in *PLK1* gene and protein expression of up to 96% in Huh-7 cells, and a reduction in cell proliferation by 68% and 92% in MTS and BrdU cell proliferation assays, respectively. There was a 3-fold increase in apoptosis events, and TUNEL staining and caspase-3 assays suggested that this was caspase-independent. The pan-caspase inhibitor Z-VAD-FMK was unable to rescue the apoptotic cells. Immunofluorescence co-localized endonuclease-G to fragmented chromosomes, implicating it in apoptosis. Huh-7 cells transplanted subcutaneously into nude mice showed tumor regression in siPLK1-treated mice, but not in controls.

CONCLUSION: Knockdown of PLK1 overexpression in HCC was shown to be a potential therapeutic target, leading to apoptosis through the endonuclease-G pathway.

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Key words: RNA; Polo-like kinase 1; Apoptosis; Endonuclease G; Forkhead box transcription factors; Nude mice

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common solid tumor cancer in the world, and is ranked third in terms of mortality according to GLOBALCAN 2002^[1]. Treatments for HCC other than liver transplantation, can benefit patients but are often plagued by recurrence of the tumor^[2,3]. Those with advanced HCC have a poor prognosis. Recently, vascular endothelial growth factor inhibitors showed a survival benefit^[4], albeit of only 3 mo, in the treatment of HCC. Therefore, this finding and the paucity of effective treatment options is a strong indicator for the investigation of new genomic targets that can provide better treatment outcomes for HCC patients. One genomic target that has been reported in many other tumors but only recently in HCC is polo-like kinase 1 (PLK1)^[5-8].

PLK1 is a cell cycle protein that plays multiple roles in promoting cell cycle progression^[9]. However, the most prominent role of PLK1 probably lies in regulating the spindle checkpoint at the M-phase^[10]. Mutated PLK1 alleles induce abrupt spindle formation resulting in polyploid cells^[11], and in mammalian cells depleted of PLK1, mitotic arrest with dysfunctional spindle assembly occurs^[10]. PLK1 is a highly conserved protein that has unique polo-box binding domains that can bind to phosphopeptide and render it to be regulated spatially and temporally during the cell cycle by proteins that carry the optimal phosphopeptide motif^[12].

PLK1 is overexpressed in many cancers and serves as a significant prognostic factor in cancers such as small-cell lung cancer, colon cancer and ovarian cancer^[13]. In addition, high expression levels of PLK1 in melanoma and breast cancer correlate well with the metastatic potential of these tumors^[14,15]. PLK1 overexpression may contribute to the deregulation of cell proliferation during oncogenesis by overcoming mitotic checkpoints^[16]. In this study, we sought to determine if PLK1 was a potential therapeutic target for HCC.

MATERIALS AND METHODS

Patients and samples

Tumor and surrounding non-tumor liver tissues were obtained from 56 primary HCC patients who underwent liver resection in the National University Hospital from 1998 to 2002. The study was approved by the National University of Singapore Institutional Review Board, and National Healthcare Group Domain Specific Review Board. Informed consent was obtained from every patient for the use of excised tissue. The human hepatoma cell line HepG2 was obtained from American Type Culture Collection, Huh-7 cells from the Japanese Collection

of Research Bioresources Cell Bank and HepG2.2.15 was kindly provided by Dr. Acs (Mt. Sinai Medical College, New York, NY, United States). HepG2 and Huh-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 0.1 mmol/L sodium pyruvate, 0.1 mmol/L L-glutamine, 0.1 mmol/L non-essential amino acids, 10% fetal calf serum, and 1X antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA, United States). HepG2.2.15 cells were cultured in similar media with the addition of 0.4 mg/mL G418 (Sigma-Aldrich, St Louis, MO, United States). All cell lines were maintained in an incubator with 5% CO₂ at 37 °C.

RNA extraction and cDNA synthesis

Total RNA was extracted from cell lines or patient tissues using RNeasy Mini kits (Qiagen, Hilden, Germany) or Trizol (Invitrogen), respectively according to the manufacturer's protocol. Reverse transcription was carried out in 26 µL aliquots of solution containing 5 µg of total RNA, 2.5 ng Oligo d(I)₁₈ and RNase-free water, which was then incubated for 15 min at 72 °C in a thermal cycler. After 10 min of cooling at 4 °C, 24 µL of the RT-enzyme mixture containing 5 µL 10 mmol/L dNTPs, 5 µL 0.1 mol/L dithiothreitol, 10 µL 5 × first-strand buffer, 2 µL SuperScript II reverse transcriptase (Invitrogen) and 2 µL recombinant RNasin ribonuclease inhibitor (Promega, Madison, WI, United States) was added to obtain a final volume of 50 µL. The mixture was then incubated for 90 min at 42 °C followed by 15 min incubation at 72 °C, and was subsequently cooled to 10 °C.

Real-time quantitative reverse transcription polymerase chain reaction

Primers and probes for PLK1 and FOXM1 were from TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, United States). In brief, 20 µL reaction was set up containing 4 µL cDNA, 10 µL 2 × TaqMan Universal PCR Master Mix (Applied Biosystems), 1 µL 20 × TaqMan Gene Expression Assay Mix of corresponding target gene, 1 µL 20 × human hypoxanthine-guanine phosphoribosyltransferase endogenous control (Applied Biosystems)^[17] and 4 µL RNase-free water. The reaction was run on the ABI Prism 7000 Sequence Detection System (Applied Biosystems) with the following profile: 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, then 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. No-template control and TaqMan Control Total RNA (Human) (Applied Biosystems) as reference control were also included where applicable. Relative quantification was done using the 2^{-ΔΔC_t} method.

Short interfering RNA transfection

Short interfering RNA (siRNA) targeting human PLK1 (si-PLK1) and FOXM1 were from siGENOME On-TargetPlus Set of 4 duplex (Dharmacon, Chicago, IL, United States) and On-Target plus siCONTROL Non-targeting pool (si-NT) (Dharmacon) was used as negative control. The mixture containing 4 siRNA duplexes was transfected

into cells using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's protocol. No-treatment and transfection-reagent-only controls were also included where suitable. The transfection period was 48 h for all assays or tests unless otherwise specified.

Cell proliferation assays

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium (MTS) assay was performed using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's protocol. The BrdU (5-bromo-2-deoxyuridine) assay was performed using Cell Proliferation enzyme-linked immunosorbent assay (ELISA), BrdU (chemiluminescent) (Roche Applied Science, Indianapolis, IN, United States) according to the manufacturer's protocol.

Western blot analysis

Cells were lysed using Complete Lysis-M (Roche Applied Science) according to the manufacturer's protocol. The protein concentration was determined using BCA Protein Assay Kit (Pierce, Rockford, IL, United States). Protein (30 μ g) was mixed 1:1 with Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, United States) supplemented with 5% β -mercaptoethanol and heated at 95 °C for 5 min followed by 5 min incubation on ice. The sample was then loaded onto a 10% Tris-HCl Ready Gel (Bio-Rad) and subsequently electrotransferred to a Hybond-P polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). The membrane was blocked for an hour at 22 °C with 5% Blotting-Grade Blocker and non-fat dry milk (Bio-Rad). After blocking, the membrane was incubated with mouse anti-human Plk1 (F-8) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, United States) at 1:500 for 1 h at 22 °C followed by incubation with Cruz Marker Compatible goat anti-mouse IgG antibody (Santa Cruz) at 1:50 000 for 1 h at 22 °C. The reaction was detected using the ECL plus system (Amersham) and developed using Hyperfilm ECL (Amersham). Mouse anti-human β -actin (C4) antibody (Santa Cruz) diluted at 1:10 000 was used as loading control. The blots were analyzed with the Quantity One software (Bio-Rad).

Immunofluorescence imaging

Cells were cultured on Lab-Tek II 2-chambered glass slides (Nunc, Rochester, NY, United States). Twenty 4 h after siRNA transfection, the cells were fixed in 4% buffered paraformaldehyde for 30 min at 4 °C. The cells were then permeabilized with 0.5% Triton X-100 in phosphate-buffered solution for 10 min and subsequently blocked in 2% bovine serum albumin for 10 min. The cells were incubated with primary antibody diluted at 1:100 for 1 h at 22 °C followed by an hour's incubation at 22 °C with fluorescein isothiocyanate (FITC)-conjugated secondary antibody diluted at 1:400. The cells were subsequently mounted using Vectashield with 4',6-diamidino-

2-phenylindole (Vector Laboratories, Burlingame, CA, United States) for nuclei staining. Primary antibodies used were FITC-conjugated mouse anti- α -tubulin antibody (clone DM1A) (Sigma-Aldrich), goat polyclonal anti-human endonuclease G (Santa Cruz) and goat polyclonal anti-human apoptosis-inducing factor (Santa Cruz). The secondary antibody used was donkey anti-goat IgG-FITC (Santa Cruz). Fluorescence images were captured using Olympus Fluoview FV500 or BX60F5 (Olympus, Center Valley, PA, United States).

Chromosome fragmentation detection assay, terminal deoxynucleotidyl transferase dUTP nick end labeling assay and caspase-3 activity assay

Chromosome fragmentation was detected using Cell Death Detection ELISA^{PLUS} (Roche) according to the manufacturer's protocol. Thapsigargin (Sigma-Aldrich) treatment was used as positive control. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was carried out after 24 h of transfection using the DeadEnd Fluorometric TUNEL system (Promega) according to the manufacturer's protocol. Fluorescence images were captured using Olympus BX60F5 (Olympus). The caspase-3 activity assay (Roche) was performed according to the manufacturer's protocol.

Caspase inhibition assay

Cells were transfected with siRNA in the presence of cell permeable pan-caspase inhibitor Z-VAD-FMK (Sigma-Aldrich) at 100 μ mol/L for 48 h. Cell proliferation was subsequently determined with MTS assay. Camptothecin (Sigma-Aldrich) at 5 μ mol/L was used to induce apoptosis in HepG2 cells. For camptothecin treatment, HepG2 cells were incubated with the caspase inhibitor for an hour before camptothecin was added and remained till the end of incubation. An equal amount of DMSO was used in the negative control where appropriate.

In vivo nude mice experiment

Nude mice were transplanted subcutaneously with 2×10^6 Huh-7 cells in 100 μ L Matrigel (Sigma-Aldrich). The treatment was started 1 wk after the transplantation. Eighteen tumor-bearing nude mice were divided equally into a group ($n = 6$) that received si-PLK1 treatment, a group that received si-NT treatment ($n = 6$), and a control group that received no treatment ($n = 6$). Treatment groups received intratumoral injections of 1 nmol siRNA coupled with Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) every alternate day. The control group was injected with saline instead. Tumor sizes recorded before treatment were calculated by the formula: volume (mm^3) = (width)² \times length/2. Animal experiments were carried out in compliance with the guidelines of the Laboratory Animals Centre, National University of Singapore and were approved by the National University of Singapore Institutional Animal Care and Use Committee.

Statistical analysis

Statistical analysis was carried out using Microsoft Excel

Table 1 Correlation between polo-like kinase-1 gene expression and clinicopathological parameters in 56 patients with hepatocellular carcinoma

Clinicopathological parameters	n	Relative <i>PLK1</i> gene expression (mean ± SE)	P value
Age (yr)			
< 65	42	18.35 ± 2.95	0.570 ¹
≥ 65	14	16.15 ± 5.11	
Sex			
Male	49	17.24 ± 2.74	0.436 ¹
Female	7	21.72 ± 6.83	
Histologic type of tumor			
Well differentiated	2	14.00 ± 13.10	0.846 ²
Moderately differentiated	41	17.39 ± 2.92	
Poorly differentiated	13	19.69 ± 5.87	
Liver cirrhosis			
Present	44	16.05 ± 2.42	0.497 ¹
Absent	12	24.22 ± 7.83	
Pathological stage			
I	1	5.68	0.849 ²
II	5	14.11 ± 6.67	
III	32	17.68 ± 2.93	
IV	18	19.72 ± 5.74	

¹P value was determined by the Mann-Whitney *U* test; ²P value was determined by the Kruskal-Wallis test. *PLK1* gene expression in every hepatocellular carcinoma tissues was quantified relative to the non-tumor tissues counterparts using 2^{-ΔΔCt} method. PLK1: Polo-like kinase-1.

or SPSS. *P* values of less than 0.05 were deemed significant. All data was expressed as mean ± SE unless otherwise specified.

RESULTS

Baseline characteristics of the patients

The patients, 49 male, 7 female, age range 32-82 years (mean, 56 years), were hepatitis B-positive and were Asian (Table 1).

PLK1 gene expression in HCC patients and correlation with clinicopathological parameters

Gene expression of 10 candidate genes (*PLK1*, forkhead box M1, pituitary tumor-transforming gene 1, ubiquitin specific peptidase 21, reticulocalbin 2, dual specificity phosphatase 12, ubiquitin specific peptidase 1, S100 calcium binding protein P, ubiquitin specific peptidase 5, X-box binding protein 1) indentified from a literature survey of genes shown to be upregulated in tumors but not well documented in HCC, were chosen for real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis in 10 sets of paired HCC tumor/adjacent non-tumor tissue (data not shown). *PLK1* was found to be consistently upregulated in HCC tumor tissue. Gene expression of *PLK1* was then investigated in 46 other patient samples (a total of 56 HCC tumors) using real-time quantitative RT-PCR. The median *PLK1* gene expression was about 12 times higher in 50% of the HCC tumors when compared with non-tumor tissues (Figure 1A).

PLK1 siRNA successfully silenced *PLK1* gene expression in Huh-7 cells

PLK1 gene expression in Huh-7 cell-line was about eight times higher than other human hepatoma cell lines (HepG2 and HepG2.2.15) as determined by real-time quantitative RT-PCR (data not shown). Hence, it was selected as *in vitro* model to study the effect of silencing *PLK1* gene expression. *PLK1* knockdown with 1 nmol/L, 50 nmol/L and 100 nmol/L si-*PLK1* was able to silence of *PLK1* gene expression by 83%, 95% and 96%, respectively, compared with the Huh-7 cells transfected with an equal concentration of si-NT (Figure 1B). The reduction in *PLK1* gene expression by si-*PLK1* corresponded to the reduction observed in *PLK1* protein expression level. Using 50 nmol/L si-*PLK1*, *PLK1* protein expression was reduced by 95% when compared with the si-NT transfected Huh-7 cells (Figure 1C). Therefore, si-*PLK1* was shown to be efficient and specific in silencing *PLK1* gene and protein expression in Huh-7 cells.

Silencing of *PLK1* reduced cell proliferation in Huh-7 cells

Transfection of si-*PLK1* caused a significant reduction in Huh-7 cells proliferation as measured by the MTS cell proliferation assay (Figure 2A) and BrdU cell proliferation assay (Figure 2B), but with no apparent dose-response. On average, si-*PLK1*-treated Huh-7 cells showed 68% and 92% reductions in cell proliferation in MTS and BrdU cell proliferation assays, respectively. In addition, Huh-7 cells that were transfected with si-*PLK1* appeared to be binucleated (Figure 2C, left panel) while Huh-7 cells transfected with si-NT completed mitosis with functional spindle assembly (Figure 2C, right panel), indicative of its role in establishing functional spindle assembly.

Silencing of *PLK1*-induced apoptosis in Huh-7 cells

Nuclear fragmentation expressed as the enrichment factor (sample absorbance/absorbance of the non-transfected control) > 1, indicates enrichment of mono- and oligonucleosomes in the cytoplasm of the apoptotic cells due to DNA breakdown. The enrichment factor in Huh-7 cells that were transfected with si-*PLK1* was 3-fold higher than in the Huh-7 cells transfected with si-NT (Figure 3A). In addition, TUNEL staining of si-*PLK1*-transfected Huh-7 cells helped to identify and visualize apoptotic cells with fragmented chromosomes (Figure 3B). To examine the apoptosis pathway, caspase-3 activity was analyzed in si-*PLK1* transfected Huh-7 cells at three different time points (Figure 3C). There was activation of caspase-3 to over 500 U (compared with 2350 U in positive controls), but this was only detected at 48 h after transfection (Figure 3C). This was an unexpected finding as the TUNEL assay had already showed prominent nuclear fragmentation in apoptotic cells 24 h after transfection, and suggests that the apoptosis pathway induced by *PLK1* knockdown in Huh-7 cells may be caspase-independent.

Apoptosis induced by silencing *PLK1* gene expression is caspase-independent

A cell permeable pan-caspase inhibitor Z-VAD-FMK was

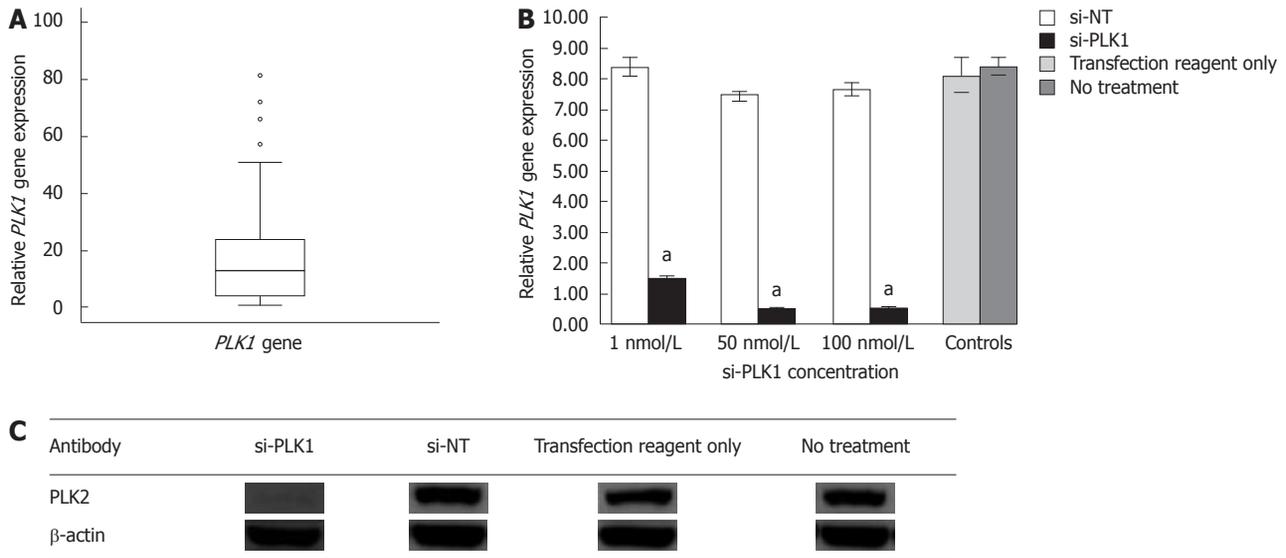


Figure 1 Upregulation of polo-like kinase 1 gene expression in 56 hepatocellular carcinoma tumors, efficiency of short-interfering RNA in silencing the polo-like kinase 1 gene, and protein expression in Huh-7 cells. A: Boxplot showing the minimum, 25th percentile, median, 75th percentile and maximum relative polo-like kinase 1 (*PLK1*) gene expression. Circles represent statistical outliers. *PLK1* gene expression in all hepatocellular carcinoma (HCC) tissue was quantified relative to the non-tumor tissue counterpart using the $2^{-\Delta\Delta Ct}$ method; B: Knockdown with short interfering *PLK1* (si-*PLK1*) at 1 nmol/L, 50 nmol/L and 100 nmol/L successfully silenced *PLK1* gene expression (using the $2^{-\Delta\Delta Ct}$ method by quantitative real-time RT-PCR) by 83%, 95% and 96% respectively, compared with short interfering non-targeting (si-NT), or controls. Data shown as mean \pm SE, using the Student *t*-test ($^aP < 0.05$); C: Western blotting showing reduction of *PLK1* protein expression in Huh-7 cells, at si-*PLK1* and si-NT (non-targeting si-RNA) concentrations of 50 nmol/L compared with si-NT or controls.

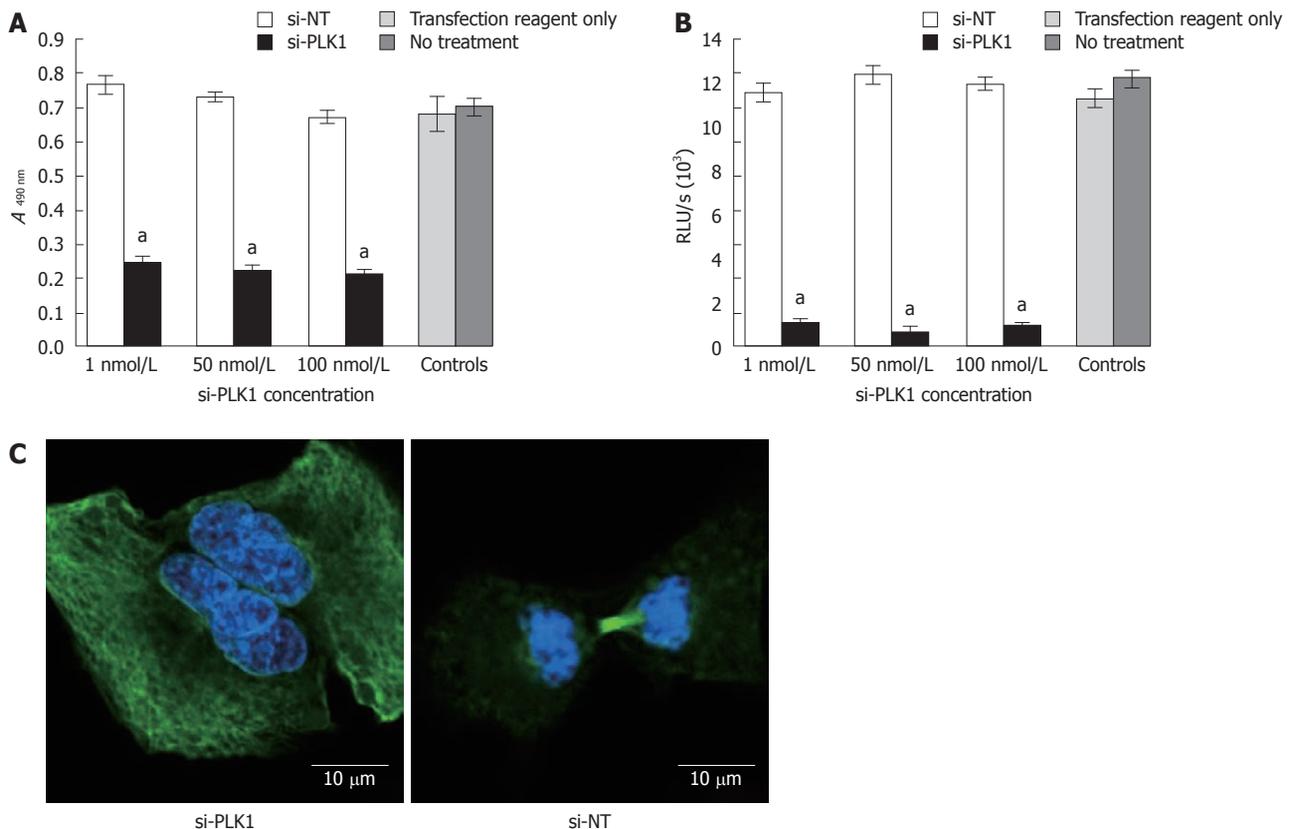


Figure 2 Reduction of cell proliferation by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay and bromodeoxyuridine assay after silencing of polo-like kinase 1, and failure of mitosis after knockdown of polo-like kinase 1. A: Knockdown of polo-like kinase 1 (*PLK1*) reduced cell proliferation in Huh-7 cells in the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell proliferation assay by a mean of 65% compared with short interfering non-targeting (si-NT) or controls; B: Knockdown of *PLK1* reduced cell proliferation in the bromodeoxyuridine cell proliferation assay in Huh7 cells by a mean of 93% with 50 nmol/L short-interfering *PLK1* (si-*PLK1*) compared with si-NT or controls; C: Confocal fluorescence images show the si-*PLK1* transfected Huh-7 cells (left panel) were binucleated, depicting failure in completing mitosis due to the lack of a functional spindle assembly. The right panel shows a functional spindle assembly in Huh-7 cells transfected with si-NT. Huh-7 cells were processed for confocal imaging after 24 h of transfection either with 50 nmol/L si-*PLK1* or si-NT; α -tubulins were stained with fluorescein isothiocyanate-conjugated antibody and nuclei were counterstained with 4',6-diamidino-2-phenylindole. Data are shown as mean \pm SE, using the Student *t*-test ($^aP < 0.05$).

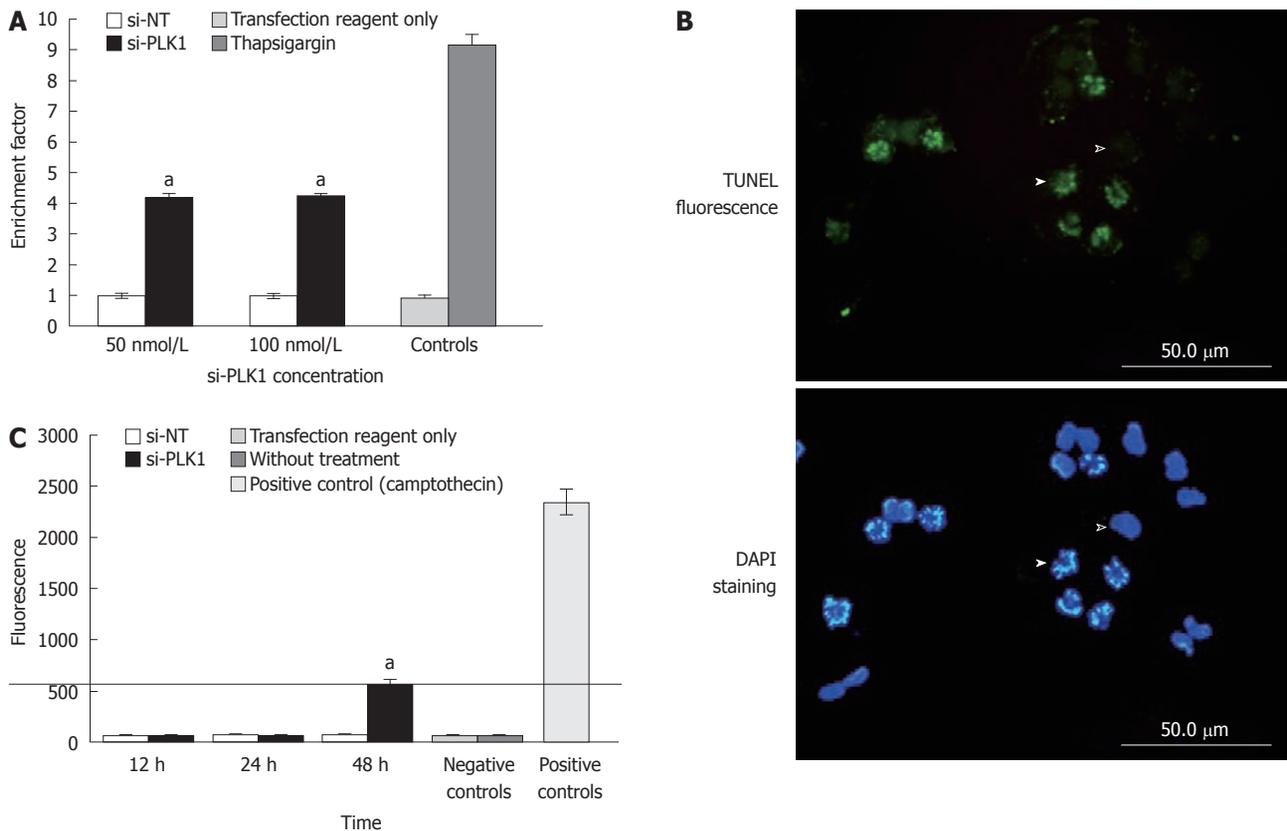


Figure 3 Increased apoptosis, apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling staining and caspase 3 activity after knockdown of polo-like kinase 1. **A:** Increased apoptosis; apoptosis measured by enrichment factor [enrichment factor (sample absorbance/control absorbance) > 1 indicates nuclear fragmentation]. Huh-7 cells transfected with short-interfering polo-like kinase 1 (si-PLK1) showed a 4-fold increase in enrichment factor compared with short-interfering non-targeting (si-NT) or the negative control, no difference was seen between the 50 nmol/L and 100 nmol/L si-PLK1. Thapsigargin (5 μ mol/L) treated Huh-7 cells were positive controls. Data are shown as mean \pm SE, using the Student *t*-test ($^{\ast}P < 0.05$); **B:** Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) fluorescence images showing fragmented DNA labeled with fluorescein-12-dUTP using recombinant terminal deoxynucleotidyl transferase (upper image). The lower image is the corresponding 4',6-diamidino-2-phenylindole (DAPI) stain showing an apoptotic Huh-7 cell (solid arrowhead) and a non-apoptotic Huh-7 cell (hollow arrowhead). The TUNEL staining colocalized the fragmented chromosomes to apoptotic cells while is absent in non-apoptotic cells with intact chromosomes. Huh-7 cells were processed for fluorescence imaging after 24 h of transfection with 50 nmol/L si-PLK1; **C:** Caspase 3 activity was measured using a fluorometric immunosorbent enzyme assay from Huh-7 cells transfected with 50 nmol/L si-PLK1 showed no change after 12 and 24 h, with an increase to just over 500 U at 48 h compared with si-NT and negative controls. Positive controls showed values over 2350 U (cell lysates from U937 cells treated with camptothecin, supplied with the kit). Data are shown as mean \pm SE, using the Student *t*-test ($^{\ast}P < 0.05$).

applied to validate the requirement of caspase in apoptosis induced by silencing *PLK1* gene expression in Huh-7 cells. By using the MTS cell proliferation assay as the functional end-point indicator for apoptosis, the pan-caspase inhibitor was unable to rescue the reduction in cell proliferation of Huh-7 cells transfected with si-PLK1 (Figure 4A). The finding clearly demonstrates that apoptosis in si-PLK1-transfected Huh-7 cells was caspase-independent. Two exclusively mitochondrial pro-apoptotic proteins, endonuclease G (EndoG) and apoptosis-inducing factor (AIF), have been identified to be involved in the caspase-independent apoptosis pathway^[18]. In Figure 4B, EndoG was released from the mitochondria and colocalized in the fragmented chromosomes in apoptotic Huh-7 cells, indicating that EndoG was likely to be the main caspase-independent apoptotic effector. AIF was found to be absent and therefore was probably not involved in this particular apoptosis event (Figure 4B).

Targeting *PLK1* in vivo impeded tumor growth

Huh-7 cells were subcutaneously transplanted into the

flanks of nude mice and allowed to develop into HCC tumors. These tumors were later treated with repeated intratumoral injections of si-PLK1 every alternate day to evaluate the anti-tumor effect by targeting PLK1. The mean tumor volume of the si-PLK1 treatment group was significantly lower than the si-NT treatment group starting from Day 11 after five si-PLK1 treatments (Figure 5A and B). At the end of the treatment period, the mean tumor volume in the si-PLK1 treatment group was about 33% lower than in the si-NT treatment group.

DISCUSSION

PLK1 gene overexpression was seen in a majority of the HCC patients in this study, suggesting that PLK1 may have a role in the HCC tumorigenesis. Recently, PLK1 expression was significantly correlated with a higher recurrence rate in HCC patients^[19]. Moreover, PLK1 overexpression has also been recently reported to be correlated with metastatic HCC disease^[6] and poor prognosis^[5] in HCC. More recently, Pelligrino *et al.*^[8] found that PLK1

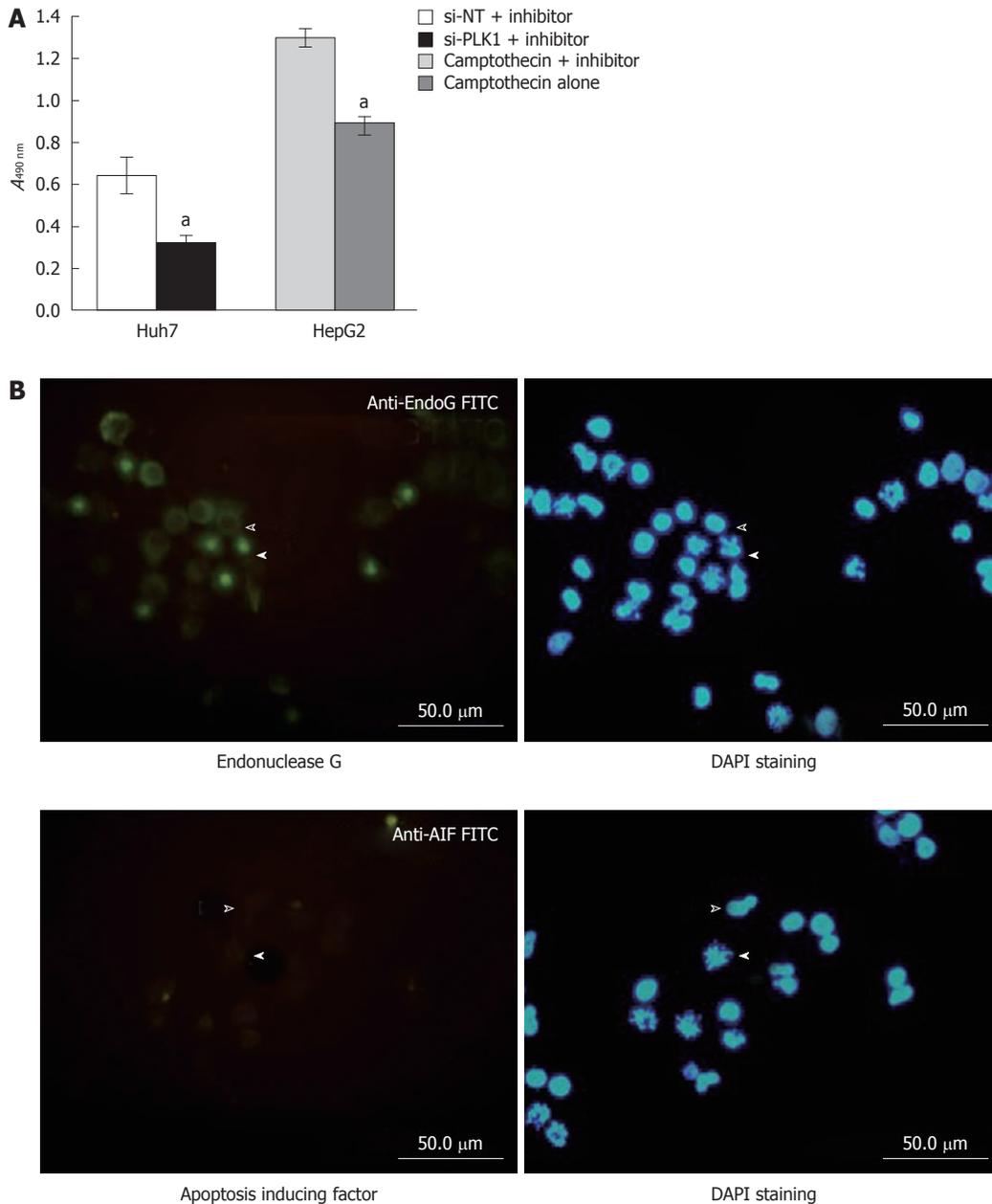


Figure 4 Lack of apoptosis protection and caspase-independent apoptosis. A: Lack of apoptosis protection by pan-caspase inhibitor Z-VAD-FML after knockdown of polo-like kinase 1 (PLK1). The MTS cell proliferation assay in Huh-7 cells transfected with either short interfering non-targeting (si-NT) or short interfering-PLK1 (si-PLK1) in the presence of the pan caspase inhibitor Z-VAD-FMK showed that the pan caspase inhibitor failed to protect si-PLK1 transfected Huh-7 cells from apoptosis. In contrast, HepG2 that were treated with camptothecin and Z-VAD-FMK were protected from apoptosis induced by camptothecin while those treated with camptothecin without Z-VAD-FMK were not protected from apoptosis. Data are shown as mean \pm SE, using the Student *t*-test ($^aP < 0.05$); B: Caspase-independent apoptosis likely due to endonuclease G. Huh-7 transfected with 50 nmol/L si-PLK1 for 24 h was probed with antibody against endonuclease G or apoptosis-inducing factor (AIF) and subsequently visualized with fluorescein isothiocyanate-conjugated secondary antibody (left panels). The corresponding 4',6-diamidino-2-phenylindole (DAPI)-stained images are shown in the right panels. Block arrowheads and hollow arrowheads mark apoptotic and non-apoptotic Huh-7 cells respectively. Endonuclease G was positively stained in apoptotic cells and colocalized to the fragmented chromosomes (upper left panel), while AIF was found to be absent (lower left panel).

silencing was associated with suppression of cell growth, while PLK2-4 silencing had the opposite effect. These studies indicate that PLK1 may have an important role in HCC.

We have shown that Huh-7 cell proliferation requires PLK1. Moreover, PLK1 knockdown in Huh-7 cells results in nuclear fragmentation, indicating apoptosis is present. Intriguingly, caspase activation is found to be unnecessary for PLK1-induced apoptosis. Consequently,

the caspase-independent pathway is likely to be active, and the apoptotic effector appears to be endoG, which is a DNase that is exclusively mitochondrial. EndoG is released from mitochondria and translocates to the nucleus to cleave DNA independently of caspase upon apoptosis induction^[20]. AIF, another potential effector candidate in the caspase-independent apoptosis pathway^[21] was found not be involved in PLK1-induced apoptosis. It is not an essential requirement for the presence of both endoG

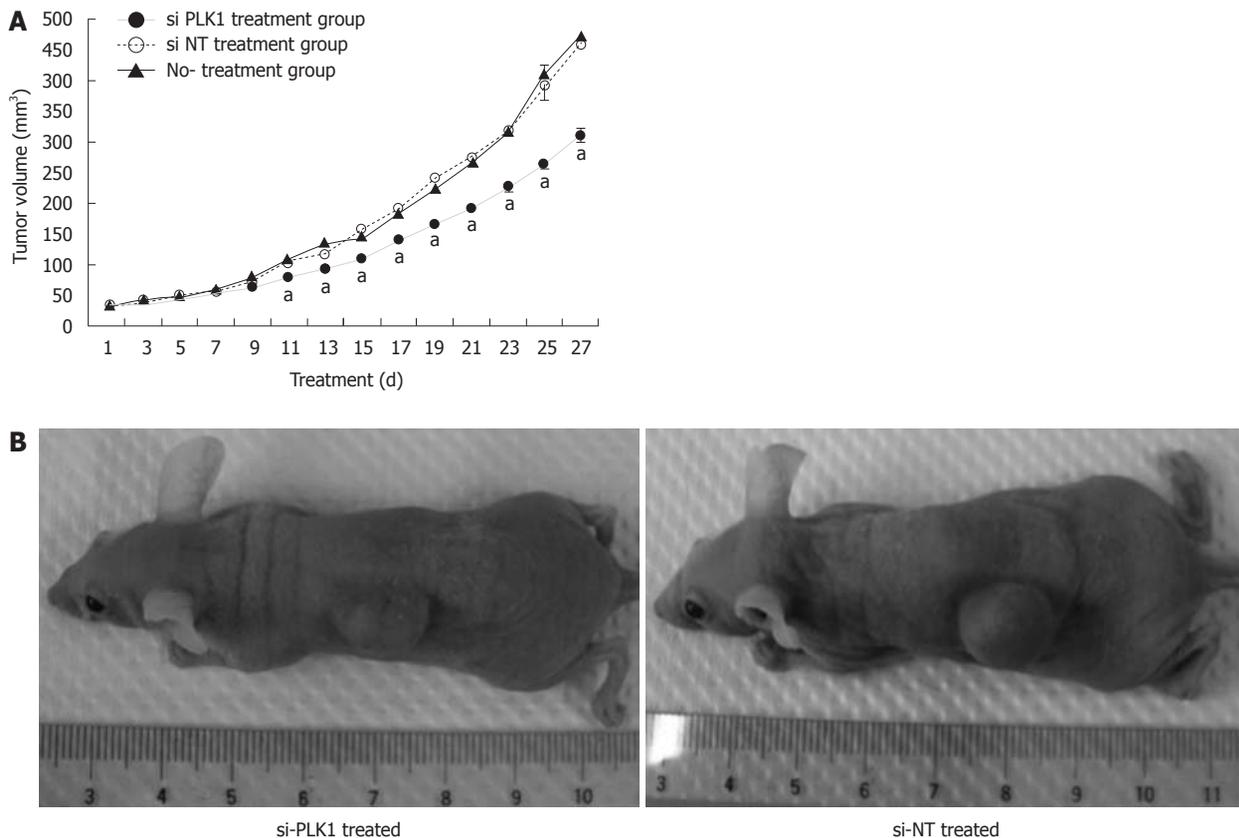


Figure 5 Reduction in hepatocellular carcinoma tumor size with knockdown of polo-like kinase 1. A: Treatment of short-interfering RNA (siRNA) against polo-like kinase 1 (PLK1) (si-PLK1, $n = 6$) in nude mice impeded the tumor growth compared with short interfering non-targeting (si-NT) ($n = 6$) or the non-treatment group ($n = 6$). Huh-7 tumor-bearing nude mice were treated with 1 nmol/L si-PLK1 intratumoral injections every alternate day for 27 d. Data are shown as mean \pm SE, using the Student *t*-test ($^*P < 0.05$); B: Images of nude mice with their tumors at day 27. The left panel shows a reduction in tumor size of subcutaneous tumors in nude mice treated with si-PLK1, while the right panel shows similar mice treated with si-NT.

and AIF to induce caspase-independent apoptosis. In galectin-1 induced T-cell apoptosis, which is independent of caspase, AIF and cytochrome *c*, only endoG was shown to be involved^[22]. The induction of apoptosis that is independent of caspase by silencing *PLK1* gene expression in Huh-7 cells has significant implications in the treatment of HCC. It is reported that caspase-1 and caspase-3 expressions are downregulated in HCC and this could contribute to the lack of efficacy of chemotherapy^[23]. Consequently, a caspase-directed strategy is unlikely to be successful, and thus targeting PLK1 may be a more effective strategy in treatment of HCC patients.

Similar to other reports of PLK1 involvement in the proliferation of cell types such as breast cancer, lung cancer, prostate cancer and esophageal cancer^[24-26], we have also shown that silencing of PLK1 significantly reduced cell proliferation and also impeded HCC growth in nude mice. *PLK1* is therefore a promising gene candidate for targeted therapy in treating not only HCC but other solid tumors as well. The requirement of PLK1 in the tumorigenesis of HCC and other cancers implies it has a critical role in the final pathway for cancer cell proliferation. Overexpression of PLK1 may help to inactivate the spindle checkpoint persistently by targeting early-mitotic inhibitor 1, which is an inhibitor of anaphase promoting complex/cyclosome, that is degraded by ubiquitin ligase

complexes^[27]. However, the exact role of PLK1 overexpression in the tumorigenesis of HCC or other cancers is still unclear since the physiological role of PLK1 is not fully defined.

The results of our study suggest that targeting PLK1 in HCC treatment is a promising therapeutic option. In addition, normal diploid cells such as hTERT-RPE1 and MCF10A cells can survive similar PLK1 depletion that otherwise causes mitotic arrest and apoptosis in cancer cell lines^[28]. Normal diploid cells still require PLK1 for survival, but at a much lower PLK1 level compared with cancer cells^[29]. Consequently, controlling the extent of PLK1 depletion could minimize the deleterious effects on HCC patients. Currently, there are a number of PLK1 inhibitors in development such as ON 01910, DAP81, TAL, BTO-1/cyclopolin, and compound 1^[30]. However, the most advanced agent is a novel small molecular inhibitor, BI2536, which inhibits the enzymatic activity of PLK1 protein with high selectivity (10 000-fold selectivity over other kinases) and high potency^[31,32]. BI2536 has now completed phase I clinical trials in patients with advanced solid tumors with reported anti-tumor activity, and neutropenia as a dose-limiting toxicity^[33]. Phase II clinical trials of BI2536 are currently ongoing for various tumor indications^[34]. Such inhibitors could probably be suitable in treating HCC or could be used in combina-

tion with other drugs, a strategy that is being explored in many cancers, for example, breast cancer^[35].

In conclusion, PLK1 is overexpressed in HCC and the silencing experiments have shown significant antiproliferative effects *in vitro* and *in vivo*, through induction of a caspase-independent apoptosis pathway that could lead to increased sensitivity of chemotherapy in HCC patients. Thus, PLK1 is a promising therapeutic target for HCC.

COMMENTS

Background

The pathogenesis of hepatocellular carcinoma (HCC) is likely to be a multi-gene process. One gene that has been described in many cancers but only recently in HCC is polo-like kinase 1 (PLK1), a kinase that is critical to spindle formation during the metaphase.

Research frontiers

PLK1 is emerging as an important therapeutic target as a number of new agents are currently undergoing clinical trials.

Innovations and breakthroughs

The authors show that PLK1 knockdown results in a reduction in PLK1 gene and protein expression with resultant reduction in cell proliferation and increase in apoptosis. The authors also show that the increase in apoptosis is caspase-independent and involves EndoG and apoptosis-inducing factor. Knockdown of PLK1 in subcutaneous tumors transplanted into nude mice showed impairment of tumor growth.

Applications

The study indicates that PLK1 inhibitors should be evaluated in clinical trials of HCC, either as monotherapy or combination therapy.

Terminology

PLK1: Polo-like kinase, an important kinase required for spindle formation in metaphase; caspase-induced apoptosis is a key pathway for apoptosis; cell proliferation is a classic method to evaluate tumor cell growth - blocking proliferation implies that tumor growth can be blocked; si-RNA knockdown: Short interfering RNAs are short complementary RNAs that bind specifically to the mRNA produced by the gene of interest, hence blocking the signal for protein expression.

Peer review

This is a good descriptive study in which authors investigate the role of PLK1 as a therapeutic target for HCC. The results are interesting and suggest knockdown of PLK1 overexpression in HCC was shown to be a potential therapeutic target, leading to apoptosis through the endonuclease-G pathway.

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Zinc finger protein A20 protects rats against chronic liver allograft dysfunction

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Abstract

AIM: To investigate the effect of zinc finger protein A20 on chronic liver allograft dysfunction in rats.

METHODS: Allogeneic liver transplantation from DA rats to Lewis rats was performed. Chronic liver allograft dysfunction was induced in the rats by administering low-dose tacrolimus at postoperative day (POD) 5. Hepatic overexpression of A20 was achieved by recombinant adenovirus (rAd.)-mediated gene transfer administered intravenously every 10 d starting from POD 10. The recipient rats were injected with physiological saline, rAdEasy-A20 (1×10^9 pfu/30 g weight) or rAdEasy (1×10^9 pfu/30 g weight) every 10 d through the tail vein for 3 mo starting from POD 10. Liver tissue samples were harvested on POD 30 and POD 60.

RESULTS: Liver-transplanted rats treated with only tacrolimus showed chronic allograft dysfunction with severe hepatic fibrosis. A20 overexpression ameliorated

the effects on liver function, attenuated liver allograft fibrosis and prolonged the survival of the recipient rats. Treatment with A20 suppressed hepatic protein production of tumor growth factor (TGF)- β 1, interleukin-1 β , caspase-8, CD40, CD40L, intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin. A20 treatment suppressed liver cell apoptosis and inhibited nuclear factor- κ B activation of Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs) and hepatic stellate cells (HSCs), and it subsequently decreased cytokine mRNA expression in KCs and LSECs and reduced the production of TGF- β 1 in HSCs.

CONCLUSION: A20 might prevent chronic liver allograft dysfunction by re-establishing functional homeostasis of KCs, LSECs and HSCs.

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Key words: Chronic allograft dysfunction; Liver transplantation; Zinc finger protein A20; Rat

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INTRODUCTION

Although the incidence of chronic rejection at 5 years after transplantation has decreased from 15%-20% in the 1980s to an expected incidence of 3%-5% in current recipients, probably because of the introduction of the novel drug tacrolimus^[1,2], the incidence of chronic liver

allograft dysfunction is still high in long-surviving recipients. Chronic liver allograft dysfunction, which results in the loss of approximately 2000 liver grafts every year, has a significant impact on liver graft function and long-term survival. It has been reported that a significant proportion of long-term liver allografts presented features of a hepatitis-like reaction that was not attributable to known viral agents or other agents^[3,4]. Chronic liver allograft dysfunction is now considered to be a result of various causes of hepatic injury, including immune and non-immune factors. Currently, research is focused on non-immune factors that may lead to chronic liver allograft dysfunction, including the use of donor organs of marginal quality, the use of organs from brain injury/brain-dead donors, the presence of ischemia-reperfusion injury, Kupffer cell (KC) activation, interleukin and growth factor production, damage caused by immunosuppressive drugs (chronic toxicity of damage) and cytomegalovirus infection. Because of the numerous causative factors, the prevention and treatment of chronic liver graft dysfunction represents a considerable challenge.

Pathological changes observed in cases of chronic liver allograft dysfunction include arterial proliferative occlusive disease and/or bile duct disappearance, liver cell death and eventually liver fibrosis. Bile duct disappearance is considered to be a result of arterial proliferative occlusive disease. Molecular changes include increased hepatic expression of tumor growth factor (TGF)- β , interleukin (IL)-1 β , caspase-1 and caspase-8^[5]. IL-1 has been shown to contribute to chronic rejection^[6]. IL-1 is produced by activated macrophages and many other cell types, including injured endothelial cells (ECs), and it stimulates smooth-muscle proliferation *in vitro* and increases the adhesive properties of the vascular endothelium. Overproduction of TGF- β is a chief cause of tissue fibrosis in various organs^[7]. TGF- β induces the phenotypic transition of hepatic stellate cells (HSC) into proliferating myofibroblast-like cells, thus enhancing production of extracellular components^[8]. The cellular and molecular mechanisms of chronic liver allograft dysfunction are still not completely clear, and the currently available drug treatments are ineffective.

The process of liver fibrosis is well-understood, and the basic steps can be summarised as follows: (1) various sources of liver damage induce KC activation; (2) activated KCs express and produce a variety of cytokines and co-stimulating molecules, such as TNF- α , IL-6, TGF- β , IL-1 β and CD40L^[9-11]; and (3) cytokines and co-stimulating molecules stimulate HSC activation and stimulation of myofibroblasts, which synthesize a large amount of extracellular matrix, resulting in liver fibrosis. In the process of hepatic fibrosis, nuclear factor (NF)- κ B may play an important central regulatory role by regulating functional changes of hepatocytes, KCs and HSCs^[9,11,12].

NF- κ B is a key nuclear factor involved in the regulation of KC activation. In addition to the production of pro-inflammatory cytokines, such as TNF- α , IL-1 β , TGF- β , IL-6 and IL-8, activated KC also express the co-stimulatory molecule CD40L, which is an important char-

acteristic of chronic liver allograft dysfunction^[13]. Expression of inflammatory mediators can stimulate the nuclear translocation of NF- κ B in KCs *via* autocrine or paracrine pathways and induce the production of additional inflammatory mediators, leading to an "inflammatory cascade", which results not only in liver damage, but also leads to the rapid stimulation of HSC activation and proliferation. Thus, inhibition of NF- κ B activation in KCs may down-regulate the expression of inflammatory mediators, such as TNF- α , TGF- β , IL-1 and CD40L, and thereby suppress the liver inflammatory response.

Although the role of NF- κ B in liver graft arterial lesions is not completely clear, NF- κ B plays a key regulatory role in non-organ transplant atherosclerosis. In 1996, Brand demonstrated the presence of activated NF- κ B in human atherosclerotic tissue for the first time^[14]. Activation of NF- κ B was identified in smooth muscle cells, macrophages and ECs in their study. Previous studies had shown that atherosclerosis involves activation of vascular ECs and proliferation of vascular smooth muscle cells which are subject to the regulation of NF- κ B activation^[15-19].

A20 is a zinc finger protein that was originally identified as a TNF-responsive gene in ECs^[20]. A20 is expressed in multiple cell types, including fibroblasts, B cells, T cells and β cells, in response to a variety of stimuli that activate NF- κ B, including IL-1, LPS, phorbol 12-myristate 13-acetate, H₂O₂ and CD40 ligand. In ECs and hepatocytes, A20 has a dual cytoprotective function^[21-26]. A20 is anti-inflammatory, due to inhibition of NF- κ B through a negative feedback loop, and it is antiapoptotic, due to inhibition of the caspase cascade at the level of initiator caspase-8^[21-23]. A20 can also inhibit NF- κ B activation induced by LPS, IL-1 and CD40 cross-linking through the negative feedback loop^[24-26]. A20 curtails inflammation by inhibiting NF- κ B activation, either through its association with I κ B kinase- γ /NF- κ B essential modifier within the signalosome or through its ubiquitin-editing functions^[21,22,27,28].

A previous study indicated that reduced expression of A20 might be an important pathogenic contributor to an increased susceptibility to liver allograft ischemia/reperfusion (I/R) injury^[29]. Ramsey *et al.*^[30] recently reported that A20 could protect mice from lethal liver I/R injury by increasing peroxisome proliferator-activated receptor- α expression. In addition, it has been shown that A20 expression is up-regulated in human renal allografts in response to immune injury inferred by acute rejection, and the result suggests that A20 could limit graft injury^[31]. Our previous studies indicated that A20 expression was up-regulated in immature dendritic cells derived from rat liver allografts undergoing acute rejection^[32]. Furthermore, A20 overexpression could inhibit NF- κ B activation of liver sinusoidal endothelial cells (LSECs) in rat liver allografts and suppress acute rejection^[32]. These results suggest that A20 may protect liver allografts from I/R injury and acute rejection.

Although the effects of A20 on lipopolysaccharide-

induced acute toxic lethal hepatitis, liver regeneration, hepatic I/R injury and liver allograft rejection have been investigated, little is known about the effect of A20 on chronic liver allograft dysfunction. In this work, the effect of A20 on liver allograft chronic dysfunction induced by postoperative low-dose tacrolimus administration was investigated.

MATERIALS AND METHODS

Recombinant adenoviruses

The rAdEasy-A20 and the empty control rAdEasy containing green fluorescent protein were generated in our laboratory^[33]. The *Nco*I → *Sal*I fragment (2332 bp) of the A20 gene, which was obtained from the plasmid pCAGGS-FLAGmA20 (a kind gift from Dr. Rudi Beyaert from Department of Molecular Biology, Flanders Interuniversity Institute of Biotechnology, University of Ghent, Belgium) and carries the entire mouse A20 cDNA sequence, was cloned into the shuttle plasmid pAdTrack-CMV. Homologous recombination took place between the resultant plasmid and the backbone plasmid pAdEasy-1 in *Escherichia coli* BJ5183, and the recombinant adenoviral plasmid was generated. The adenovirus was packaged in 293 cells, and the recombinant adenovirus rAdEasy-A20 was generated. The empty Ad vector (rAdEasy) was generated following the same principle.

Animal model

Inbred male DA (RT1^a) and Lewis (RT1^b) rats weighing 260–320 g were used as liver donors and recipients, respectively. The animals were maintained under standard conditions and treated according to the Guidelines for the Care and Use of Laboratory Animals of Sichuan University. Orthotopic liver transplantations (OLT) were performed with the two-cuff technique. All operations were performed under ether anesthesia under sterile conditions. Cefazolin (40 mg/kg, injected intramuscularly) was given after the implantation operation for 5 d to prevent infection. More than 90% of the rats survived this operative procedure. To induce chronic liver allograft dysfunction, a low dose of tacrolimus (0.1 mg/kg per day) was administered intramuscularly for 5 d after the implantation operation^[5]. To examine chronic liver allograft dysfunction, recipient rats were given physiological saline (PS), rAdEasy-A20 (1×10^9 pfu/30 g weight) or rAdEasy (1×10^9 pfu/30 g weight) through the tail vein once every 10 d from postoperative day (POD) 10 for 3 mo. Five recipient rats per group were allowed to survive until they died. Ten recipient rats per group were killed on POD 30 and POD 60 before rAdEasy-A20 or rAdEasy injection. Blood samples were harvested from the inferior vena cava. The left lateral lobes and caudate lobes from 5 liver allografts per group were harvested for Western blotting, Masson staining and immunohistochemistry, and the other liver lobes were harvested for KC and LSEC isolation. Another 5 liver grafts per group were harvested for HSC isolation.

Histological analysis

Liver fibrosis was analyzed on POD 30 and POD 60. The liver tissue lobes were fixed in 10% neutral-buffered formalin embedded in paraffin. For histological analysis, the sections were stained with hematoxylin-eosin. For fibrosis analysis, the sections were stained with Masson stain.

Hepatic A20 expression

Liver graft specimens were harvested on POD 30 and POD 60. Immunohistochemistry was performed for hepatic A20 protein expression in five sections from per graft after the samples were fixed in 10% neutral-buffered formalin embedded in paraffin. The liver sections were incubated with a 1:100 dilution of anti-rabbit polyclonal A20 antibody (Abcam, ab45366) for 45 min and EnvisionTM for 45 min. The sections were counterstained with hematoxylin. Positive cells were counted at 400 × magnification. Ten random fields were observed in each of the liver portal tract areas.

Liver function assay

Serum samples from POD 30 and POD 60 were also analysed for alanine aminotransferase (ALT) and total bilirubin (TBIL) levels as indices of hepatocellular injury. The levels of ALT and TBIL were measured with an automatic biochemical analyser using diagnostic kits from Sigma Chemical Co.

Hepatic protein production study

TGF- β_1 , IL-1 β , caspase-1, caspase-8, CD40, CD40L, Intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and E-selectin protein levels in the liver allografts were analysed by Western blotting. Liver tissue lysates were electrophoresed on sodium dodecylsulfate polyacrylamide gel electrophoresis gels and transferred to polyvinylidene chloride (PVDC) membranes for Western blotting analysis. Briefly, the PVDC membranes were incubated in a blocking buffer for 1 h at room temperature followed by incubation for 2 h with Abs raised against TGF- β_1 , IL-1 β , caspase-1, caspase-8, CD40, CD40L, ICAM-1, VCAM-1 and E-selectin. The membranes were washed and incubated for 1 h with horseradish peroxidase-labelled immunoglobulin G. Immunoreactive bands were visualised using enhanced chemiluminescence detection reagent. The bands were quantified using a scanning densitometer from Bio-Image Analysis System. The results were expressed as the relative optical density.

Hepatic cells apoptosis

The paraffin sections of liver grafts on POD 30 and 60 were analysed for apoptotic cells using the terminal transferase dUTP nick end-labelling (TUNEL) method (*in situ* Cell Death Detection Kit, Roche Biochemicals, Mannheim, Germany). For all staining procedures, positive and negative cells were counted in 3 randomly selected fields under a light microscope. Quantitative analysis was performed

using the Coulter EPICS Elite ESP cell sorting system, United States.

Isolation of KC and LSECs

KCs and LSECs were isolated using a modified method of Braet and colleagues^[34]. In brief, the liver graft was perfused with Ca²⁺-Mg²⁺-free Hanks' balanced salt solution followed by 0.6% collagenase A (Sigma type 1) *via* a polyethylene catheter inserted into the portal vein trunk. After incubation of the fragmented tissue in the same solution, the resulting cell suspension was centrifuged at 100 r/min for 10 min to remove the parenchymal cells. The supernatant containing a mixture of the hepatic nonparenchymal cell fraction was subsequently layered on top of a two-step Percoll gradient (25% to 50%) and centrifuged for 10 min at 900 r/min. The intermediate zone located between the two density layers, which was enriched with LSECs and KCs, was cultured for 20 min in plastic flasks, and the LSECs and KCs subsequently were further isolated based on the selective adherence of KCs to plastic flasks and the spreading of the LSECs on collagen.

Isolation of HSCs

Hepatic stellate cells were isolated from the liver allografts by a modified method that has been described previously^[35]. Briefly, HSCs were isolated from the liver grafts by sequential *in situ* perfusion with collagenase and digestion with pronase. Suspensions of liberated HSCs were prepared by centrifugation on a double-layered (17%/11.5%) metrizamide solution (Sigma). After centrifugation at 1700 *g* for 15 min, the HSCs were harvested from the top of the upper layer. More than 90% pure and viable HSCs were routinely obtained using this procedure, as determined by ultraviolet-excited fluorescence microscopy and Trypan blue dye exclusion, respectively. Isolated HSCs were used for nuclear protein or RNA extraction.

NF- κ B activation of LSECs, KCs and HSCs

The NF- κ B activity of the LSECs, KCs and HSCs was analysed with the electrophoretic mobility shift assay (EMSA) as previously described^[33]. Nuclear proteins were extracted from LSECs, KCs and HSCs. The protein concentration of the nuclear extracts was determined by Bradford assay^[36]. Nuclear extracts were frozen on dry ice and stored at -80 °C until they were assessed in the EMSA. The double-stranded NF- κ B consensus oligonucleotides (5'-AGTTGAGGGGACITTTCCAGGC-3', and 3'-TCAACTCCCCTGAAAGGGTCCG-5')^[37] used in EMSA were end-labelled with γ -³²P adenosine triphosphate (3.7 × 10⁵ Bq/L at 5 μ L) using T4 polynucleotide kinase. The reaction products were separated in 6% non-denaturing polyacrylamide gels subjected to gamma autoradiography at -70 °C for 48 h and were analysed with a gel imaging system.

mRNA expression of cytokines in LSECs and KCs

Analysis of mRNA expression of ICAM-1, VCAM-1,

E-selectin, IL-1 β and CD40 in LSECs and mRNA expression of TGF- β 1, IL-1 and CD40 L in KCs was performed by semiquantitative reverse transcription polymerase chain reaction (RT-PCR) amplification and compared with the expression of the house-keeping gene β -actin using the one-step PCR Kit. Total RNA from LSECs and KCs was extracted using the TripureTM reagent. PCR was performed in a 25 μ L reaction system. The PCR reaction produced a 513-bp product for ICAM-1, a 257-bp product for VCAM-1, a 239-bp product for E-selectin, a 388-bp product for CD40, a 395-bp product for CD40L, a 378-bp product for IL-1 β , a 383 bp product for TGF- β 1, and a 813-bp product for β -actin. The PCR products from each sample were subjected to electrophoresis in a 15 g/L agarose gel containing 0.5 mg/L ethidium bromide. Densitometrical analysis using NIH imaging software was performed for semiquantification of the PCR products. The mRNA expression of each target was evaluated by determining the ratio of the band intensity to β -actin and was presented as the percent of β -actin (%).

TGF- β 1 protein level in HSCs

Supernatant samples from the HSCs were analysed for TGF- β 1 using enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions.

Statistical analysis

SPSS 13.0 statistical software (SPSS Inc., Chicago, IL) was used to analyse the relevant data. The results are expressed as the means \pm SD. Significant differences between two groups or more were identified by the paired Student *t* test. *P* values less than 0.05 were considered statistically significant.

RESULTS

A20 protein over-expression in liver grafts by successful venous adenoviral gene transfer

Immunohistochemical staining confirmed significant hepatic A20 protein expression on POD 30 (data not shown) and POD 60 in the group of rats that received venous A20 adenovirus, whereas only some hepatic A20 protein expression was shown in the rats treated with rAdEasy and PS POD 30 (data not shown) and POD 60 (Figure 1A-C).

Survival study

The survival days of the liver-grafted rats are shown in Table 1. The results suggested that the rats in the A20 treatment group survived longer than the rats in the PS and rAdEasy groups.

Liver fibrosis

Fibrosis of the liver allografts was detected on POD 30 and POD 60 with Masson staining. The histological findings showed that postoperative administration of low-dose tacrolimus without A20 treatment resulted in marked liver fibrosis on POD 60. However, tacrolimus

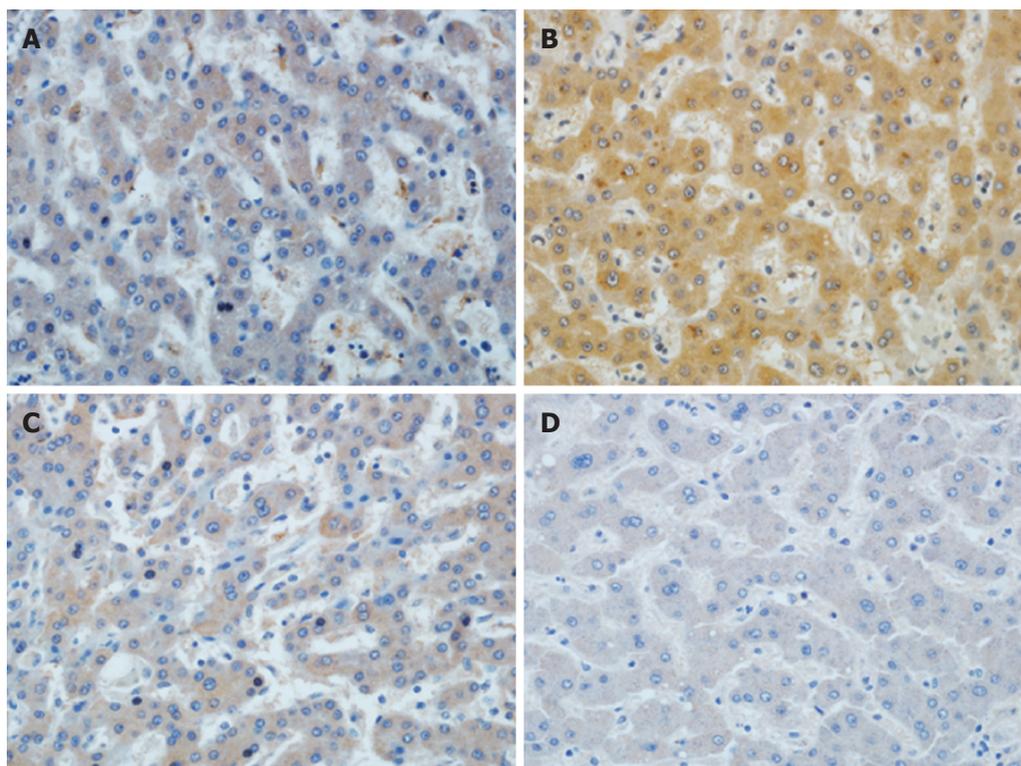


Figure 1 Immunohistochemical analysis of A20 expression in liver grafts on postoperative day 30. Representative immunohistochemical sections from FK506 + physiological saline (A) treated livers demonstrate lower A20 expression compared to the substantial number of A20-positive liver cells in the FK506 + A20 treated liver grafts (B). Furthermore, the expression of FK506 + rAdEasy (C) is also low. D: The results of the normal control group (brown staining) (original magnification, 400 ×).

Table 1 Effect of A20 on the survival of Lewis rats transplanted with Dark Agouti livers

Treatment ¹	Survival (d)	mean ± SD
FK506 + PS	52, 57, 60, 72, 78	63.80 ± 10.83
FK506 + rAdEasy A20	117, 121, 123, 128, 136	125.00 ± 7.31 ^a
FK506 + rAdEasy	44, 50, 57, 58, 67	55.20 ± 8.70

¹Lewis rats transplanted with Dark Agouti livers were given 0.1 mg/kg FK506 for 5 d after transplantation. Lewis rats receiving transplants were given rAdEasy-A20 (1×10^9 pfu/30 g weight) or rAdEasy or physiological saline (PS) once every 10 d from postoperative day (POD) 30 to POD 90. ^a $P < 0.01$ vs FK506 + PS and FK506 + rAdEasy treatment.

combined with A20 treatment resulted in reduced hepatic fibrosis (Figure 2).

Serum ALT and TBIL levels

As shown in Table 2, the results showed that postoperative administration of low-dose tacrolimus led to a significant increase of TBIL and ALT levels on POD 30 and POD 60. A20 treatment markedly decreased serum TBIL and ALT levels on POD 30 and POD 60.

A20 decreases hepatic protein production of TGF-β1, IL-1β, caspase-8, CD40, CD40L, ICAM-1, VCAM-1 and E-selectin

High levels of TGF-β1, IL-1β, caspase-1, caspase-8, CD40, CD40L, ICAM-1, VCAM-1 and E-selectin protein were detected in liver grafts from rats that did not receive

Table 2 Serum total bilirubin and alanine aminotransferase levels after liver transplantation (*n* = 5)

Group	ALT (IU/L)		TBIL(μmol/L)	
	POD 30	POD 60	POD 30	POD 60
FK506 + PS	163.41 ± 35.28	257.35 ± 42.78	85.72 ± 16.47	165.43 ± 24.63
FK506 + A20	66.79 ± 17.56 ^b	90.28 ± 22.37 ^{b,d}	37.61 ± 8.06 ^b	63.71 ± 11.38 ^{b,d}
FK506 + rAdEasy	187.66 ± 43.54	282.75 ± 53.64 ^d	106.37 ± 21.35	182.93 ± 28.75 ^d

^b $P < 0.01$ vs FK506 + physiological saline (PS) or FK506 + rAdEasy; ^d $P < 0.01$ vs postoperative day (POD) 30. ALT: Alanine aminotransferase; TBIL: Total bilirubin.

A20 treatment liver graft on POD 30 (data not shown) and POD 60. However, A20 treatment markedly down-regulated the protein levels of TGF-β1, IL-1β, caspase-8, CD40, CD40L, ICAM-1, VCAM-1 and E-selectin in liver allografts (Figure 3).

A20 treatment suppresses liver cell apoptosis

Liver cells apoptosis on POD 30 and POD60 were measured with the TUNEL assay. TUNEL staining revealed a decreased apoptosis index among the liver cells in the A20 group (11.83% ± 3.52% on POD 30, 14.66% ± 3.84% on POD 60) compared with that of the PS group (20.62% ± 5.36% on POD30, 32.78% ± 6.74% on POD 60) and rAdEasy group (21.58% ± 6.17% on POD 30, 35.27% ± 7.38% on POD 60) ($P < 0.01$) (Figure 4).

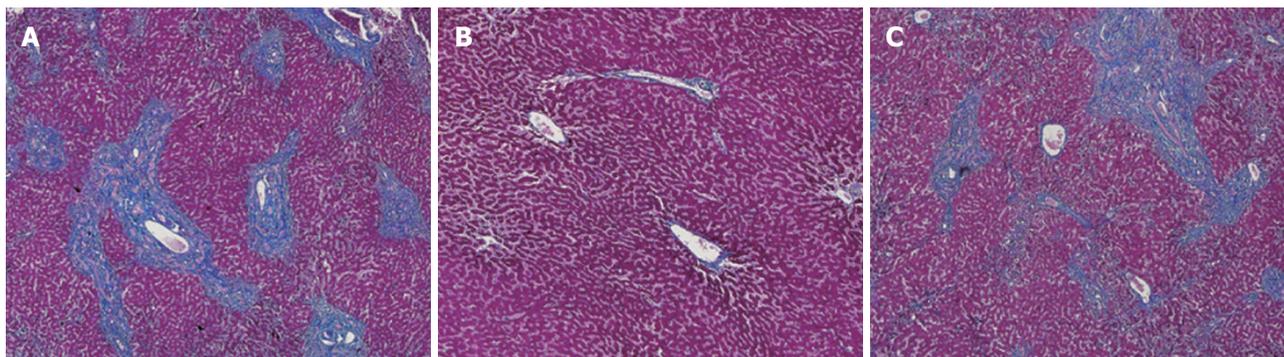
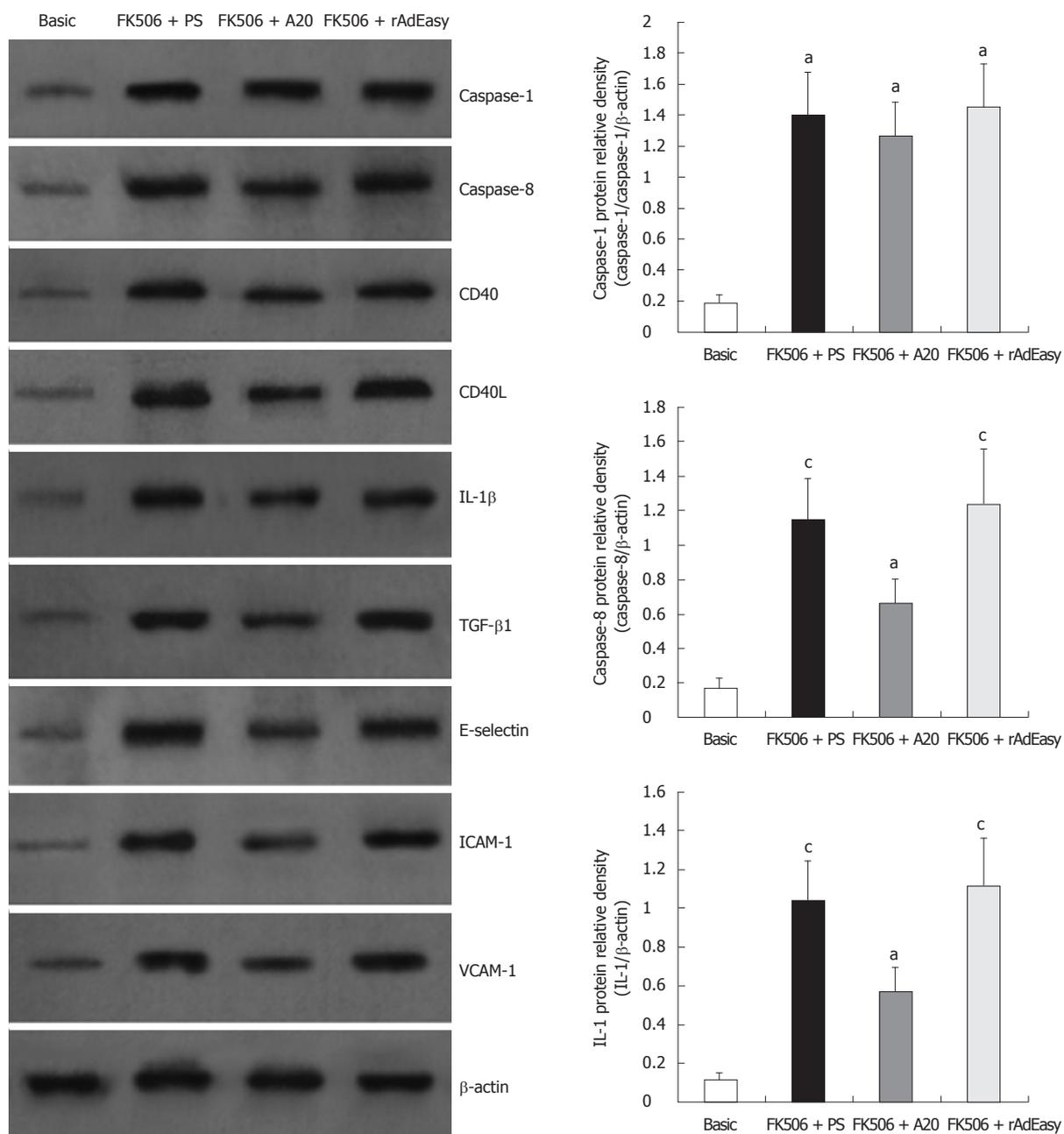


Figure 2 Representative liver fibrosis samples from FK506 + physiological saline treated group (A), FK506 + A20 treated group (B) and FK506 + rAdEasy-treated group (C) on postoperative day 60 (100 ×).



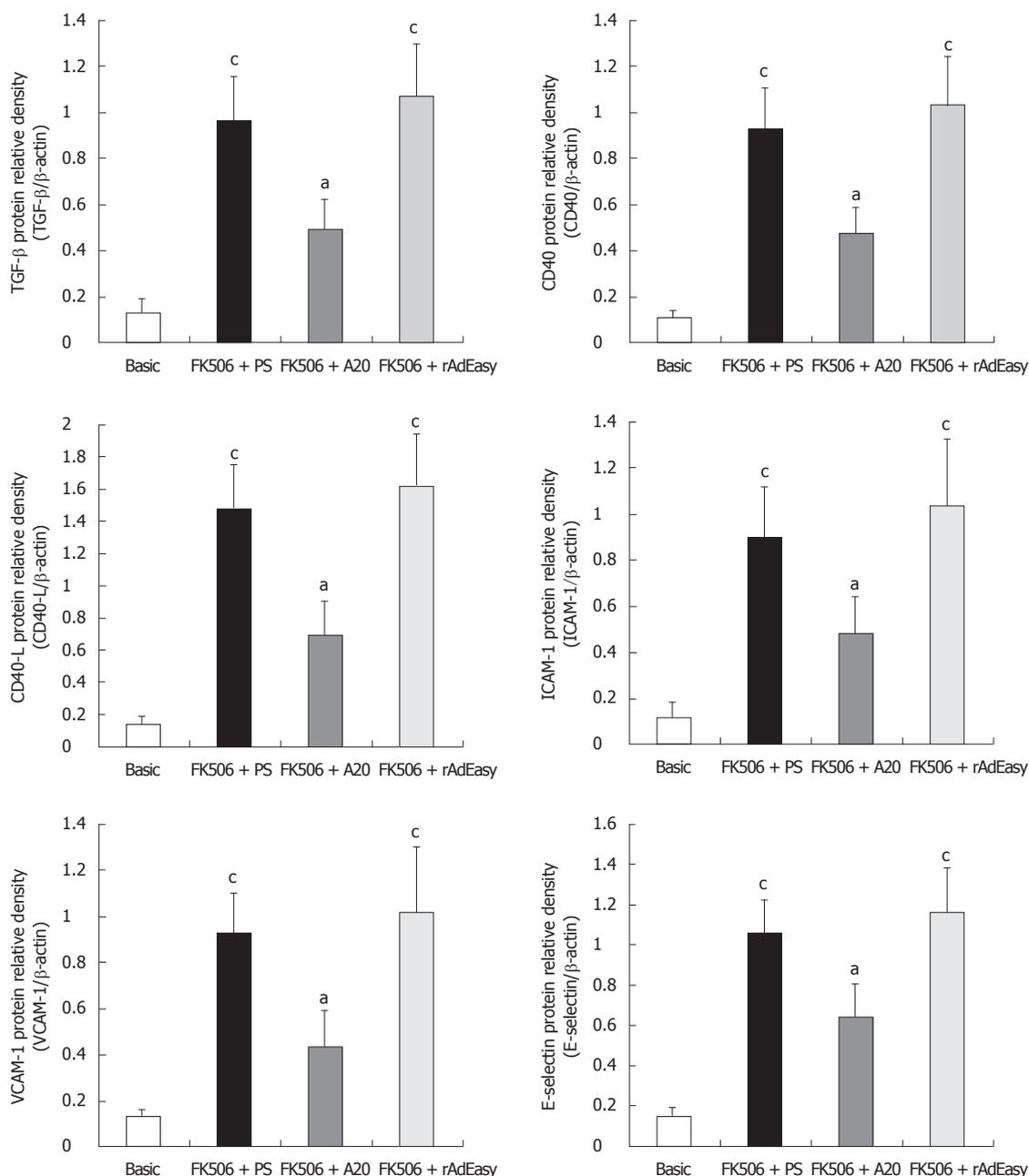


Figure 3 Western blotting analysis and densitometric analysis of associated cytokines in liver graft tissue. Data were expressed as the relative intensity vs β -actin. ^a*P* < 0.05 vs basic control; ^c*P* < 0.05 vs FK506 + A20. IL: Interleukin; TGF: Tumor growth factor; ICAM: Intercellular adhesion molecule; VCAM: Vascular cell adhesion molecule; PS: Physiological saline.

A20 suppresses NF- κ B activation of LSECs, KCs and HSCs

The EMSA showed that postoperative low-dose tacrolimus treatment led to a significant activation of NF- κ B in LSECs, KCs and HSCs on POD 30 (data not shown) and POD 60, and A20 treatment markedly inhibited NF- κ B activation in these cells (Figure 5).

A20 suppresses cytokine mRNA expression in LSECs and KCs

High levels of ICAM-1, VCAM-1, E-selectin, IL-1 β and CD 40 mRNA in LSECs, as well as high levels of TGF- β ₁, IL-1 β and CD40L, in KCs on POD 30 and POD 60 were detected by RT-PCR. The increased mRNA expression

levels of these cytokines were significantly reduced by A20 treatment (Figure 6).

A20 suppresses TGF- β ₁ protein production in HSCs

The ELISA showed that A20 overexpression significantly reduced TGF- β ₁ protein production in HSCs from liver allografts (Table 3).

DISCUSSION

To improve the survival of OLT patients, it is particularly important to protect liver grafts from chronic dysfunction. In the present study, we demonstrated that the zinc

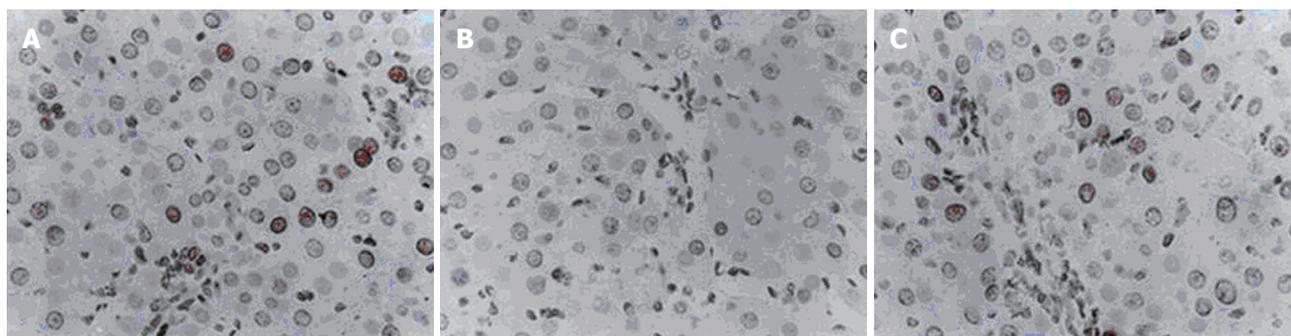


Figure 4 Terminal transferase dUTP nick end-labelling analysis of liver cells apoptosis (red stained) (original magnification, $\times 400$). Overexpressed A20 in liver graft resulted in a significantly decreased number of transferase dUTP nick end-labelling-positive liver cells (B) compared with physiological saline group rats (A) and rAdEasy group rats (C) ($P < 0.01$).

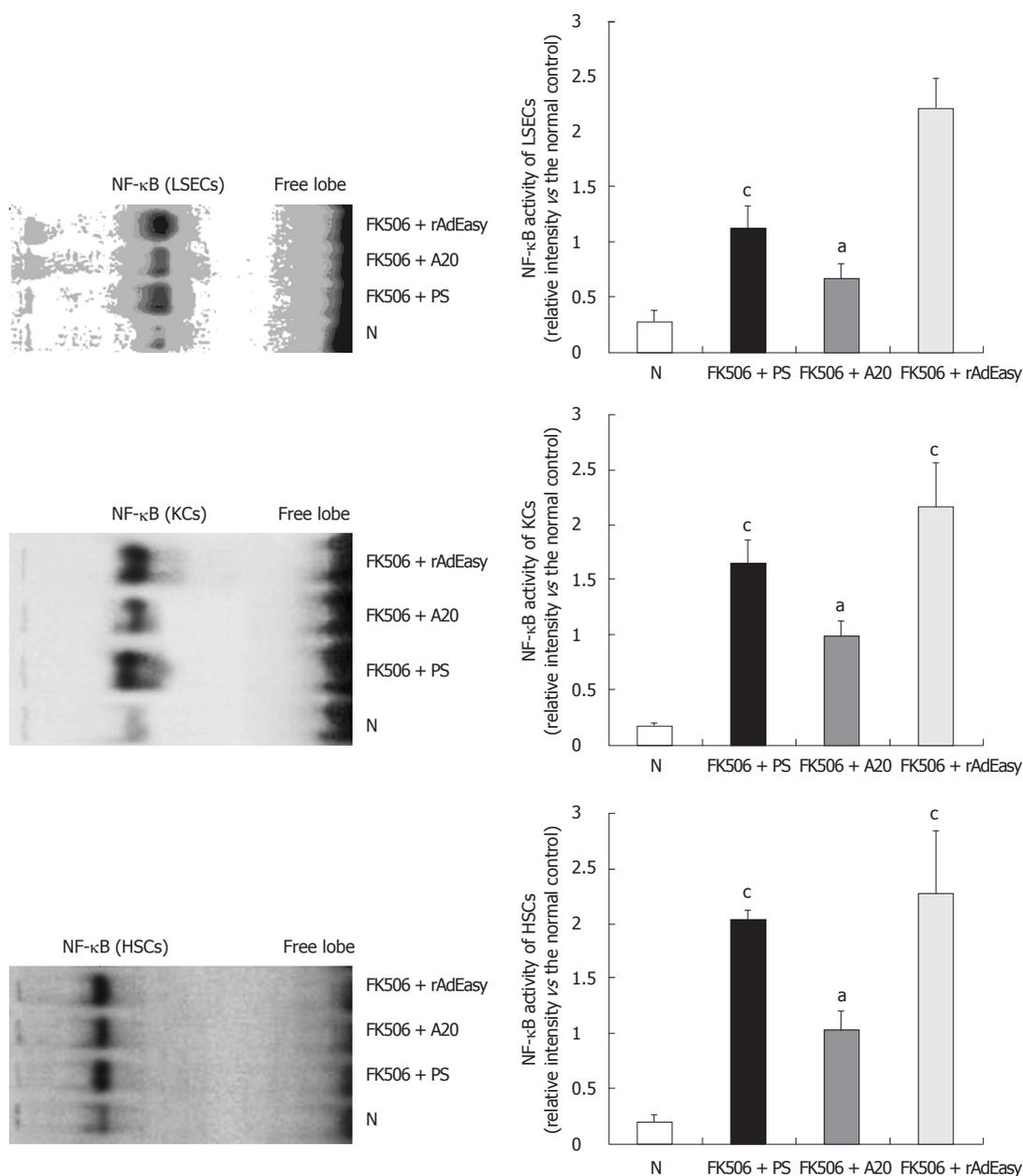
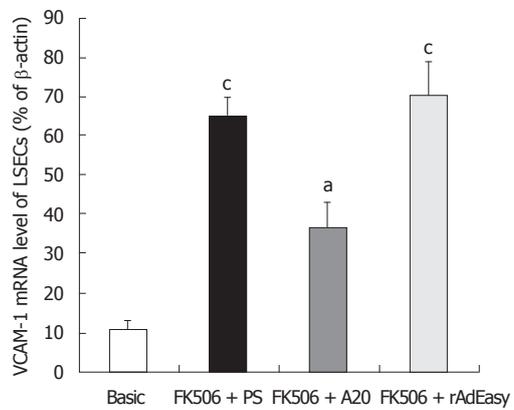
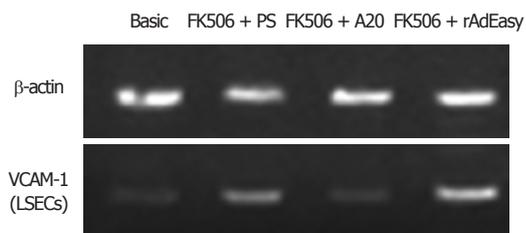
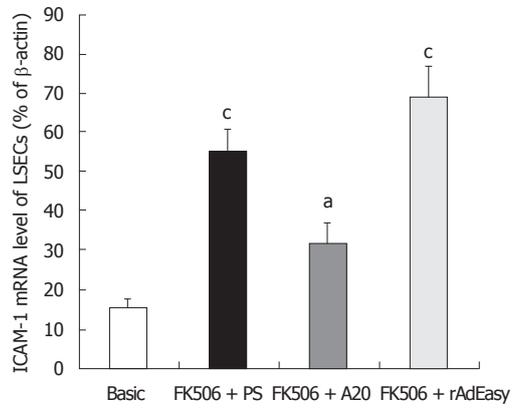
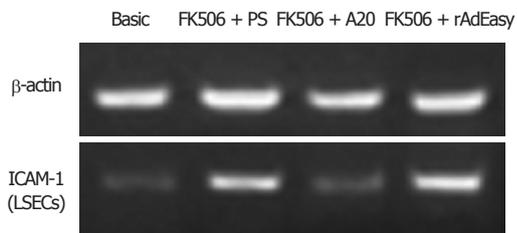
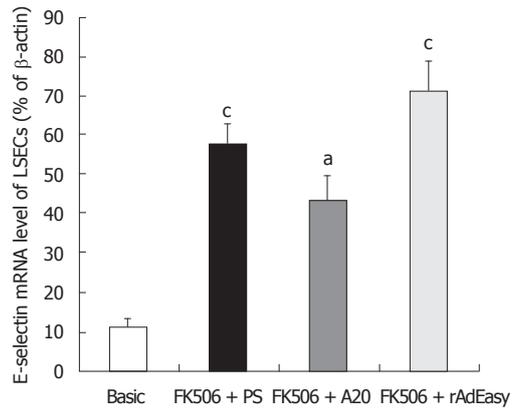
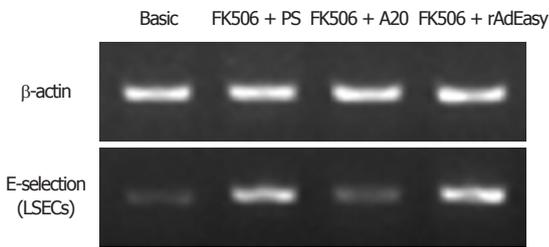
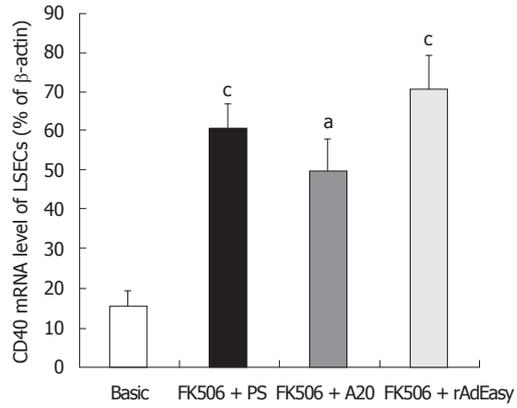
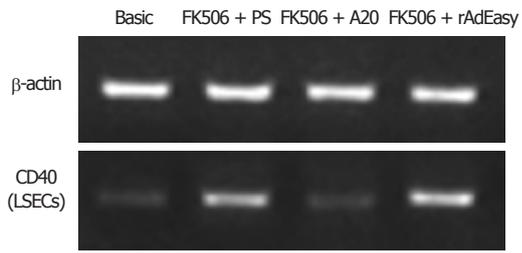


Figure 5 Effects of A20 on nuclear factor- κ B activation in liver sinusoidal endothelial cells, Kupffer cells and hepatic stellate cells. Nuclear factor- κ B (NF- κ B) activation on postoperative day (POD) 60 was determined by electrophoretic mobility shift assay (EMSA). Data were expressed as the relative intensity vs the normal basic control (N). ^a $P < 0.05$ vs basic control; ^c $P < 0.05$ vs FK506 + A20. LSECs: Liver sinusoidal endothelial cells; KCs: Kupffer cells; HSCs: Hepatic stellate cells.



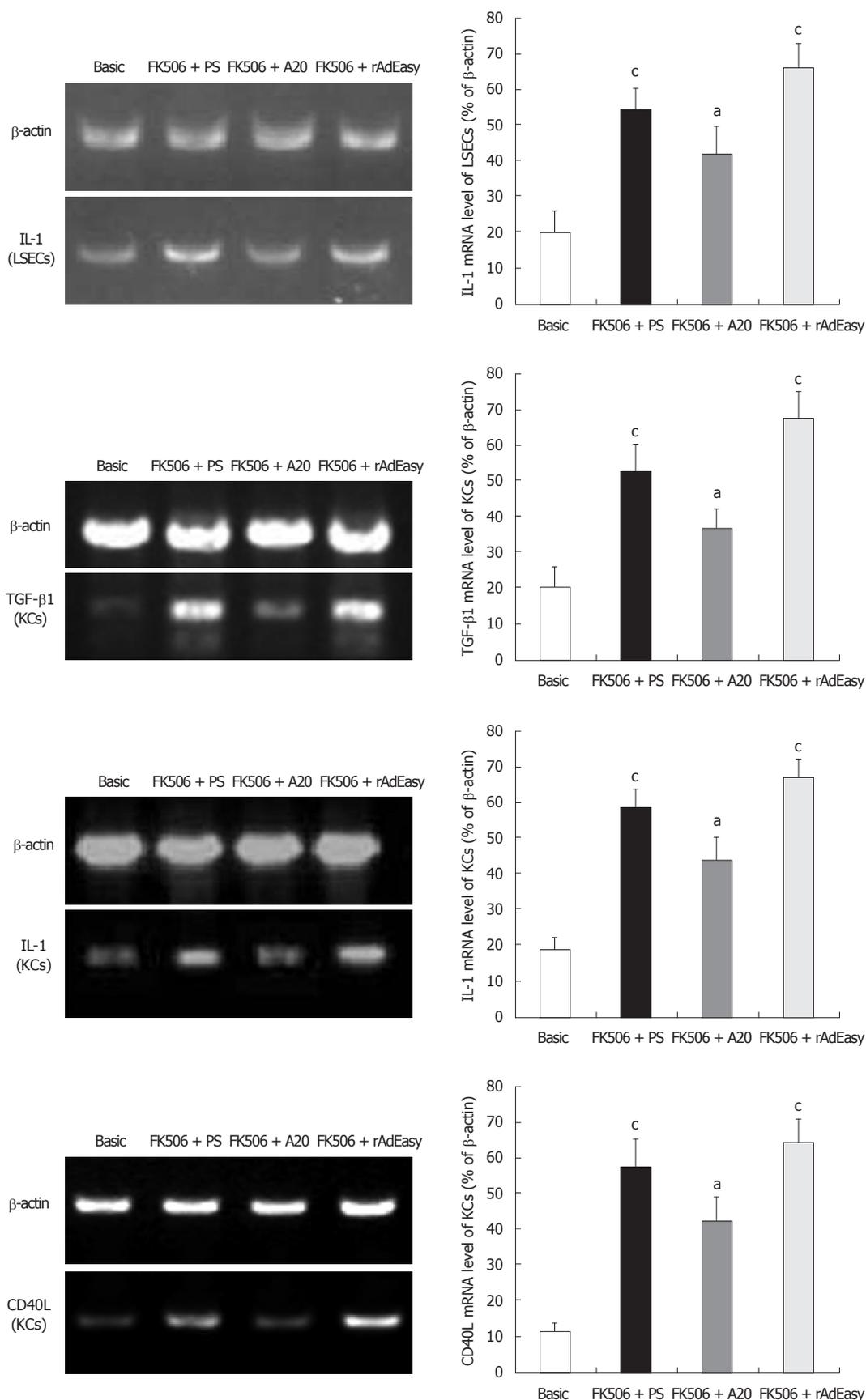


Figure 6 Cytokine mRNA expression in hepatic nonparenchymal cells on postoperative day 60 detected by reverse transcription polymerase chain reaction analysis. Data were expressed as the relative intensity vs β-actin. ^a*P* < 0.05 vs basic control; ^c*P* < 0.05 vs FK506 + A20. LSECs: Liver sinusoidal endothelial cells; ICAM: Intercellular adhesion molecule; KCs: Kupffer cells; VCAM: Vascular cell adhesion molecule; TGF: Tumor growth factor; IL: Interleukin.

Table 3 Tumor growth factor- β 1 levels in the conditioned media of cultured hepatic stellate cells ($n = 5$)

Group	TGF- β 1 (pg/mL, mean \pm SD)	
	POD 30	POD 60
FK506 + PS	1385.80 \pm 186.84 ^b	2368.62 \pm 211.58 ^{b,d}
FK506 + A20	595.76 \pm 85.71	718.28 \pm 97.12
FK506 + rAdEasy	1525.32 \pm 202.66 ^b	2475.33 \pm 194.93 ^{b,d}

^b $P < 0.01$ vs FK506 + A20; ^d $P < 0.01$ vs postoperative day (POD) 30. TGF- β 1: Tumor growth factor- β 1.

finger protein A20, a potent negative feedback inhibitor of NF- κ B activation and a hepatoprotective gene, could suppress chronic liver allograft dysfunction in rats.

The identification of NF- κ B as a key factor for the pathogenesis of allograft rejection suggests that NF- κ B-targeted therapeutics might be useful in transplant patients. Although many drugs, such as corticosteroids and cyclosporin, can inhibit NF- κ B activation^[38-40], these immunosuppressants have few effects on chronic liver allograft dysfunction. Therefore, novel effective agents for chronic liver allograft dysfunction should be investigated.

Previous studies have identified A20 as a critical component of the physiologic hepatoprotective role of hepatocytes. The effects of A20 on lipopolysaccharide-induced acute toxic lethal hepatitis^[22,23], liver regeneration^[22,23,33], hepatic I/R injury^[60] and liver allograft rejection^[33] have been investigated. Furthermore, as a role for NF- κ B is inferred in the pathological changes involved in chronic liver allograft dysfunction, such as liver cell death, arterial proliferative occlusive disease and/or bile duct disappearance, and eventually liver fibrosis, we reasoned that A20 would probably attenuate chronic liver allograft dysfunction. In the present study, we found that A20 is also an effective agent for chronic liver allograft dysfunction by showing that fibrosis was markedly attenuated in A20-overexpressing liver allografts compared with the controls. The suppressed NF- κ B activation in LSECs, KCs and HSCs, the decreased production of TGF- β 1, IL-1 β , caspase-8, ICAM-1, VCAM-1, E-selectin, CD40 and CD40L, as well as the suppressed level of liver cell apoptosis, are possible mechanisms for these effects.

Overproduction of TGF- β 1 is a chief cause of liver fibrosis. TGF- β 1 is mainly produced by HSCs and KCs. HSC has been affirmed to be the main effector cell of liver fibrosis. As the main macrophage and proinflammatory cell, KCs not only perform phagocytosis, but they also release many proinflammatory cytokines, including TNF- α , IL-1, IL-6 and TGF- β 1, meaning that the role of KCs in liver grafts may change during different phases, including the early phase of induction of hepatic I/R injury^[41], the acute rejection phase in human liver allografts^[42] and during the establishment of tolerance in the OLT model of transplantation from one Sprague-Dawley (SD) rat to another SD rat^[43]. However, the role of KCs in chronic liver graft dysfunction has not been investigated. IL-1 has been shown to contribute to chronic

rejection^[6]. IL-1 produced by activated macrophages and many other cell types, including injured ECs, increases smooth muscle proliferation *in vitro* and the adhesive properties of the vascular endothelium. LSECs provide a barrier against the infiltration of the liver graft infiltrating mononuclear cells (LIMCs), and blocking of adhesion molecules, such as ICAM-1, on ECs interferes with recruitment of sinusoidal NK-like cells into the rat liver^[44]. NF- κ B activation is the key regulatory factor of nuclear transcription of TGF- β and IL-1 in these cells; therefore, inhibition of NF- κ B activation could inhibit transcription of TGF- β and IL-1. In our previous study, we showed that A20 could inhibit NF- κ B activation and apoptosis in LSECs, which subsequently suppresses ICAM-1 mRNA expression and recruitment of LIMCs, including T cells and NK/NKT cells^[33]. Thus, we reasoned that LSECs and KCs could play important effect on the development of chronic liver allograft dysfunction. Therefore, functional changes of KCs and LSECs in liver allografts, including NF- κ B activation and transcription of TGF- β and IL-1, were investigated in the present study.

Low-dose tacrolimus was administrated post-OLT for 5 d to induce liver allograft dysfunction in the present study. Short-term postoperative administration of low-dose tacrolimus resulted in significant liver fibrosis on POD 60 and reduced liver function on POD 30 and POD 60. Excessive NF- κ B activation and a high level of mRNA expression of ICAM-1, VCAM-1, E-selectin, IL-1 β and CD40 in LSECs were found on POD 30 and POD 60. Similar changes were detected in KCs and HSCs, including marked NF- κ B activation and high levels of TGF- β 1, IL-1 β and CD40L mRNA expression in KCs, as well as elevated TGF- β 1 protein levels in HSCs. Increased hepatic protein production of TGF- β 1, IL-1 β , ICAM-1, VCAM-1, E-selectin, CD40 and CD40L were also detected on POD30 and POD 60. These results suggested that excessive NF- κ B activation and the associated production and secretion of cytokines by LSECs, KCs and HSCs may be the important steps inducing chronic liver allograft dysfunction. This hypothesis was confirmed by the results obtained with the combined A20 treatment. Liver fibrosis and liver function were significantly improved by the combined A20 treatment. Furthermore, the NF- κ B activity in LSECs, KCs and HSCs was significantly down-regulated by A20 treatment, and consequently the elevated cytokines mRNA expression and protein production were more suppressed. Suppression of ICAM-1, VCAM-1 and E-selectin production in LSECs could inhibit the recruitment of LIMCs into the liver graft^[33], reducing the hepatic injury caused by LIMCs. The down-regulated IL-1 β secretion in LSECs and KCs might theoretically suppress chronic rejection^[6]. The CD40/CD40 L signalling pathway is a potent activator of ECs and a promoter of atherosclerosis. A20 works at multiple levels to protect ECs from CD40/CD40L-mediated activation and apoptosis. A20-based therapy could be beneficial for the treatment of vascular diseases, such as atherosclerosis and transplant-associated vascu-

lopathy^[15]. A20 can also inhibit NF- κ B activation induced by LPS, IL-1 and CD40 cross-linking through a negative feedback loop^[24-26]. Previous data suggested that FasL expression on APCs and phagocytosis of apoptotic T cells by FasL⁺ KCs were indicators of acute and chronic rejection activity in human liver allografts^[41]. In the present study, the A20-induced decrease in the expression of IL-1 and CD40 in LSECs, as well as IL-1 and CD40L in KCs, might inhibit NF- κ B activation in LSECs through a negative feedback loop and protect LSECs from apoptosis, subsequently inhibiting recruitment of LIMCs into the liver graft. Suppression of NF- κ B activation in KCs could inhibit hepatic ischemia/reperfusion injury, which represents an important cause of chronic liver allograft dysfunction. The decreased expression of TGF- β 1 in KCs and the suppressed NF- κ B activation in HSCs by A20 might inhibit the transition from HSCs to myofibroblast-like cells and consequently suppress TGF- β 1 protein production in HSCs. The present study also revealed suppressed TGF- β 1 production and reduced fibrosis in combined A20-treated liver grafts.

A20 has a dual cytoprotective function in ECs and hepatocytes. In addition to its anti-inflammatory function, A20 is also antiapoptotic through inhibition of the caspase cascade at the level of initiator caspase 8^[21-23]. A20 can also protect hepatocytes from TNF-mediated apoptosis^[21,22]. Furthermore, it has been well-established that hepatocytes undergo apoptotic cell death in the course of rejection of a liver graft, and apoptosis is a mechanism of cell death in liver allograft rejection^[45,46]. FasL expression on activated NK cells was augmented^[47,48], and FasL ligation to Fas expressed on hepatocytes could mediate hepatocyte apoptosis^[49,50]. In the present study, high levels of caspase-8 and caspase-1 protein were demonstrated in the liver grafts with chronic dysfunction, and caspase-8 but not caspase-1 production was markedly decreased by A20 treatment. This result was the opposite of the findings presented in the previous study^[5] in which hepatocyte growth factor significantly suppressed the production of caspase-1 but not caspase-8 in liver allografts with chronic dysfunction. However, the A20-induced decrease in hepatic caspase-8 production observed in the present study was consistent with the report by Daniel *et al.*^[23], who demonstrated that A20 could protect ECs from TNF-, Fas-, and NK-mediated cell death by inhibiting caspase-8 activation. Our previous study^[33] also showed a marked down-regulation of the number of LIMCs by A20, including a more prominent decrease in the subproportion of NK and NKT cells in the liver allograft. Thus, we could hypothesise that A20 could inhibit infiltration of LIMC (including NK cell) by suppression of LSEC caspase-8 activation in the liver allograft and consequently attenuate hepatic injury, including acute rejection and chronic dysfunction. In the present study, liver cell (including hepatocyte) apoptosis increased chronic dysfunction progressed, and the apoptosis indices in the PS group and the rAdEasy group on POD 60 were markedly higher than on POD 30 in the same groups. However,

A20 treatment significantly inhibited liver cell apoptosis, and the results showed that the apoptosis index on POD 30 and POD 60 in A20 group were similar.

In summary, A20 could protect the liver allograft from chronic dysfunction, which might be caused by the re-established functional homeostasis of KCs, LSECs and HSCs, as well as the suppressed liver cell apoptosis.

COMMENTS

Background

In spite of the introduction of the use of tacrolimus in liver transplantation recipients, the incidence of chronic liver allograft dysfunction is still high in long-surviving recipients. Chronic liver allograft dysfunction, which results in the loss of approximately 2000 liver grafts every year, has a great impact on liver graft function and long-term survival. The mechanisms of chronic liver allograft dysfunction are still unclear, and there is no effective treatment. The possible factors that induce chronic liver allograft dysfunction include immune and non-immune events.

Research frontiers

Many important events, such as activation of liver graft sinusoidal endothelial cells (LSECs), activation of Kupffer cells and hepatic satellite cells, atherosclerosis, hepatic fibrosis and liver cells' apoptosis through nuclear factor- κ B (NF- κ B) activation, were reproduced in the rat model of chronic liver allograft dysfunction, and the effects of the zinc finger protein A20 on chronic liver allograft dysfunction were investigated, including the effects of A20 on recipient survival, hepatic fibrosis and liver function, NF- κ B activation in LSECs, Kupffer cells and hepatic stellate cells, liver cell apoptosis, and the expression of the related cytokines.

Innovations and breakthroughs

This study sought to elucidate the cellular mechanisms of chronic liver allograft dysfunction. This study also intended to provide a theoretical basis for the use of A20 as a treatment of liver allograft chronic dysfunction.

Applications

The study results suggest that the zinc finger protein A20 may be an effective tool for the prevention and treatment for chronic liver dysfunction after liver transplantation.

Terminology

A20 is a zinc finger protein originally identified as a tumor necrosis factor-responsive gene in endothelial cells (ECs). A20 is expressed in multiple cell types, including fibroblasts, B cells, T cells, and β cells, in response to a variety of stimuli that activate NF- κ B, including interleukin-1, lipopolysaccharides, phorbol 12-myristate 13-acetate, H₂O₂ and CD40 ligand. In ECs and hepatocytes, A20 has a dual cytoprotective function. It has been shown that A20 is a protective gene for the liver.

Peer review

This is an original study in which the authors provide strong evidence demonstrating the important finding that the zinc finger protein A20 might protect liver allografts from chronic dysfunction. These results are easily comprehensible and convincing. Additionally, the study explores the cellular and molecular mechanisms of chronic liver allograft dysfunction.

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Quality audit of colonoscopy reports amongst patients screened or surveilled for colorectal neoplasia

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Abstract

AIM: To complete a quality audit using recently published criteria from the Quality Assurance Task Group of the National Colorectal Cancer Roundtable.

METHODS: Consecutive colonoscopy reports of patients at average/high risk screening, or with a prior colorectal neoplasia (CRN) by endoscopists who perform 11 000 procedures yearly, using a commercial computerized endoscopic report generator. A separate institutional database providing pathological results. Required documentation included patient demographics, history, procedure indications, technical descriptions, colonoscopy findings, interventions, unplanned events, follow-up plans, and pathology results. Reports abstraction employed a standardized glossary with 10% independent data validation. Sample size calculations determined the number of reports needed.

RESULTS: Two hundreds and fifty patients (63.2 ± 10.5 years, female: 42.8%, average risk: 38.5%, personal/family history of CRN: 43.3%/20.2%) were scoped in June 2009 by 8 gastroenterologists and 3 surgeons (mean practice: 17.1 ± 8.5 years). Procedural indication and informed consent were always documented. 14% provided a previous colonoscopy date (past polyp removal information in 25%, but insufficient in most to determine surveillance intervals appropriateness). Most procedural indicators were recorded (exam date: 98.4%, medications: 99.2%, difficulty level: 98.8%, prep quality: 99.6%). All reports noted extent of visualization (cecum: 94.4%, with landmarks noted in 78.8% - photodocumentation: 67.2%). No procedural times were recorded. One hundred and eleven had polyps (44.4%) with anatomic location noted in 99.1%, size in 65.8%, morphology in 62.2%; removal was by cold biopsy in 25.2% (cold snare: 18%, snare cautery: 31.5%, unrecorded: 20.7%), 84.7% were retrieved. Adenomas were noted in 24.8% (advanced adenomas: 7.6%, cancer: 0.4%) in this population with varying previous colonic investigations.

CONCLUSION: This audit reveals lacking reported items, justifying additional research to optimize quality of reporting.

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Key words: Colonic-disorders; Endoscopy-general; Oncology-clinical; Colonoscopy; Endoscopic reporting system

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INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of death from cancer in Canada^[1]. Screening of asymptomatic average-risk persons for this type of cancer is strongly recommended^[2-6]. Colonoscopy is one of the most accurate screening tests for CRC. It is used for primary CRC screening but also for surveillance of patients with prior colorectal neoplasia (CRN), including cancer, and diagnosing patients with lower gastrointestinal (GI)-tract symptoms. The effectiveness and safety of colonoscopy depends, However, on the quality of examination in what is a high-volume procedural setting. A growing body of evidence suggests that the quality of clinical practice varies^[7-12].

In 2007, the Quality Assurance Task Group of the National CRC Roundtable developed a reporting and data system for colonoscopy (CO-RADS) to assist endoscopists in establishing standards that permit the monitoring of quality indicators in their practice. The Quality Assurance Task Group created a standardized reporting system that represents a consensus among experts in gastroenterology, diagnostic radiology, primary care and health care delivery^[2]. A national US study was recently conducted, using the standardized reporting system, and uncovering lacks in the colonoscopy reports. Yet in Canada, to our knowledge, no such initiative has been published to date^[13].

The objective of this study was therefore to assess the level of adherence of a sample of colonoscopy reports from an academic university-based endoscopy unit using the criteria set out by the Quality Assurance Task Group CO-RADS, and to determine reporting of quality indicators with the poorest adherence.

MATERIALS AND METHODS

Patient population

We selected consecutive colonoscopy reports completed from procedures performed in June 2009. We only considered procedures carried out for the screening or surveillance of patients with prior CRN, excluding colonoscopy reports completed for other reported indications.

Electronic reporting system and institutional database

The Montreal General Hospital site of the McGill University Health Centre (MUHC-MGH) is a tertiary care institution with a 4-room endoscopy unit staffed by 12 medical and surgical endoscopists. Patients can access the services of the unit both through a same-day consultation and procedural critical path of care at the request of a referring physician providing screened information, or on a subsequent date, after the specialist endoscopist has initially assessed the patient in the office. On average, 11 000 procedures are performed at the MUHC-MGH per year, of which 75% were colonoscopies in 2008. Average waiting time between the indication of the colonoscopy and the colonoscopy is currently around 2-3 mo. All patients receive an information sheet on the procedure

prior to colonoscopy and consent is obtained by the endoscopist. Patients also receive written instructions after the colonoscopy is performed.

The unit is equipped with a structured, computerized endoscopic report generator allowing for image and video capture (Endoworks, Olympus Corporation, Center Valley, PA, United States). It is used for all cases performed during and outside regular hours by all endoscopists. The data file from the report is electronically transmitted to a central data repository housed at the MUHC-MGH. The information is then securely locked in an MUHC Endoworks database.

The routine colonoscopy report at the MUHC-MGH endoscopy unit includes some compulsory fields, default population of certain fields included in the final report for which the endoscopist needs to approve or choose alternatives, drop down menus for selecting other components of the report, and data acquisition fields for free text entries. Endoscopists were not aware we would be carrying out the audit at the time the reports were entered in Endoworks. Any *post-hoc* amendment of a report can be identified through a review of the electronic log entries.

We also accessed pathology results from an institutional electronic medical file software (OACIS, Telus, Vancouver, Canada) which is not part of Endoworks as the current practice is not to link directly the pathology results as part of the actual colonoscopy report. These latter data provided us with the prevalences of adenomas, advanced adenomas, and cancer detection rate.

Quality indicators

Based upon continuous quality improvement indicators established by the Quality Assurance Task Group of the National CRC Roundtable^[2], we developed a specific list of quality indicators (Table 1) that should be explicitly addressed in the colonoscopy reports, and made available to the referring physician. Unplanned interventions for adverse events included only those interventions that were reported at the time of colonoscopy since no specific mechanism or manpower support currently exists at the MUHC-MGH digestive endoscopy unit to allow for the reliable capture of downstream adverse events once the patient has returned home.

Data collection

The current study is a retrospective review of all consecutive eligible reports using a standardized checklist developed using the Quality Assurance Task Group of the National CRC Roundtable publication^[2]. We dichotomized screened patients into those for whom the indication for colonoscopy was average or increased-risk (patients with a family or personal history of CRC or polyps). Data were compiled and individually analyzed by a trained research assistant using a specially developed electronic data abstraction form. Using a standardized glossary of study variables, 10% of all entered data was reviewed by an independent observer and validated.

Table 1 Colonoscopy quality indicators

Patient demographics and history
Age
Sex
MRN
Management plans
Informed consent documentation
Previous GI procedures: documented date (yes/no)
Documentation of ASA classification
Indications for procedure
Average risk
Increased risk
Incomplete colonoscopy
Post adenoma resection
Procedure: Technical description
Date and time
Sedation
Level of difficulty of the procedure
Bowel preparation
Type and dosage
Quality
Actual extent of examination
Cecal intubation (yes/no)
Documentation of cecal landmarks
Appendiceal orifice
Ileocecal valve
Total and withdrawal time recorded (yes/no)
Colonoscopic findings
Colonic polyp(s):
Number
Size
Morphology
Morphology anatomic location
Method of removal
Completeness of removal (yes/no)
Retrieved (yes/no)
Sent to pathology (yes/no)
Interventions/unplanned events
Unplanned interventions and complications
Documentation of discharge plans (info to patient, info to referring MD)
Pathology
Documentation of pathology results to the patient and the physician
Adenoma detection (yes/no)
Cancer detection (yes/no)

MRN: Medical record number; ASA: American Society of Anesthesiology; GI: Gastrointestinal.

Statistical analysis

The sample size was based on a preliminary analysis of the first 111 consecutive reports. The widest point estimate for presence (or absence) of documentation of a quality indicator was for that of polyp removal (51.1%; 95% CI: 35.9%-63.3%). We estimated the number of reviewed reports, needed to narrow the range of uncertainty around this point estimate to 10%. Assuming an identical projected point estimate of 51%, we calculated that we would need to audit 250 scope reports to narrow a 95% CI down to 45.5%-55.8%. We therefore completed the audit up to this consecutive number of patients.

Descriptive variables are presented as means and standard deviations for continuous variables and proportions with 95% confidence intervals for categorical variables. All analyses were performed by using SAS software version 9.1 (SAS Institute Inc, Cary, NC, United States).

Table 2 Patient population and endoscopists description *n* (%)

	Patients (<i>n</i> = 250) endoscopists (<i>n</i> = 11)
Mean age (yr)	63.2 ± 10.5
Sex	
Women	107 (42.8)
Men	143 (57.2)
Procedure indications	
Average risk	85 (38.5)
Past personal history	90 (43.3)
Past family history	42 (20.2)
HNPCC	1 (1.4)
FAP	1 (1.4)
Specialty of endoscopists	
Surgical	3 (27.3)
GI	8 (72.7)
Average years of endoscopists practice (yr)	17.1 ± 8.5

HNPCC: Hereditary nonpolyposis colorectal cancer syndrome; FAP: Familial adenomatous polyposis; GI: Gastrointestinal.

RESULTS

From June 1st to June 30th 2009, 250 reports on 250 consecutive patients were audited for the frequency of reporting of patient demographics and history, procedure indications, technical descriptions, colonoscopy findings, interventions, unplanned events, follow-up plan, and we reviewed the corresponding histological information. These 250 colonoscopy reports were reported by 11 different physicians including 2 colorectal surgeons, 1 general surgeon, and 8 gastroenterologists. Not all endoscopists were included since they do not all perform screening colonoscopies. The average number of years of practice of the 11 endoscopists was 17.1 ± 8.5 years.

Patient demographics and endoscopists' description

The overall patient population and endoscopists' description of the reports are presented in Table 2. The mean age of the patient population was 63.2 ± 10.5 years with 42.8% of the patients being women. The procedure indication pertaining to the risk of the patient was indicated in every report. Overall, 38.5% of examinations were performed on average risk individuals, 43.3% of patients had a past personal history of prior CRN, while 20.2% of patients had first-degree relatives with CRC or a CR adenoma. Only one patient had a hereditary nonpolyposis CRC syndrome, while another had familial adenomatous polyposis.

Pre-procedure indicators

The American Society of Anesthesiology (ASA) classification field was not completed in any of the reports. The documentation of informed consent was noted in all reports. Overall, 9.6% of patients had had previous colonoscopies, but the date of the prior examination was only noted in 14% of reports with details about previous polyp resection in 25%. In most cases, the colonoscopy report lacked sufficient information to determine wheth-

Table 3 Pre-procedure indicators *n* (%)

Quality indicator sought in the report	(<i>n</i> = 250)
Consent documentation	250 (100.0)
Management plan for anticoagulation ¹	1 (0.5; 95% CI: 0.0-1.5)
Previous GI colonoscopy date ¹	24 (14.0; 95% CI: 8.7-19.2)
ASA classification	0 (0)
Previous polyp resection	20 (12.7; 95% CI: 7.4-17.9)
Details available	5 (25.0; 95% CI: 4.2-45.8)
1-2 tubular adenoma < 1 cm	2 (33.3; 95% CI: 0.0-87.5)
3-10 tubular adenoma > 1 cm	1 (16.7; 95% CI: 0.0-59.9)
10 adenomas	1 (16.7; 95% CI: 0.0-59.9)
Sessile adenoma > 2 cm	1 (16.7; 95% CI: 0.0-59.9)

¹Usually documented elsewhere, but not in the endoscopy report. ASA: American Society of Anesthesiology; GI: Gastrointestinal.

er the surveillance interval respected published guidelines (Table 3).

Procedural indicators

The date of the examination was recorded in 98.4% of reports. Administered medications and dosage were indicated in 99.2%, while the level of difficulty of the procedure was reported in 98.8%. The quality of the bowel preparation was not recorded in 0.1% of the reports. When reported, the quality was described as good in 85.1%, fair in 10.8% and poor in 4%. All reports included information about cecal intubation. The cecum was reached in 94.4% of examinations, while cecal landmarks (appendiceal orifice and/or ileocecal valve) were noted in 78.8% when the cecum had been reached. Photo-documentation was present in 67.2% of reports. Retroflexion in the rectum was performed in 70.8% of procedures. Total procedural and withdrawal times were never recorded. Intra-procedural complications were reported in 0.4% (Table 4).

Colonoscopic findings

Polyps were found in 111 procedures (44.4%). Amongst all patients with polyps, polyp size was recorded in 65.8%, and morphology in 62.2%. The mean polyp size was 17.6 ± 33.1 mm. The anatomic location of the polyp(s) was documented in 99.1%. The method of polyp removal was not mentioned in 20.7% of the reports. When specified, 25.2% of the polyps were removed by cold biopsy, 18% by cold snare, and 31.5% using hot snare cautery. Eighty-four point seven percent of all polyps were retrieved and 76.5% were sent to pathology (Table 4).

Of all retrieved polyps (44.4% of all patients), 70% were adenomas (24.8% of all patients), 21% (7.6% of all patients) were advanced adenomas, and 1% (0.4% of all patients) were cancerous.

Post-procedural indication

Ninety-nine point six percent of all endoscopy reports included documentation of discharge plans. Although documented elsewhere, none of the reports included post-discharge precautions to patients nor the documentation of pathology.

Table 4 Procedural indicators and colonoscopic findings *n* (%)

Procedural indicators	<i>n</i> = 250
Quality indicator sought in the report	
Date of exam	246 (98.4, 95% CI: 96.8-100.0)
Medications with dosage	248 (99.2, 95% CI: 98.1-100.0)
Level of difficulty	247 (98.8, 95% CI: 97.4-100.0)
Bowel preparation quality	
Poor	10 (4.0, 95% CI: 1.6-6.5)
Fair	27 (10.8, 95% CI: 7.0-14.7)
Good	212 (85.1, 95% CI: 80.7-89.6)
Actual extent of examination	
Cecum	236 (94.4, 95% CI: 91.7-97.3)
Ascending colon	6 (2.4, 95% CI: 0.5-4.3)
Transverse colon	2 (0.8, 95% CI: 0.0-1.9)
Descending colon	2 (0.8, 95% CI: 0.0-1.9)
Recto sigmoid	4 (1.6, 95% CI: 0.0-3.2)
Cecal intubation	236 (94.4, 95% CI: 91.5-97.3)
Photodocumentation	186 (74.4, 95% CI: 69.0-79.8)
Documentation of cecal landmarks	
Appendiceal orifice	168 (67.2, 95% CI: 61.2-73.2)
Ileocecal valve	103 (41.2, 95% CI: 35.1-73.1)
Retroflexion in rectum	177 (70.8, 95% CI: 65.1-76.5)
Withdrawal time	0 (0)
Total time	0 (0)
Intra-procedural complications	1 (0.4, 95% CI: 0.0-1.2)
Colonoscopic findings: polyps	
Polyp findings	111 (44.4, 95% CI: 38.2-50.6)
Mean polyp number	2.2 ± 2.5
Polyp size documented	73 (65.8, 95% CI: 56.8-74.7)
Mean polyp size (mm)	17.6 ± 33.1
Morphology	
Documented	69 (62.2, 95% CI: 53.0-71.3)
Pedunculated	17 (23.9, 95% CI: 13.8-34.1)
Sessile	56 (80.0, 95% CI: 70.4-89.6)
Anatomic location documented	110 (99.1, 95% CI: 97.3-100.0)
Method of removal	
Cold biopsy	28 (25.2, 95% CI: 17.0-33.4)
Cold snare	20 (18.0, 95% CI: 10.8-25.3)
Snare cautery	35 (31.5, 95% CI: 22.8-40.3)
Not mentioned	23 (20.7, 95% CI: 13.1-28.4)
Retrieved	72 (84.7, 95% CI: 76.9-92.5)
Sent to pathology	85 (76.5, 95% CI: 67.3-85.7)

DISCUSSION

The effectiveness of colonoscopy in reducing cancer prevalence cannot be improved if procedural reports do not include critical quality indicators to track performance in colonoscopy. In other words, the potential benefits of colonoscopy depend on the quality of the examination^[14], and thereby its reporting. The final version of the Standardized Colonoscopy Report includes important elements that can be measured in diverse clinical practice settings. Patient demographics and history, assessment of patient risk and comorbidities, procedure indications, procedure technical description, colonoscopy findings, assessment, interventions and unplanned events, follow-up plan, and pathology are the main variables proposed by the Standardized Colonoscopy Report established by the Quality Assurance Task Group of the National CRC Roundtable, requiring recording^[2].

The current study revealed that even with a computerized endoscopic report generator, some key quality fields were lacking, some often. Several of these fields are

Table 5 Comparison between national United States study and the current audit

	%	Current audit (%)
Patient characteristics		
Women	49	42.8, 95% CI: 36.6-49.0
Men	51	57.2, 95% CI: 51.0-63.4
Average risk	29.6	38.5, 95% CI: 32.0-44.9
Past family history	13.4	20.2, 95% CI: 14.7-25.7
Past personal history	19	43.3, 95% CI: 36.5-50.1
Presence of recorded variables		
ASA classification	89.9	0
Bowel preparation quality	86.1	99.6, 95% CI: 98.8-100.0
Previous GI colonoscopy date	33.9	14.0, 95% CI: 8.7-19.2
Cecal landmarks	85.9	67.2, 95% CI: 61.3-73.1
Polyp size	90	65.8, 95% CI: 56.8-74.7
Polyp morphology	85.3	62.2, 95% CI: 53.0-71.3
Polyp retrieval	95.5	84.7, 95% CI: 76.9-92.5
Endoscopic outcomes		
Cecal intubation	96.3	94.4, 95% CI: 91.5-97.3
Polyp findings	36.3	44.4, 95% CI: 38.2-50.6

ASA: American Society of Anesthesiology; GI: Gastrointestinal.

important in determining the quality of the examination including photo documentation of cecal landmarks present in only (67.2%; 95% CI: 61.3%-73.1%). Of course, the absence of these data does not allow us, to infer about a poor examination quality, but makes its tracking difficult, and even impossible for certain aspects. Assuming all past procedures were indicated, the current reports should include documentation of the prior colonoscopy examinations and their findings. In most cases, we found this documentation lacking (86%; 95% CI: 81.5%-90.5%), and, therefore, it was often not possible to determine the appropriateness of the screening interval. Additional important missing information in the report included historical data which, according to current local practice, may be present in a separate consultation report. Nonetheless, the Quality Assurance Task Group of the National CRC Roundtable has mandated that, to facilitate adequate benchmarking, this information should be found in the endoscopic report.

The omission of key polyp descriptors like polyp size absent in (34.2%; 95% CI: 25.3%-43.2%), the number of polyps found, and the morphology lacking in (37.8%; 95% CI: 28.7%-47.0%) of reports can impact subsequent decisions on surveillance colonoscopy intervals^[15], although a more accurate determination of polyp size is available from the histological reports, when available. This information should eventually find its way back to the endoscopy report for benchmarking purposes of endoscopists and for good clinical practice to ensure a copy is sent to the referring physician^[16]. Here too, these data may have been documented in a separate follow-up form. However once again, Lieberman *et al.*^[2] have suggested that these data be present in the actual (follow-up) endoscopy report. Indeed, any subsequent quality initiative would otherwise be limited with various pieces of information being present in different places-i.e., not all documented in the electronic report. Furthermore,

if the documentation of whether the polyp was sent to pathology or not was omitted in many (23.5%; 95% CI: 14.3%-32.7%) of examinations, and affects the immediate patient care, that could lead to risks of undiagnosed cancers if this information is not efficiently retrieved and integrated in overall management. We also had no way of validating whether post-procedural complications were noted without reviewing a patient's file (and even then, such information may be lacking) These data should also find their way back to the endoscopy report. Perhaps data cross links or integrated data management will help future enhancement of reporting quality across pre, intra- and post-procedural domains of quality reporting.

Procedure durations (withdrawal and total times) were never recorded. Although, somewhat of a controversial subject, there is evidence that there exists a significant correlation between withdrawal time and adenoma detection rates^[15,17]. It is thus recommended as a quality indicator and has been found useful in previous audits^[7].

We noted other lacks in reporting of selected variables which are recommended but do not directly impact examination quality including the ASA classification which was absent in all reports in this audit. Although, this indicator does not reflect examination quality, it can be an important surrogate of co-morbidity^[18], and better defines the screened population, aiding the explanation of possible subsequent morbidity and the interpretation of medications dosing and the interpretation of reported patient satisfaction.

Another controversial variable was retroflexion in the rectum (performed in 70.8% of examinations); it provides additional data that can be added to complete an accurate colonoscopy report, and its recording may be useful either in demonstrating its need in identifying pathology; it remains a controversial quality indicator^[12,13].

A number of the variables were recorded in the great majority of the reports such as the date of the exam (98.4%; 95% CI: 96.8%-100.0%), used medications with dosage (99.2%; 95% CI: 98.1%-100.0%), the level of difficulty of the procedure (98.8%; 95% CI: 97.4%-100.0%) and the bowel preparation quality. The compulsory nature of some fields, pre-formatted text, and drop-down menus in the electronic reporting system no doubt participated in this high level of reporting, and should serve to guide improvement in areas of in which the recording of variables was lacking. Indeed, these fields likely need to be developed for other variables which are less frequently reported yet needed.

We compared the findings of the current study with the one conducted by Lieberman *et al.*^[13]. The study using a national CORI database^[13] included 73 US gastroenterology practice sites, and 43 8521 reports. Some differences in patient characteristics existed (Table 5). Quality outcomes from the procedure were similar or superior in the Canadian study polyp detection rate (44.4% vs 36.3%; 95% CI: 38.2%-50.6%), while the documentation of patient or procedural variables was poorer in many instances [such as for ASA classification, withdrawal times, previous colonoscopy date, photo-documentation of

cecal landmarks, and polyp description, (Table 5)]. They also used the proportion of patients with polyp(s) > 9 mm or with suspected malignant tumour as a surrogate end point for advanced neoplasia.

Although not our primary aim, this quality initiative also allowed us to benchmark the quality of the colonoscopies performed in this successive sample, and compare them to established consensus thresholds. In total, 111 polyps were found (44.4%). The adenoma detection rate was of (24.8%; 95% CI: 19.4%-30.2%) which suggests that even in this population with a varying colonoscopy screening history, adenoma pick-up rates were excellent, since respective recommended thresholds are > 25% in men older than 50% and > 15% in women according to current recommendations by the United States Multi-Society Task Force on CRC^[7,15] and a recent meta-analysis^[19]. Furthermore, the recommended benchmark for cecal intubation rate is 95% which is comparable to the cecal intubation rate achieved in this study (94.4%; 95% CI: 91.5%-97.3%)^[7,15,20,21]. These recommendations are part of a series of recent studies published in the world literature aimed at improving the quality of colonoscopy^[22-24] in an attempt to optimize patient outcomes in CRC screening^[25].

In summary, the overall quality of the reports was good (considering the location of reported information pre- and post-procedures), although not optimal. Indeed, the MUHC-MGH group appears to perform within the threshold set by the Quality Assurance Task Group of the National CRC Roundtable^[2] for most indicators, although improvement is required for some documentation (for e.g., ASA score, and withdrawal time). It is now imperative to continue to improve the appropriate use of the reporting system and revise the user-interface of the software accordingly to optimize the quality of colonoscopies and CRC screening care. Moreover, further improvements are needed in linking databases for optimal consolidation of information on past procedures, post-procedural complications, and pathology results such that they all appear in a single report that can be provided to referring physicians and patients.

COMMENTS

Background

Colonoscopic quality is critical in colorectal cancer screening. Formal quality assessments of colonoscopy reporting are few. The authors completed a quality review according to criteria of the Quality Assurance Task Group of the National Colorectal Cancer (CRC) Roundtable.

Research frontiers

Prospective studies assessing the completeness of colonoscopic reporting for CRC screening are few in the literature.

Innovations and breakthroughs

The authors audited reports of 250 patients (63.2 ± 10.5 years, 42.8% female) scoped in June 2009 by 8 gastrointestinal and 3 surgeons (mean practice years = 15.3). While some quality indicators were routinely reported, others were systematically lacking.

Applications

Modification of the electronic reporting software for colonoscopy reporting is required to optimize quality indicator reporting.

Peer review

This is a good descriptive study in which authors complete a quality audit using

recently published criteria from the Quality Assurance Task Group of the National CRC Roundtable. The results are interesting and suggest two hundreds and fifty patients were scoped in June 2009 by 8 gastroenterologists and 3 surgeons.

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Natural orifice transluminal endoscopic surgery vs laparoscopic ovariectomy: Complications and inflammatory response

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Abstract

AIM: To compare natural orifice transluminal endoscopic surgery (NOTES) vs standard laparoscopic ovariectomy in mini pigs with respect to technical aspects, complications and parameters of systemic inflammatory response.

METHODS: This was a randomized, experimental,

survival study. Ten female mini pigs underwent NOTES transgastric ovariectomy (NOTES group) and ten female mini pigs underwent laparoscopic ovariectomy (LAP group). A "percutaneous endoscopic gastrotomy" approach with guidewire and sphincterotome was used for gastrotomy creation. The ovary was resected using standard biopsy forceps and a snare. The access site was closed using a "KING" closure with a single endoloop and several clips. In the laparoscopic group, a three-port laparoscopy and an ovariectomy were performed with the use of standard laparoscopic devices. C-reactive protein (CRP), white blood count and interleukin (IL)-6 plasma levels were used as indicators of systemic inflammatory response. All animals were euthanized 28 d after surgery.

RESULTS: All animals survived without complications. The mean procedure time was 41.3 min \pm 17.6 min (NOTES group) and 25.7 min \pm 5.25 min (LAP group, $P < 0.02$). Postmortem examinations demonstrated that 50% and 70% of animals were free of any complications in the NOTES and LAP groups, respectively. The remaining animals developed minor complications (adhesions) in a comparable frequency between the two groups. In the NOTES group, one animal developed a small intramural gastric abscess close to the gastrotomy site. A minor serous exudate that was present in 50% and 40% of the animals in the NOTES and laparoscopy groups, respectively, was not considered a complication. In both groups CRP levels increased significantly on the 2nd and 7th postoperative days (POD) and returned to normal after 28 d. On POD 2, an increase of CRP level was significantly higher in the NOTES group compared to the LAP group. Values of IL-6 did not differ from baseline values in either of the groups postoperatively. Interestingly, the platelet count decreased significantly on POD 2, but returned close to baseline values on POD 7 and PODs 28-30.

CONCLUSION: Both NOTES and laparoscopic ovariec-

tomies had a similar frequency of minor complications. However, the NOTES technique produced an increased systemic inflammatory response on POD 2.

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Key words: Natural orifice transluminal endoscopic surgery; Laparoscopy; Ovariectomy; Systemic inflammatory response

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INTRODUCTION

Natural orifice transluminal endoscopic surgery (NOTES) is a surgical procedure that enables intraperitoneal surgical interventions with the elimination of abdominal wall incisions. The potential benefits of NOTES include no remnant scars and obviating the possibility of abdominal wall infection and hernia formation. Although several NOTES procedures have already been performed in humans^[1], NOTES still remains an experimental surgical technique. Since laparoscopy serves as a golden standard for mini-invasive surgery, NOTES should be compared to laparoscopy in all imaginable aspects. If NOTES is to be used in the future, it must at least reach standards comparable to the existing procedures.

Several small experimental studies have compared NOTES with laparoscopic procedures. These included peritoneoscopy, transvaginal cholecystectomy, colon injury repair and distal pancreatectomy^[2-6]. NOTES procedures were feasible but they usually lasted longer. Most studies found a comparable frequency of abdominal complications. Only a few studies tested the differences in systemic inflammatory response, however, no finding has ever suggested a clear difference between NOTES and laparoscopy^[3,4,7]. Only one study found a potentially significant relative thrombocytopenia in NOTES as compared to laparoscopy during a simple peritoneoscopy^[8].

The aim of our randomized experimental survival study was to compare both the outcome criteria (mortality, morbidity and complications such as peritonitis or adhesion formations) and indicators of postoperative systemic inflammatory response and platelet count in NOTES and laparoscopic ovariectomy in mini pigs. There is currently no data available comparing laparoscopic and NOTES

experimental ovariectomy. In addition, ovariectomy can serve as a model for appendectomy from a technical point of view since pigs do not have an appendix. The main working hypothesis was the non-inferiority of NOTES to standard laparoscopic procedures.

MATERIALS AND METHODS

Twenty female domestic mini pigs weighing between 10-31 kg were used for this study. Ten animals underwent a NOTES procedure and 10 animals underwent a laparoscopic procedure. The animals were randomly assigned to one of the two groups using a block randomization allowing an equal number of animals in each group.

Before the procedure, the animals were fed with a liquid diet for 2 d and consequently left fasting overnight. Premedication with Ketamine 10 mg/kg (Narkamon 1%, SPOFA, Czech Republic) and Atropine 0.2 mg (Atropine Biotica 0.5 mg, BB Pharma, Czech Republic) was given IM 30 min before the procedure. Oral intubation of the mini pigs was performed, a marginal ear vein cannula was placed and anesthesia was maintained using 1.5% isoflurane and fentanyl (3-5 mL/h). Antibiotics were administered neither before nor after the procedure.

The protocol was approved by The Committee for the Protection of Animals of The Czech Academy of Sciences and the experiment was performed in accordance with Act No. 246/1992.

Natural orifice transluminal endoscopic surgery procedure

All procedures were performed with a double-channel endoscope (GIF 2T160; Olympus Medical Co., Tokyo, Japan). The percutaneous endoscopic gastrostomy was used to gain access into the peritoneal cavity^[9]. After transillumination, the stomach was punctured with a needle and the guidewire (Jagwire; Boston Scientific, Natick, Mass) was introduced through the needle into the stomach. The guidewire was then locked-in with a snare and pulled out through the endoscope. Consequently, the sphincterotome (KD-V411M-0330 Papillotome, Olympus Medical Co.) was introduced with the guidewire to the gastric wall. A gastric wall incision measuring approximately 15-20 mm was then performed with a tightened sphincterotome, and the endoscope was advanced into the peritoneal cavity. Afterwards, a brief exploration of the peritoneal cavity was performed with the endoscope.

Air was used for moderate insufflation, but intraperitoneal pressure was not measured during the procedure.

The ovary was then exposed and resected using both standard biopsy forceps and a snare with a coagulation current.

The endoscope with a resected ovary using a tightened snare was then pulled back to the stomach and the peritoneal cavity was completely desufflated. The desufflation was possible without reintroducing the scope into the abdominal cavity since a double-channel endoscope was used. After the ovary was withdrawn from the mouth, the endoscope was reintroduced for the gastric wall closure.

The gastric wall closure was performed with one en-

doloop (HX-400U-30 Aset PolyLoop Colo5; Olympus Medical Co.) and 4-5 clips. This technique provides a reliable full-thickness closure^[10]. An open endoloop with a diameter of 30 mm (MAJ-340, Olympus Medical Co.) was fixed around the gastrotomy site with 4-5 clips (HX-610-90L, Olympus Medical Co.). A big endoscopic grasper (Olympus FG-48L, 026235, Olympus Medical Co.) was then advanced through an open endoloop. Both edges of the incision were grasped and pulled through the endoloop toward the endoscope. Subsequently, the endoloop was closed and released.

Laparoscopic procedure

The laparoscopic group consisted of ten animals undergoing a standard three-trocar laparoscopic ovariectomy. After the induction of anesthesia, a pneumoperitoneum was established with CO₂ using a standard laparoscopic insufflator. The insufflation pressure was set to 10 mmHg and gas flow was initiated. After pneumoperitoneum was established, the intra-abdominal pressure was monitored throughout the procedure. After the exposure of the fallopian tube, the ovary was resected using scissors with a monopolar coagulation current, and the ovary was grasped and withdrawn using grasping forceps. The pneumoperitoneum was evacuated and all skin incisions were closed with a single absorbable suture.

Postoperative period and blood drawing

All pigs recovered well after extubation and were placed in an animal facility unit where they were monitored twice a day for any signs of complications, whether in feeding or in general well-being. All pigs resumed pig chow on the first postoperative day. Their survival was assessed on the 28-30th postoperative day, at which point the animals were euthanized and a necropsy was performed. The adhesions were assessed using the Adhesion Scoring Group system (none; minor = avascular, flimsy; major = dense and/or vascular or cohesive)^[11].

Blood samples for the blood count, C-reactive protein (CRP)-levels, and interleukin 6 (IL-6) levels were taken before the procedure (15-60 min) and on days 2, 7 and 28-30 after the procedure. The blood count was analyzed immediately using a fully automatic hematology analyzer (Scil ABC Vet 16p, ABX Diagnostics, France). Blood samples for CRP and IL-6 analyses were centrifuged and plasma kept frozen (-80 °C) until the final analysis.

Analysis of C-reactive protein and IL-6

Both CRP and IL-6 were analyzed in duplicate with one control. A commercially available enzyme-linked immunosorbent assay kit Quantikine Porcine IL-6 Immunoassay (R and D Systems, Inc.) was used to measure IL-6 levels. The dynamic range of the assay was 39.1-2500 ng/L. An Immunoperoxidase Assay for Determination of C-Reactive Protein in Pig Samples (Gen Way Biotech, Inc.) was used for CRP level assessment. Its dynamic range was 6.25-200 µg/L. An analysis was performed according to the manufacturer's instructions by a researcher blinded to the operative procedure.

Table 1 Necropsy findings 4 wk after the procedure *n* (%)

	Laparoscopy	Notes	
Adhesions	3 (30)	4 (40)	NS
Abscess	0	1 (10) ¹	NS
Peritonitis	0	0	NS
Pigs without any complication	7 (70)	5 (50)	NS

¹Small intramural abscess (8 mm) at the healed gastrotomy site. NS: Not significant.

Statistical analysis

For descriptive statistics, the data is presented as median values with appropriate percentiles. As all variables confirmed the normality and equal variance assumption, a Student *t*-test was performed for testing the differences between and within the groups. Binary variables (e.g., presence or absence of complications) were assessed with the Fisher exact test. Statistical significance was defined as a *P*-value of less than 0.05 and all *P*-values were two-sided. For multiple comparisons, a Bonferroni correction was used.

RESULTS

Procedure

Access into the peritoneal cavity was gained within 3-7 min in all animals in the NOTES group. A peritoneoscopy did not show any injuries to adjacent organs. Seven right-sided and three left-sided ovariectomies were performed without major technical problems. All gastrotomies were successfully closed using the technique described above.

All laparoscopies were performed without major technical problems. Nine right-sided and one left-sided ovariectomies were performed.

The NOTES procedure took significantly longer than the laparoscopy procedure - medians were 42.5 min [interquartile (IQ) range: 30-45 min] vs 25 min (IQ range: 25-29 min), *P* = 0.02.

Survival and complications

All animals had lived and had been fed normally without any clinical signs suggesting complications until they were euthanized. They gained the desirable weight after the procedure. At necropsy no signs of organ damage or peritonitis were discovered in either group. The gastrotomy site healed completely in all pigs. Only minor complications were found (minor adhesions and one small abscess) in similar frequencies in both groups (Table 1). A minor serous exudate that was present in 50% and 40% of the animals in the NOTES and laparoscopy groups, respectively, was not considered a complication.

Systemic inflammatory response and other laboratory parameters

White blood count and platelets: White blood count (WBC) had a tendency to increase after the procedure (on days 2 and 7 after the procedure) and to return to base-

Table 2 Parameters of systemic inflammatory response

	NOTES (<i>n</i> = 10)				Laparoscopy (<i>n</i> = 10)			
	Before	POD 2	POD 7	PODs 28-30	Before	POD 2	POD 7	PODs 28-30
CRP (mg/L)	14.5 (12-17)	52.6 (47-73) ^{abc}	51.8 (31-88) ^{abc}	22.1 (14-38)	16.1 (11-35)	37.1 (23-70) ^c	51.4 (33-55) ^c	20 (16-31)
WBC ($\times 10^9$ /L)	14.9 (14-17)	19.6 (15-21)	19.7 (17-23) ^a	15.3 (15-20)	18.3 (14-23)	23.5 (19-24) ^c	20 (18-26) ^c	15.4 (14-18)
IL-6 (ng/L)	108 (105-118)	115 (110-121)	110 (100-123)	104 (97-129)	92 (79-117)	103.5 (90-146)	98.6 (85-178)	111 (67-130)
Platelets ($\times 10^9$ /L)	414 (400-465)	314 (261-351) ^{abc}	366 (336-442)	381 (325-479)	424 (313-540)	273 (253-412) ^{abc}	433 (381-641)	389 (382-498)

Data is median with interquartile range. ^a*P* < 0.05 vs before the procedure; ^c*P* < 0.05 vs POD 28-30; ^b*P* < 0.05 vs post-operative day (POD) 7 and PODs 28-30, no significant differences between the groups [natural orifice transluminal endoscopic surgery (NOTES) vs laparoscopy]. CRP: C-reactive protein; WBC: White blood count; IL-6: Interleukin 6.

Table 3 Diversion ratios from preoperative values

	POD 2		POD 7		POD 28	
	NOTES	Laparoscopy	NOTES	Laparoscopy	NOTES	Laparoscopy
CRP (%)	288 (207-357)	64 (4-172) ^a	154 (70-436)	172 (60-273)	31.6 (3-193)	25 [(-57)-76]
WBC (%)	16 (0.3-43)	21 [(-6)-66]	34 (13-42)	33 [(-8)-60]	8 [(-0.3)-26]	-15 [(-23)-10]
IL-6 (%)	3.7 (1.6-9)	6 (3-15)	-1.6 [(-8)-0.9]	6.6 (0.9-19)	-9.8 [(-21)-(-1.6)]	-6.7 [(-21)-44]
Platelets (%)	-23.5 [(-24)-(-13)]	-17.6 [(-35)-(-10)]	1.5 [(-13)-19]	21 (9-30)	2 [(-11)-7]	3 [(-2)-12]

Diversion rate (%): (Postoperative value-preoperative value/preoperative value) \times 100. Data is median with interquartile range. ^a*P* = 0.01 vs natural orifice transluminal endoscopic surgery (NOTES), no other significant differences between NOTES and laparoscopy groups. POD: Post-operative day; CRP: C-reactive protein; WBC: White blood count; IL-6: Interleukin 6.

line values (on days 28-30) in both groups (Table 2). No significant differences were detected between the groups (Table 3).

CRP: CRP levels increased on days 2 and 7 after the procedure and returned close to the baseline values on day 28-30 (Table 2, Figure 1A). Changes in CRP levels were similar among the two groups on PODs 7 and 28. However, on POD 2, according to the diversion rate analysis, an increase of CRP level was significantly higher in the NOTES group compared to the LAP group (Table 3).

IL-6: No significant changes with respect to IL-6 within the groups or between the groups were discovered (Tables 2 and 3, Figure 1B).

Platelets: The platelet count decreased significantly on POD 2, but it returned close to baseline values on POD 7 and PODs 28-30. The values of platelet count on POD 2 were, however, not dangerously low from a clinical point of view (Tables 2 and 3, Figure 1C).

DISCUSSION

Currently NOTES still represents an experimental surgical technique. If NOTES is to be introduced into clinical practice, it must show advantages in comparison with commonly available procedures. One of the potential advantages of NOTES is a “scar-less” surgery, whilst other real advantages are more or less speculative. Since laparoscopy is a golden standard for several surgical procedures, NOTES should be compared to laparoscopy in different experimental settings.

Given the potential advantages of the NOTES method in comparison with the classical laparoscopic techniques, our survival experimental study compared a standard laparoscopic ovariectomy with a NOTES ovariectomy in a sample of mini pigs. At the moment, there are no studies comparing NOTES and laparoscopic techniques involving ovariectomy.

All procedures were performed without any significant technical problems. The NOTES procedure lasted longer than the laparoscopy. However, the duration of the fastest NOTES procedure was 18 min, which was very close to the time of the fastest laparoscopic ovariectomy (14 min). Most studies comparing NOTES with laparoscopy also showed a better technical feasibility of laparoscopy^[5,6]. This can be explained by the absence of appropriate equipment currently available for NOTES. We believe that an improvement of technical performance would reflect the implementation of new “NOTES” equipment.

Apart from the procedure duration, no significant differences between the two techniques regarding any other outcome criteria were found. All animals survived without any clinical signs of complications. At necropsy only minor adhesions were found in a small number of pigs in both groups. One pig in the NOTES group developed a small intramural abscess next to the gastrotomy site. This complication was most likely related to our otherwise safe gastric-closure technique rather than the NOTES procedure itself. Importantly, there were no signs of peritonitis in either group. Based on this finding, NOTES is not an inferior procedure to laparoscopy with respect to main outcome criteria despite lower aseptic conditions (an endoscope cannot be sterile as opposed to laparoscopic equipment), the absence of antibiotics administration and

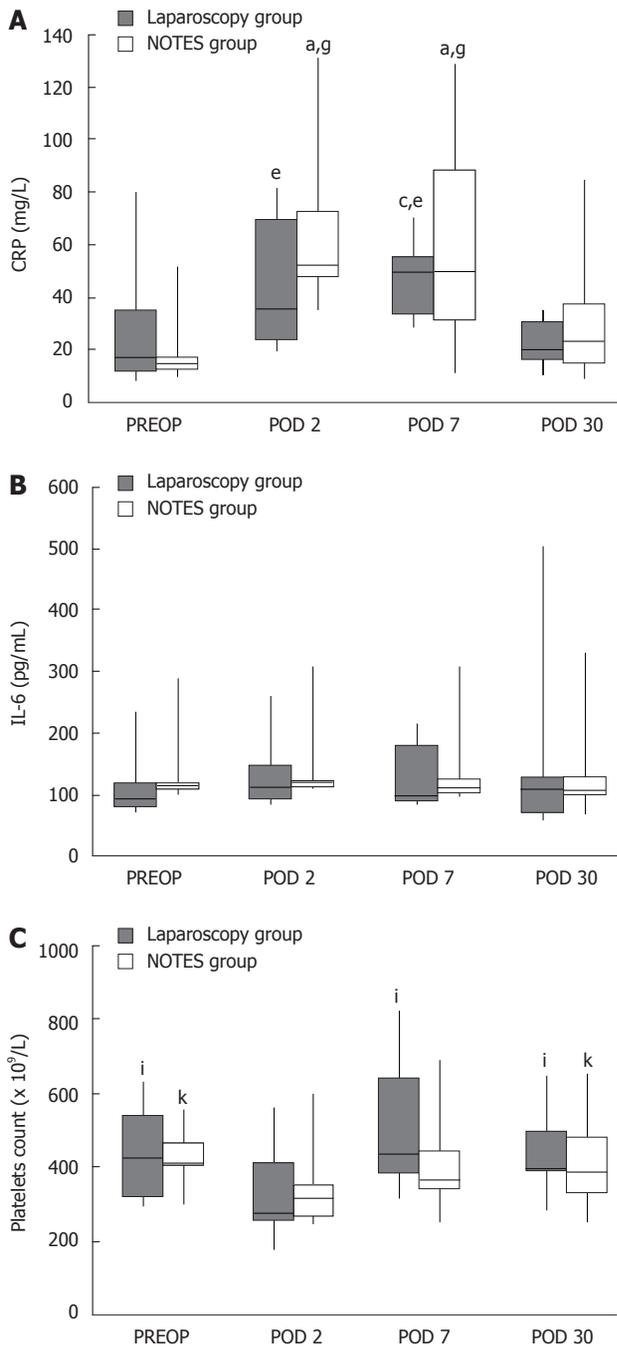


Figure 1 Box-whisker plot of C-reactive protein (A), interleukin 6 (B) and platelets (C) response in Natural Orifice Transluminal Endoscopic Surgery and the laparoscopic group before the procedure and on post-operative days 2, 7 and 28-30. Horizontal bars represent medians, ^a*P* < 0.05 vs before the procedure [natural orifice transluminal endoscopic surgery (NOTES) group]; ^c*P* < 0.05 vs before the procedure [laparoscopy (LAP) group]; ^e*P* < 0.05 vs postoperative days (POD) 30 (LAP group); ^g*P* < 0.05 vs POD 30 (NOTES group); ⁱ*P* < 0.05 vs POD 2 (LAP group); ^k*P* < 0.05 vs POD 2 (NOTES group). IL-6: Interleukin 6.

the absence of disinfection of the gastric lumen. Similar outcome criteria were used in several other studies comparing NOTES with laparoscopy^[2,4-8,12]. A majority of these studies did not show significant differences between the two techniques regarding the frequency of major complications. This involved procedures such as peritoneoscopy^[2,3,7,8], transvaginal cholecystectomy^[4], colon injury

repair^[5], salpingectomy^[12] and distal pancreatectomy^[6].

However, in some cases the occurrence of adhesions or histological peritonitis was higher in NOTES as opposed to laparoscopy^[2,13]. The slightly higher frequency of adhesions or intraperitoneal infections is possibly related to the quality of the access site closure. Safe closure is thus considered a prerequisite for any study comparing NOTES with laparoscopy. For example, von Renteln *et al*^[14] showed a higher frequency of infectious complications, including one fatal case of peritonitis with an incomplete gastrotomy closure using endoclips. In our study a single-loop and clips technique providing full-thickness closure was used.

In order to assess the physiological impact of NOTES compared to laparoscopic surgery, changes in interleukin-6 levels, white-blood cells, platelet count and CRP levels were analyzed. IL-6 has been extensively studied as an indicator demonstrating a lower invasiveness of laparoscopy in comparison to open surgery^[15-17]. IL-6 alterations have been directly correlated to the operation duration^[15]. IL-6 levels culminate 24 h after surgery and its serum level increases later than the serum level of other cytokines (tumor necrosis factor- α and IL-1 β). It also remains longer in circulation. This cytokine was hence chosen for the purpose of our study.

No significant changes with respect to IL-6 values were detected in either group. Furthermore, no increase in IL-6 levels after either procedure was observed. It can be argued that measuring IL-6 levels on POD 2 is too late to detect such an increase. Freeman *et al*^[18] showed a peak serum concentration of IL-6 levels 2 h after NOTES ovariectomy in dogs. IL-6 levels returned to baseline values within 18 h. The possible explanation for this increase is that the procedures lasted an average of 157 min, which is considerably more than in our study. Other studies investigating IL-6 levels showed elevated levels even on POD 5 in a group of patients undergoing laparoscopic colorectal resection or laparoscopic cholecystectomy^[19,20]. On the other hand, Fan *et al*^[4] did not show any significant changes with regard to IL-6 levels after transvaginal cholecystectomy on PODs 1 and 2. Their study, though, was rather small with only 2 pigs in the laparoscopic control group.

We did not assess the peritoneal inflammatory response as measured by IL-6 levels in the peritoneal fluid. It has been demonstrated that the degree of such a response correlated with the extent of adhesions^[2,13]. Since we detected similar occurrence of adhesions in both groups we did not expect to find a differing local inflammatory response.

Several studies have investigated the way in which laparoscopic surgery affects the acute-phase response by assessing CRP. CRP-levels have been found to be elevated in a majority of laparoscopic procedures as late as on POD 14^[21,22]. In agreement with these findings, we observed an increase in CRP-levels on POD 2 and 7 in our laparoscopic group. CRP-levels returned to the baseline values on PODs 28-30. In the NOTES group a quite sim-

ilar behavior in CRP response was found. We detected a significantly higher increase of CRP levels in the NOTES group compared to the LAP group on POD 2; however, on POD 7 this difference was no longer present. This suggests that an inflammatory response in the NOTES procedure is not lower than that in the LAP group.

An increase of WBC after both laparoscopic and NOTES peritoneoscopy on PODs 2 and 7 has been reported^[8], however, no significant changes in WBC were found after the same procedure in another study^[7]. In our study, only an insignificant trend in WBC increase on PODs 2 and 7 was observed in both groups.

Bingener *et al.*^[8] showed relative thrombocytopenia after NOTES peritoneoscopy but not after standard laparoscopy. In contrast, we found a decrease in platelet count in both groups on POD 2. However, the decrease was not clinically significant. The explanation for this phenomenon is unclear. Bingener thought that the possible reason for his finding was heparin-induced thrombocytopenia. In our study no heparin was used (for the flushing of invasive monitoring lines) as in the study conducted by Bingener. It cannot be excluded that thrombocytopenia in the porcine environment occurred due to a bacterial infection^[23]. A possible explanation could also be the Stage II of Systemic Inflammatory Response when small quantities of local cytokines are released into circulation to improve the local response. This leads to a stimulation of the growth factor and the recruitment of macrophages and platelets. We believe that the phenomenon of a decreasing platelet count in the NOTES procedure should be addressed in further studies.

There are several limitations to our study. Primarily, it is a study conducted on animals. The clinical implications and applicability of these findings in human medicine are unclear.

Secondly, the use of an infant animal model in a limited number is also a significant limitation when translating results into practice. The limited number of animals cannot exclude a type II error when interpreting our results. Thirdly, intraperitoneal pressure was not measured systematically and also microbiological contamination was not assessed. Also, for the NOTES procedures we used room air for insufflations, while for laparoscopic procedures we used a standard carbon dioxide insufflation. This could, theoretically, have influenced the results regarding the higher CRP levels in the NOTES group. Finally, IL-6 levels were not measured on the first day after the procedure.

In conclusion, both NOTES ovariectomy and laparoscopic ovariectomy were performed without any major technical problems and both were accompanied by a similar frequency of minor complications. However, the NOTES technique produced an increased systemic inflammatory response on POD 2 in comparison to the laparoscopic technique.

commonly used procedures. Natural orifice transluminal endoscopic surgery (NOTES) is an experimental surgical technique whereby "scar-less" abdominal operations can be performed with an endoscope passing through a natural orifice (mouth, anus, etc.) and then through an internal incision in the stomach or rectum. Although some NOTES procedures have already been performed on humans (e.g., transvaginal cholecystectomy), NOTES remains an experimental method.

Research frontiers

Several NOTES procedures have been examined and studied in experimental studies-NOTES peritoneoscopy, cholecystectomy, colon injury repair, etc. Should NOTES be implemented into clinical practice, it must be compared to the current surgical standards (laparoscopy and open surgery). The authors compared the technical feasibility, complications, and indicators of postoperative systemic inflammatory response in an experimental study.

Innovations and breakthroughs

The authors found that NOTES ovariectomy is not an inferior procedure to the standard laparoscopic ovariectomy in terms of technical feasibility and frequency of complications, although the NOTES procedure lasted significantly longer. The postoperative systemic inflammatory response was mild, almost identical to laparoscopy. However, C-reactive protein increase on postoperative day 2 was significantly higher in the NOTES group as compared to the laparoscopy group.

Applications

The study shows the feasibility of the NOTES procedures in the experimental setting. This is the first research study that compares laparoscopic and NOTES ovariectomy procedures and that provides evidence to the similarity between both of them. In other words, the study shows no inferiority of the NOTES ovariectomy as compared to laparoscopy with regard to the majority of variables.

Peer review

This is a rarity of published works in the literature concerning natural orifice transluminal endoscopic surgery versus laparoscopy. The manuscript is well structured. NOTES is a challenging topic for recent minimal invasive surgery. This experimental study claimed that NOTES had similar effects with laparoscopy for ovariectomy.

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COMMENTS

Background

The methods of laparoscopic and standard open surgery are currently the

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Inhibitory effects of carbon dioxide insufflation on pneumoperitoneum and bowel distension after percutaneous endoscopic gastrostomy

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Abstract

AIM: To evaluate the inhibitory effects of carbon dioxide (CO₂) insufflation on pneumoperitoneum and bowel distension after percutaneous endoscopic gastrostomy (PEG).

METHODS: A total of 73 consecutive patients who were undergoing PEG were enrolled in our study. After eliminating 13 patients who fitted our exclusion criteria, 60 patients were randomly assigned to either CO₂ (30 patients) or air insufflation (30 patients) groups. PEG was performed by pull-through technique after three-point fixation of the gastric wall to the abdominal wall using a gastropexy device. Arterial blood gas analysis was performed immediately before and after the pro-

cedure. Abdominal X-ray was performed at 10 min and at 24 h after PEG to assess the extent of bowel distension. Abdominal computed tomography was performed at 24 h after the procedure to detect the presence of pneumoperitoneum. The outcomes of PEG for 7 d post-procedure were also investigated.

RESULTS: Among 30 patients each for the air and the CO₂ groups, PEG could not be conducted in 2 patients of the CO₂ group, thus they were excluded. Analyses of the remaining 58 patients showed that the patients' backgrounds were not significantly different between the two groups. The elevation values of arterial partial pressure of CO₂ in the air group and the CO₂ group were 2.67 mmHg and 3.32 mmHg, respectively ($P = 0.408$). The evaluation of bowel distension on abdominal X ray revealed a significant decrease of small bowel distension in the CO₂ group compared to the air group ($P < 0.001$) at 10 min and 24 h after PEG, whereas there was no significant difference in large bowel distension between the two groups. Pneumoperitoneum was observed only in the air group but not in the CO₂ group ($P = 0.003$). There were no obvious differences in the laboratory data and clinical outcomes after PEG between the two groups.

CONCLUSION: There was no adverse event associated with CO₂ insufflation. CO₂ insufflation is considered to be safer and more comfortable for PEG patients because of the lower incidence of pneumoperitoneum and less distension of the small bowel.

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Key words: Percutaneous endoscopic gastrostomy; Carbon dioxide insufflation; Pneumoperitoneum; Abdominal distension; Randomized control study

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INTRODUCTION

Percutaneous endoscopic gastrostomy (PEG) has been widely accepted for enteral access since the introduction of the procedure in 1980^[1,2]. The procedure of PEG is rapid, but it requires maximal insufflation of the stomach with air in order to tightly attach the gastric wall to the abdominal wall. Percutaneous puncture into the stomach with a needle is conducted under the fully insufflated stomach and a gastrostomy tube is placed thereafter. Abdominal distension and pneumoperitoneum are frequent symptoms after PEG^[3]. Carbon dioxide (CO₂) insufflation was initially introduced for colonoscopic polypectomy in the field of gastrointestinal endoscopy^[4]. Applications of CO₂ insufflation for endoscopic procedures have also been reported for the performance of routine colonoscopy, small bowel endoscopy, endoscopic retrograde cholangiopancreatography (ERCP), peroral cholangioscopy, and endoscopic submucosal dissection in the upper and lower gastrointestinal tracts^[5-12]. These studies showed that CO₂ insufflation reduces the post-procedural abdominal distension and pain without CO₂ retention and adverse events. However, there has been no report on the safety and efficacy of CO₂ insufflation with PEG procedures. In the present study, we evaluated the inhibitory effects of CO₂ insufflation on bowel distension and pneumoperitoneum after PEG by randomized controlled trial. The safety of CO₂ insufflation was also investigated.

MATERIALS AND METHODS

Participants

Consecutive patients who were scheduled for PEG from November 2009 to March 2011 at our institution were recruited for this study. Exclusion criteria included any of the following: chronic obstructive pulmonary diseases (COPD), severe congestive heart failure (cardiothoracic ratio on chest radiography > 60%), previous upper gastrointestinal surgery, for the purpose of decompression *via* PEG, hypercapnea [arterial partial pressure of CO₂ (PCO₂) > 50 mmHg], and refusal to participate. Randomization was conducted individually into two treatment groups (1:1) using a computer-generated sequence. Sealed envelopes were used for the allocation of individual patients and opened by assistants at the endoscopy unit just before the procedure of PEG. The present study was approved by the Ethics Committee of our institution, and

written informed consent was obtained from the patients or the patients' family members.

Percutaneous endoscopic gastrostomy procedure and carbon dioxide monitoring

CO₂ was administered using a commercially available CO₂ regulator system (UCR, Olympus Optical Co., Ltd., Tokyo, Japan). The flow rate of the CO₂ insufflation was 1.5 L/min using the wide size of the connective tube, the volume of which was equivalent to the medium strength setting of the air insufflation system. Endoscopy assistants set up the insufflation system according to the allocation of the individual patients.

Conscious sedation was conducted by the intravenous administration of midazolam, the amount of which was previously determined by their primary doctor depending on the condition of the patient. An endoscope (GIF-H180, Olympus Optical Co., Ltd., Tokyo, Japan) was inserted up to the second portion of the duodenum to screen the upper gastrointestinal tract and pulled back to the stomach. Then, the stomach was fully inflated with air or CO₂ and the site for placement was determined by transillumination of the abdominal wall and finger pressure against the stomach. The abdominal skin surface of the placement area was cleansed with povidone-iodine and local anesthesia was performed by administering 1% lidocaine. After test-puncturing with a 21-gauge needle, 3-point sutures were made using a gastropexy device (Easy Tie, Boston Scientific Japan K.K., Tokyo, Japan) to fix the stomach against the abdominal wall in order to form tight fistula formation. A Seldinger needle was then punctured at the center of the 3-point sutures and a loop wire was inserted through the outer sheath of the needle. The loop wire was grasped by a snare from the endoscope, and a 20F gastrostomy tube was placed by the pull-through technique. The materials used for PEG tube placement were a Ponsky PEG (Bard Access Systems, Inc., Salt Lake City, UT, United States) or a Safety PEG kit (Boston Scientific Co., Natick, MA, United States). The endoscope was reinserted into the stomach to secure the proper placement of the gastrostomy tube. Arterial blood gas analysis was conducted just before and immediately after the PEG procedure to measure PCO₂, arterial partial pressure of O₂ (PO₂), pH and base excess.

Evaluation of post-procedural outcomes

The clinical pathway after PEG was determined as follows. Prophylactic intravenous administration of cefmetazole was conducted for 3 d following PEG. The gastrostomy tube was drained for 24 h and feeding was then started in those patients without serious events. Initial feeding was 100 mL of 5% glucose solution followed by 500 mL of glucose solution on the next day. Commercially available isotonic nutrients were administered from the third day and gradually increased up to 800 kcal/d for 7 d. Target calorie intake was achieved within 2 wk.

Abdominal computed tomography (CT) was conducted 24 h after PEG to detect pneumoperitoneum. In patients with pneumoperitoneum, the volume of the

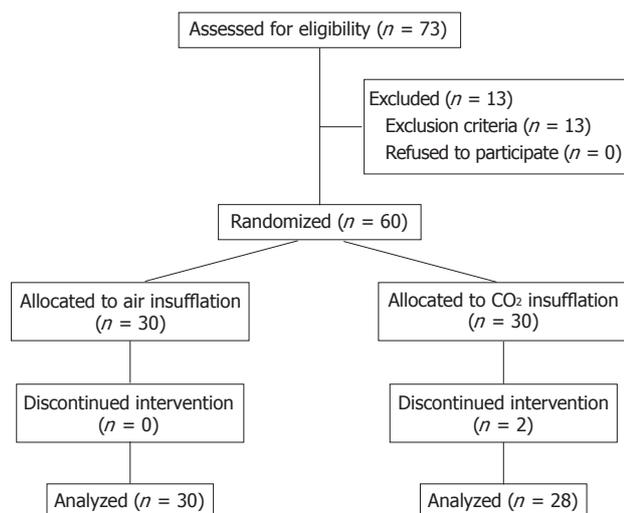


Figure 1 Flow diagram showing selection of study subjects. CO₂: Carbon dioxide.

intraabdominal free air was estimated by CT scan. Abdominal plain radiography was conducted at 10 min and 24 h after PEG to evaluate the bowel distension. The degree of bowel distension was determined by scoring the radiographic images according to the method of Bretthauer *et al*^[8]. In brief, grade I: No distension; grade II: Light distension; grade III: Moderate distension; and grade IV: Severe distension. The radiological estimation was conducted by a physician (Iwashita M) who had not been informed of the insufflation condition.

Evaluation of clinical symptoms after PEG was assessed by the frequency of body temperature elevation (> 38 °C), complications, and whether the scheduled clinical pathway had been pursued for 7 d after PEG. Blood examination [C-reactive protein (CRP) and leucocyte count] was conducted before, one day and 7 d after PEG. The treatments of the patients who had undergone PEG were conducted by individual primary doctors who were not informed of the patient's allocation. Each patient was followed-up along the clinical pathway if their condition was stable. When complications or troubles occurred for the patients, treatments other than the scheduled clinical pathway were conducted by the primary attending doctor.

Statistical analysis

The continuous values were expressed as mean ± SD and analyzed using the Student's *t*-test. The analysis of dichotomous categorical variables was performed using the χ^2 test. The analysis of the grades of bowel distension was conducted by Fisher's exact test. Statistical significance was defined as a *P* value of less than 0.05. All data were analyzed using JMP software for Windows (Version 5.1; SAS Institute Inc., Cary, NC, United States).

RESULTS

Patients

A flow diagram of this study is presented in Figure 1. A

total of 73 patients were enrolled for this study. Thirteen patients were excluded by the exclusion criteria; 6 patients for COPD, 3 patients for hypercapnea, 2 patients for severe congestive heart failure, and one each for post distal gastrectomy and for the purpose of decompression. The remaining 60 patients were equally randomized and underwent PEG with air or CO₂ insufflation. All of the PEG procedures in the air group were successfully performed, whereas those in the CO₂ group were not completed in two patients using the above-described PEG procedure. One of the reasons for failure was inability to insert an endoscope into the stomach because of benign esophageal stenosis. The other reason was failure of gastropexy, although the PEG was performed without gastropexy. These two patients were excluded from the analyses.

The demographic data of the patients are shown in Table 1. The mean age and male/female ratio were not significantly different in the two groups. The serum albumin concentration, an indicator of nutritional status, did not differ significantly.

Percutaneous endoscopic gastrostomy procedures

There were no significant differences in the amount of midazolam administered and the duration of the procedure between the two groups. The mean elevations of PCO₂ in the air and CO₂ groups were 2.67 mmHg and 3.32 mmHg, respectively (*P* = 0.408). The mean depressions of PO₂ in the air and CO₂ groups were 3.72 mmHg and 1.34 mmHg, respectively (*P* = 0.302). The mean pH after PEG of the CO₂ group was significantly lower than that of the air group (*P* = 0.018), whereas the depression of pH during the procedure was not significantly different between the two groups (*P* = 0.125). There were no significant differences in base excess between the two groups (data not shown) (Table 2).

Bowel distension

The most frequent distension grades of the small bowel at 10 min in the air and CO₂ groups were grade IV and grade II, respectively, and those at 24 h were grade II and grade I, respectively (Figure 2) (*P* < 0.001). The most frequent distension grades of the large bowel in the air and CO₂ groups were grade II at both 10 min and 24 h in the two groups (Figure 2).

Pneumoperitoneum

Pneumoperitoneum was observed in 8 patients of the air group, whereas there was no patient with pneumoperitoneum in the CO₂ group by abdominal CT (*P* = 0.003). The mean volume of the free air of the 8 patients in the air group was 36.3 mL. In addition to the 8 patients, faint extragastric air leakage around the stoma was observed in 5 patients of the air group. No such transluminal air leak was observed in any patient of the CO₂ group.

Postprocedural outcomes

The values of CRP levels and leukocyte counts were not different between the two groups at any time. The rates

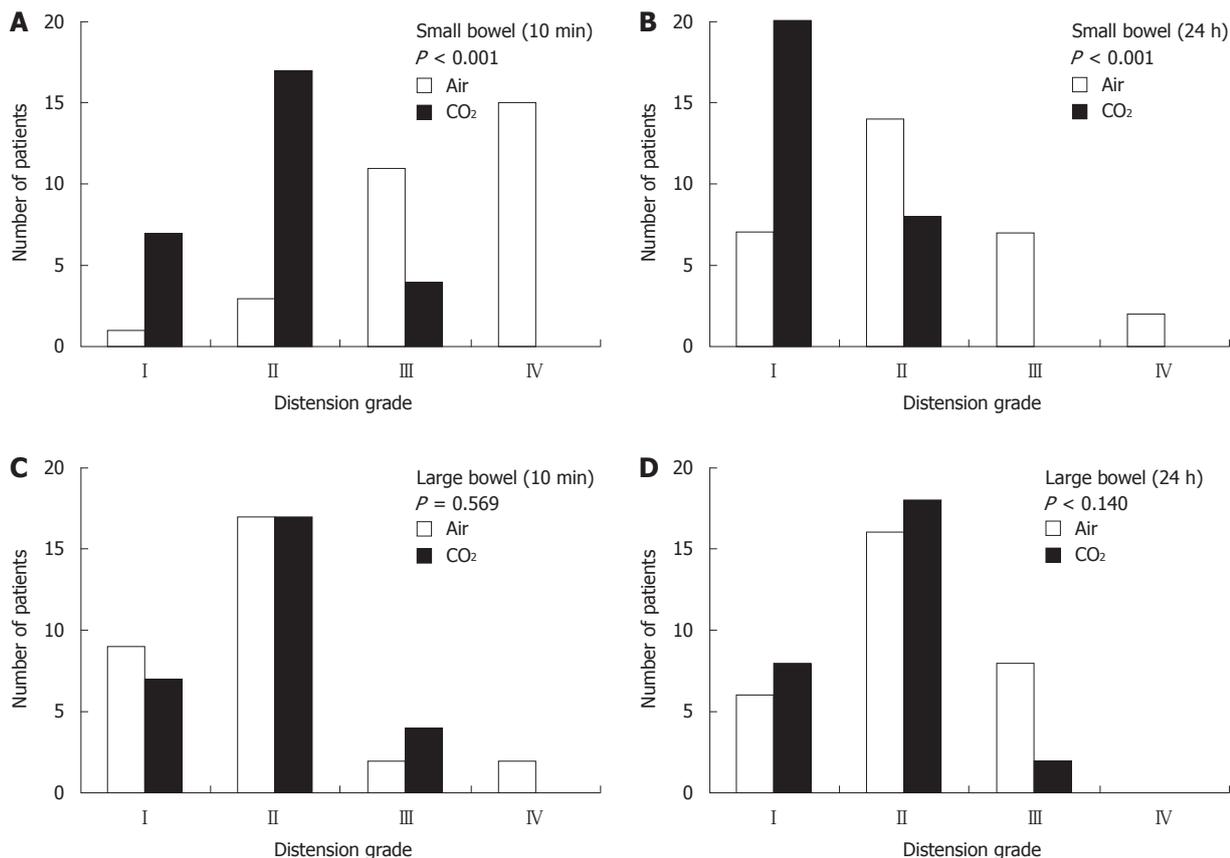


Figure 2 Radiographic evaluation of abdominal distension after percutaneous endoscopic gastrostomy. The degree of small bowel distension [A: 10 min after percutaneous endoscopic gastrostomy (PEG), B: 24 h after PEG] and large bowel distension (C: 10 min after PEG, D: 24 h after PEG) was evaluated by radiography as follows: grade I : No distension; grade II : Slight distension; grade III : Moderate distension; grade IV : Severe distension; CO₂: Carbon dioxide.

Table 1 Demographic data of the air and carbon dioxide insufflation groups

	Air group (n = 30)	CO ₂ group (n = 28)	P value
Age (mean ± SD, yr)	81.9 ± 8.8	82.3 ± 9.5	0.873
Gender (male/female)	8/22	5/23	0.421
Underlying diseases			
Cerebrovascular disease	20	16	
Dementia	5	7	
Neuromuscular disease	2	1	
Pneumonia	1	4	
Malignant tumor	1	0	
Cardiac disease	1	0	
Albumin (mean ± SD, g/dL)	3.12 ± 0.46	3.05 ± 0.69	0.645

CO₂: Carbon dioxide.

of the patients with fever of more than 38 °C in the air and CO₂ groups were 33.3% and 17.9%, respectively ($P = 0.179$). The numbers of patients who did not follow the clinical pathway during the first 7 d post-procedure were 4 each for the air and CO₂ groups (13.3% and 14.2%, respectively). The reasons for the discontinuation of the pathway were aspiration after feeding (3 and 2 patients for the air and CO₂ groups, respectively), stomal infection (1 patient each for the two groups), and hemorrhage in 1 patient in the CO₂ group. Among these complications, 1 patient in the air group and 3 patients in the CO₂ group

were interrupted for feeding until the complications recovered. There was no mortality within 30 d in this study (Table 3).

DISCUSSION

The present study is the first investigation of the effects of CO₂ insufflation during PEG. Our results clearly indicate that the use of CO₂ insufflation reduces the post-procedural abdominal distension and pneumoperitoneum compared to air insufflation. The usefulness and safety of CO₂ insufflation for colonoscopy, ERCP and double balloon enteroscopy have been reported, including effects on the respective therapeutic procedures^[5-12]. PEG is also an endoscopic operation, but the procedure is simple and of relatively short duration compared to the above-mentioned endoscopic procedures. However, the PEG procedure requires maximal insufflation of the stomach and penetration of the gastric wall by a needle and a gastrostomy tube, leading to postprocedural abdominal distension and pneumoperitoneum.

Pneumoperitoneum associated with PEG procedure is thought to be due to air leakage around the needle puncture site of the stomach during the period from the puncture to the placement of a gastrostomy tube^[13]. The reported frequencies of pneumoperitoneum range from 8.6% to 56%^[14-16]. The variation of the frequency may depend on multiple factors. Abdominal CT scan is a more

Table 2 The amount of administered midazolam, duration of percutaneous endoscopic gastrostomy procedure, and arterial blood gas analyses immediately before and after the procedure (mean \pm SD)

	Air group	CO ₂ group	P value
Amount of midazolam (mg)	2.53 \pm 1.14	2.53 \pm 1.45	0.995
Duration of procedure (s)	835 \pm 145	889 \pm 128	0.143
Arterial blood gas analysis			
PCO ₂ (mmHg)			
Before procedure	38.9 \pm 4.9	38.6 \pm 4.4	0.837
After procedure	41.6 \pm 5.6	41.9 \pm 4.8	0.778
Elevation during procedure	2.67 \pm 2.82	3.32 \pm 3.14	0.408
PO ₂ (mmHg)			
Before procedure	83.0 \pm 11.8	84.4 \pm 9.3	0.612
After procedure	79.3 \pm 10.6	83.1 \pm 9.8	0.213
Depression during procedure	3.72 \pm 8.95	1.34 \pm 7.07	0.302
pH			
Before procedure	7.479 \pm 0.035	7.471 \pm 0.023	0.295
After procedure	7.454 \pm 0.031	7.435 \pm 0.031	0.018
Depression during procedure	0.025 \pm 0.031	0.036 \pm 0.027	0.125

PCO₂: Pressure of carbon dioxide; PO₂: Pressure of oxygen.

sensitive modality to detect intraabdominal free air compared to plain radiography. The methods or devices used for the PEG procedure may also affect the frequency of pneumoperitoneum. The performance of gastropexy may have a preventive effect on pneumoperitoneum. We introduced the gastropexy technique in the present study using a T-fastener type fixation device just before the percutaneous needle puncture^[17]. Our intention with gastropexy is to prevent peritonitis if the gastrostomy tube dislodges in the early phase after PEG. In the present study, the frequency of pneumoperitoneum in the air group was 27%, the value of which was comparable to the previous reports. However, the amount of the free air was fairly small (36.3 mL on average), which would be difficult to detect by plain abdominal radiography. Although there are no reports of the preventive effects on pneumoperitoneum by the gastropexy, the procedure is supposed to reduce the amount of the free air.

Most cases of pneumoperitoneum are considered to have no clinical significance and to require no further interventions^[13,14,16]. Pneumoperitoneum after PEG usually causes no symptoms and spontaneously recovers. However, a few patients with pneumoperitoneum showed severe symptoms and underwent laparotomy in larger scale analyses. Dulabon *et al.*^[15] reported that 20% of patients with pneumoperitoneum developed peritonitis which required exploratory celiotomy. Blum *et al.*^[18] retrospectively analyzed 722 patients who had undergone PEG, and reported that pneumoperitoneum was observed in 5 out of 6 patients who had complications requiring laparotomy. They postulated that the presence of intraabdominal free fluid in addition to the free air is an indication of peritonitis requiring surgical intervention.

The inhibitory effect of CO₂ insufflation on pneumoperitoneum or pneumomediastinum at the perforation of endoscopic submucosal dissection was reported by Nonaka *et al.*^[11]. They showed 3 cases of perforation with endoscopic submucosal dissection (2 cases with es-

Table 3 Changes in laboratory data and clinical outcomes after percutaneous endoscopic gastrostomy (mean \pm SD)

	Air group	CO ₂ group	P value
CRP (mg/dL)			
Before PEG	1.58 \pm 1.63	1.51 \pm 2.71	0.906
1 d after PEG	2.92 \pm 3.23	1.66 \pm 1.58	0.068
7 d after PEG	2.12 \pm 3.23	1.14 \pm 1.75	0.158
Leucocytes (/ μ L)			
Before PEG	6330 \pm 1990	7330 \pm 2210	0.077
1 d after PEG	7760 \pm 3230	8570 \pm 2110	0.269
7 d after PEG	6690 \pm 2150	7410 \pm 2400	0.238
Fever more than 38 °C, n (%)	10 (33.3)	5 (17.9)	0.179
Discontinued clinical pathway, n (%)	4 (13.3)	4 (14.2)	0.916

CRP: C-reactive protein; PEG: Percutaneous endoscopic gastrostomy; CO₂: Carbon dioxide.

ophageal cancer and 1 case with gastric cancer) using CO₂ insufflation but no subcutaneous or mediastinal emphysema or pneumoperitoneum developed after perforation.

CO₂ insufflation has also been reported to reduce post-procedural abdominal pain as well as abdominal distension during colonoscopy and double balloon enteroscopy^[5-7]. The reduction of abdominal symptoms by CO₂ insufflation was also reported for ERCP^[8,9], although the effects remain controversial^[19]. Although the mean duration of the PEG procedure was only about 14 min in the present study, a significant reduction in small bowel distension was observed in the CO₂ group compared to the air group. The evaluation of the bowel distension was analyzed by radiography at 10 min and 24 h after PEG. The result of remarkable reduction of small bowel distension at only 10 min after the procedure was consistent with the report of Nakajima *et al.*^[20]. Almost all of the patients in our study were unable to express their symptoms due to their underlying diseases and we could not use a visual analog scale for their abdominal pain and distension, but CO₂ insufflation may reduce the abdominal symptoms after PEG.

There were no significant differences in clinical outcomes after PEG between the two groups. These results indicate that the reduction of pneumoperitoneum and bowel distension did not affect the outcomes after PEG. We experienced 8 out of 58 patients with complications, including 5 cases of aspiration, 2 cases of peristomal infection and 1 case of hemorrhage in this study. These complications were not derived from the CO₂ or air insufflation itself.

The elevation of PCO₂ using CO₂ insufflation in this study was very low and the degree of elevation was comparable to that of air insufflation. These results indicate that the elevation of PCO₂ is not derived from CO₂ insufflation itself but derived from conscious sedation by midazolam. This assumption is consistent with previous reports^[8,11,21,22]. Because the PEG procedure is of short duration, strict continuous monitoring of CO₂ status by the measurement of transcutaneous PCO₂ or partial pressure of end-tidal CO₂ would not be necessary. Suzuki *et al.*^[23] reported that the PCO₂ level increased with the duration of CO₂ insufflation for endoscopic submucosal

dissection under general anesthesia. They also found that patients with lower respiratory function showed a tendency toward CO₂ retention compared to patients with normal respiratory function. Our study excluded patients with COPD and severe chronic heart failure, but the target patients for PEG are usually elderly and often have impaired cardiorespiratory function. Further investigation of the safety of CO₂ insufflation for elderly patients will be required. In conclusion, CO₂ insufflation during the PEG procedure is considered to be safe and provides comfort by reducing pneumoperitoneum and bowel distension.

COMMENTS

Background

Carbon dioxide (CO₂) insufflation has been reported to reduce the post-procedural abdominal distension and pain in gastrointestinal endoscopy for diagnostic and therapeutic purposes. However, there has been no report on the safety and efficacy of CO₂ insufflation for percutaneous endoscopic gastrostomy (PEG) procedures.

Research frontiers

Utilizing the nature that CO₂ is rapidly absorbed from the bowel, the authors investigated the effect of CO₂ insufflation on patients undergoing PEG.

Innovations and breakthroughs

CO₂ insufflation remarkably reduced pneumoperitoneum and small bowel distension without any adverse events.

Applications

Safety of CO₂ insufflation during PEG procedure for elderly patients was demonstrated in this report. Further investigation in patients with cardiopulmonary disorders is necessary.

Peer review

This is the first randomized control study of CO₂ insufflation in PEG procedure and describes the effects of CO₂ insufflation on pneumoperitoneum and bowel distension after PEG. The presented article is a major contribution in the research field.

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Endoscopic and clinicopathologic characteristics of early gastric cancer with high microsatellite instability

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Abstract

AIM: To investigate endoscopic and clinicopathologic characteristics of early gastric cancer (EGC) according to microsatellite instability phenotype.

METHODS: Data were retrospectively collected from a single tertiary referral center. Of 981 EGC patients surgically treated between December 2003 and October 2007, 73 consecutive EGC patients with two or more microsatellite instability (MSI) mutation [high MSI (MSI-H)] and 146 consecutive EGC patients with one or no MSI mutation (non-MSI-H) were selected. The endoscopic and clinicopathologic features were compared between the MSI-H and non-MSI-H EGC groups.

RESULTS: In terms of endoscopic characteristics, MSI-H EGCs more frequently presented with elevated pattern (OR 4.38, 95% CI: 2.40-8.01, $P < 0.001$), moderate-to-severe atrophy in the surrounding mucosa (OR 1.91, 95% CI: 1.05-3.47, $P = 0.033$), antral location (OR 3.99, 95% CI: 2.12-7.52, $P < 0.001$) and synchronous lesions, compared to non-MSI-H EGCs (OR 2.65, 95% CI: 1.16-6.07, $P = 0.021$). Other significant clinicopathologic characteristics of MSI-H EGC included predominance of female sex (OR 2.77, 95% CI: 1.53-4.99, $P < 0.001$), older age (> 70 years) (OR 3.30, 95% CI: 1.57-6.92, $P = 0.002$), better histologic differentiation (OR 2.35, 95% CI: 1.27-4.34, $P = 0.007$), intestinal type by Lauren classification (OR 2.34, 95% CI: 1.15-4.76, $P = 0.019$), absence of a signet ring cell component (OR 2.44, 95% CI: 1.02-5.86, $P = 0.046$), presence of mucinous component (OR 5.06, 95% CI: 1.27-20.17, $P = 0.022$), moderate-to-severe lymphoid stromal reaction (OR 3.95, 95% CI: 1.59-9.80, $P = 0.003$), and co-existing underlying adenoma (OR 2.66, 95% CI: 1.43-4.95, $P = 0.002$).

CONCLUSION: MSI-H EGC is associated with unique endoscopic and clinicopathologic characteristics including frequent presentation in protruded type, co-existing underlying adenoma, and synchronous lesions.

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Key words: Microsatellite instability; Early gastric cancer; Endoscopic characteristic; Advanced gastric cancer

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INTRODUCTION

Microsatellites are simple repetitive DNA sequences that are scattered throughout the genome. Instability within these sequences is a marker of DNA mismatch repair deficiency^[1]. The molecular mechanism of high microsatellite instability is the accumulation of frameshift mutations in the genes containing microsatellites within coding regions^[2]. The resulting inactivation of these target genes is believed to contribute to tumor development and progression.

Previous research has focused on the analysis of microsatellite instability (MSI) in various cancers^[3-5]. In colorectal cancer, high MSI (MSI-H) phenotypes are found in most cases of hereditary nonpolyposis colorectal cancers and in 15% to 20% of sporadic colon cancers^[6]. The clinicopathologic characteristics of MSI-H colorectal cancers are younger age, lower incidence of lymph node metastasis, proximal location, and better survival rate^[7,8].

In gastric cancer, the frequency of MSI-H phenotype varies from 8.2% to 37%, depending on the number of cases investigated and the definitions used^[9,10]. The distinct features frequently reported for MSI-H gastric cancers include older age, antral location, intestinal type by Lauren classification, expanding type by Ming classification, and better survival^[10-13]. However, studies with limited numbers of patients have reported conflicting results regarding the association of the MSI-H phenotype with better survival^[14,15].

Since a limited number of previous studies have dealt with endoscopic and pathologic findings of early gastric cancer (EGC) according to MSI phenotype and most were focused on advanced gastric cancer (AGC)^[10-13,16], we set out to analyze and clarify endoscopic and pathologic characteristics of EGC according to MSI phenotype in this study.

MATERIALS AND METHODS

Patients and tissue samples

From December 2003 to October 2007, 981 patients underwent radical total or subtotal gastrectomy and lymph node dissection for EGC, and the specimens of all patients in this period were examined for MSI status. EGC was defined as gastric cancer limited to mucosa or submucosa, irrespective of lymph node status^[17]. Of 981 patients who were pathologically confirmed as having EGC, 73 (7.4%) cases were categorized as MSI high EGC. For comparison, 146 non-MSI high EGC cases from the remaining 908 patients were consecutively selected as controls. Authorization for the use of these tissues for

research purposes was obtained from the Institutional Review Board of Yonsei University College of Medicine.

Microsatellite analysis

Areas of tumor and non-tumorous tissues on the slides were examined and marked under light microscopy. DNA was extracted from the uncovered hematoxylin and eosin-stained 6 μm -thick sections from formalin-fixed, paraffin-embedded tissues, using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction.

A panel of five National Cancer Institute workshop-recommended microsatellite markers (BAT25, BAT26, D2S123, D17S250 and D5S346) was used for analysis^[18]. Polymerase chain reaction (PCR) was performed using a fluorescently labeled multiprimer, HotStar Taq polymerase (Qiagen) and the GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA). The PCR conditions comprised of an initial cycle of 15 min at 95 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 57 °C, and 1 min at 72 °C. Amplification was completed with a final 5 min at 72 °C. Automated ABI PRISM sequencer model 3100 Genetic Analyzer (Applied Biosystems) was used to analyze amplified PCR products according to the manufacturer's instructions.

MSI-H was classified as shift of two or more microsatellite markers. Microsatellite instability at only one marker was classified as MSI-low (MSI-L). A case with no microsatellite instability was classified as microsatellite stable (MSS). The PCR results of typical MSS and MSI-H cases are illustrated in Figure 1.

Analysis of endoscopic features

Two experienced endoscopists retrospectively reviewed the digital PACS images and the endoscopy database for all 219 cases. Most of the upper endoscopies were performed using GIF-Q260 or GIF-H260 (Olympus Optical, Tokyo, Japan), and a few cases were performed using GIF-Q240 in the early period of 2003. The endoscopic variables included in the analysis were the macroscopic classification of EGC by the Japanese Gastric Cancer Association, which has been internationally accepted^[19,20], location, color pattern and demarcation of the tumor. Additionally, the endoscopic degree of mucosal atrophy and intestinal metaplasia in the surrounding mucosa, and the presence of synchronous neoplasm were also included in the analysis. A synchronous lesion was defined as a distinctly separated lesion from the main EGC based on endoscopic findings. The endoscopic variables were compared between the MSI-H and non-MSI-H EGC groups.

Analysis of clinicopathologic features

Clinical information, including age and sex, was obtained from medical records. The pathologic specimens of all 219 cases were reviewed again by an experienced gastrointestinal pathologist, and categorized as intestinal, diffuse, or mixed according to the classification of Lauren. The mixed type by Lauren classification was regarded

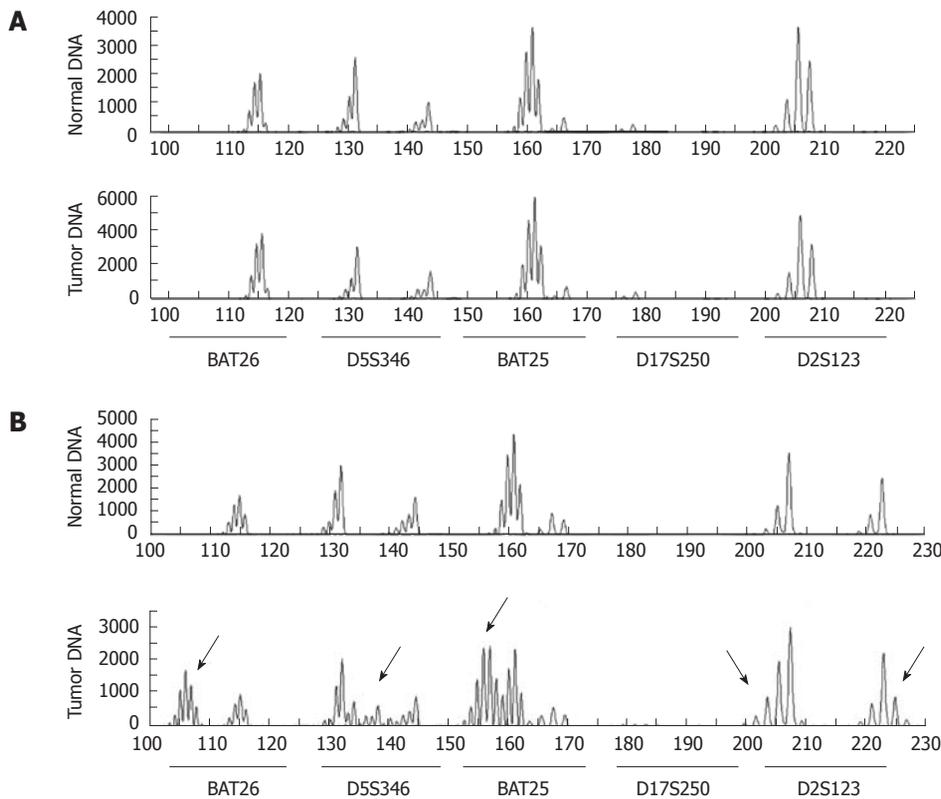


Figure 1 Polymerase chain reaction results of microsatellite analysis markers. A: Typical case of microsatellite stable early gastric cancer with no frameshift mutation; B: Typical case of high microsatellite instability showing frameshift mutations at 4 markers (BAT26, D5S346, BAT25 and D2S123, indicated as arrow).

as intestinal type on analysis. The presence of a mucinous component or a signet ring cell component were defined when these components exceeded 10% of the tumor area. Lymphoid stromal reaction was categorized subjectively as absent, mild, moderate, or severe. Other pathologic variables for analysis included differentiation by World Health Organization (WHO) classification, invasion depth, lymphovascular invasion and co-existing underlying adenoma.

Statistical analysis

The statistical analysis was performed using PASW version 18.0. (SPSS Inc., Chicago, IL, United States). The χ^2 test was performed for comparison between the MSI-H and MSS/MSI-L groups in terms of various clinicopathologic parameters. The data for age and tumor size were analyzed by Student’s *t*-test. Each parameter was also assessed by logistic regression. Differences were considered significant at $P < 0.05$. Estimated relative risks of MSI-H with endoscopic and clinicopathologic factors of EGC were expressed as odds ratios (OR) with corresponding 95% confidence intervals (95% CI).

RESULTS

Clinical and endoscopic features of MSI-H EGCs

Of 981 EGC cases, 73 cases (7.4%) showed the MSI-H phenotype. The endoscopic features of MSI-H EGCs are summarized in Table 1. MSI-H EGCs presented more frequently with elevated gross type (60.3% of type I or

II a; $P < 0.001$), antral location ($P < 0.001$), moderate-to-severe atrophy in the surrounding mucosa ($P = 0.032$), and presence of a synchronous lesion ($P = 0.018$) than non-MSI-H EGCs. The typical endoscopic findings of MSI-H EGC are illustrated in Figure 2.

The color pattern and demarcation of the tumor, and the degree of intestinal metaplasia in the surrounding mucosa, were not significantly different between the MSI-H EGC and non-MSI-H EGC groups.

Clinicopathologic features of MSI-H EGCs

The clinicopathologic features of MSI-H EGCs are summarized in Table 2. In comparison to non-MSI-H EGCs, MSI-H EGCs were characterized by more frequent female sex ($P = 0.001$), older age ($P < 0.001$), better differentiation by WHO classification ($P = 0.003$), intestinal type by Lauren classification ($P = 0.017$), presence of a mucinous component ($P = 0.017$), absence of a signet ring cell component ($P = 0.041$), moderate-to-severe lymphoid stromal reaction ($P = 0.002$) and co-existing underlying adenoma ($P = 0.001$). There was no significant difference between the MSI-H EGC and non-MSI-H EGC groups in terms of tumor size, depth of invasion, lymphovascular invasion, and lymph node metastasis. The endoscopically identified synchronous lesions of the MSI-H EGCs were diagnosed pathologically as EGC in 9.6% and adenoma in 9.6%. Synchronous lesions ($P = 0.018$) were significantly more frequent in the MSI-H EGC group when cases of EGC and adenoma were combined. However, this difference lost its significance when syn-

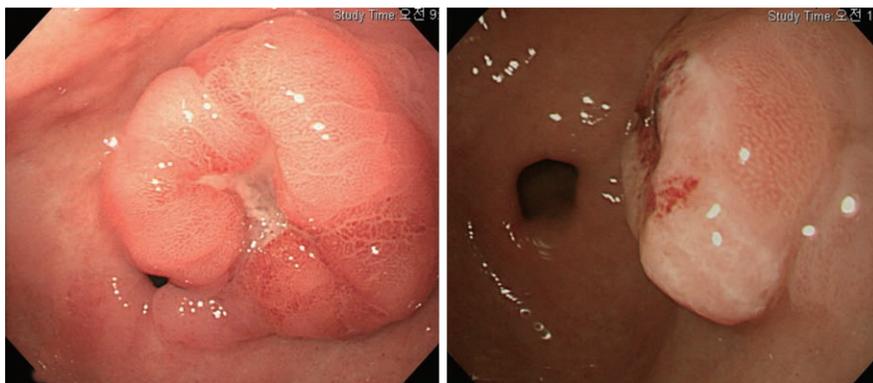


Figure 2 Typical endoscopic findings of microsatellite instability mutation early gastric cancer. Typical endoscopic features of high microsatellite instability early gastric cancers, which are grossly protruded type, arising from co-existing underlying adenoma, at an antral location.

Table 1 Comparison of endoscopic features between the high microsatellite instability and non-high microsatellite instability early gastric cancer groups *n* (%)

Endoscopic findings	MSI-H EGC (<i>n</i> = 73)	Non-MSI-H EGC (<i>n</i> = 146)	<i>P</i> value
Main gross type			< 0.001
Type I	21 (28.8)	16 (11.0)	
Type II a	23 (31.5)	22 (15.1)	
Type II b	13 (17.8)	48 (32.9)	
Type II c	9 (12.3)	51 (34.9)	
Type III	7 (9.6)	9 (6.2)	
Location			< 0.001
Antrum	56 (76.7)	66 (45.2)	
Body	15 (20.5)	77 (52.7)	
Cardia	2 (2.7)	3 (2.1)	
Color			0.117
Whitish color	2 (2.7)	9 (6.2)	
Normal mucosal color	27 (37.0)	36 (24.7)	
Erythematous color	44 (60.3)	101 (69.2)	
Demarcation			0.265
Definite	45 (61.6)	101 (69.2)	
Obscure	28 (38.4)	45 (30.8)	
Intestinal metaplasia			0.138
Absent-to-mild	41 (56.2)	97 (66.4)	
Moderate-to-severe	32 (43.8)	49 (33.6)	
Mucosal atrophy			0.032
Absent-to-mild	22 (30.1)	66 (45.2)	
Moderate-to-severe	51 (69.9)	80 (54.8)	
Synchronous lesion	14 (19.2)	12 (8.2)	0.018

MSI-H: High microsatellite instability; EGC: Early gastric cancer.

chronous EGC ($P = 0.110$) or adenoma ($P = 0.163$) were compared separately.

Correlation between MSI-H and endoscopic and clinicopathologic features of EGCs

We analyzed the interrelationship between MSI-H phenotype and other endoscopic and clinicopathologic factors of EGC by logistic regression analysis (Table 3). Upon logistic regression analysis, MSI-H EGC was significantly associated with elevated gross type (OR 4.38, 95% CI: 2.40-8.01, $P < 0.001$), antral location (OR 3.99, 95% CI: 2.12-7.52, $P < 0.001$), moderate-to-severe atrophy of the surrounding mucosa (OR 1.91, 95% CI: 1.05-3.47, $P = 0.033$), female sex (OR 2.77, 95% CI: 1.53-4.99, $P <$

Table 2 Comparison of clinicopathologic findings between the high microsatellite instability and non-high microsatellite instability early gastric cancer groups *n* (%)

	MSI-H EGC (<i>n</i> = 73)	Non-MSI-H EGC (<i>n</i> = 146)	<i>P</i> value
Patients characteristics			
Sex			0.001
Male	37 (50.7)	108 (74.0)	
Female	36 (49.3)	38 (26.0)	
Age (yr)	63.4 ± 10.4	56.8 ± 11.1	< 0.001
Pathologic findings			
Size (mm)	22.4 ± 15.7	26.5 ± 13.0	0.055
Depth of invasion			0.102
Mucosa	27 (37.0)	71 (48.6)	
Submucosa	46 (63.0)	75 (51.4)	
Histologic differentiation (by WHO classification)			0.003
Well differentiated tubular	24 (32.9)	32 (21.9)	
Moderately differentiated tubular	30 (41.1)	48 (32.9)	
Poorly differentiated tubular	6 (8.2)	29 (19.9)	
Signet ring cell	8 (11.0)	43 (24.0)	
Mucinous	5 (6.8)	2 (1.4)	
Lauren classification			0.017
Intestinal	61 (83.6)	100 (68.5)	
Diffuse	12 (16.4)	46 (31.5)	
Mucinous component	7 (9.6)	3 (2.1)	0.017
No signet ring cell component	60 (90.4)	116 (79.5)	0.041
Co-existing underlying adenoma	29 (39.7)	29 (19.9)	0.002
Lymphovascular invasion	17 (23.3)	22 (15.1)	0.134
Lymph node metastasis	12 (16.4)	14 (9.6)	0.14
Lymphoid stromal reaction			0.002
Absent-to-mild	49 (74.2)	91 (91.9)	
Moderate-to-severe	17 (25.8)	8 (8.1)	
Synchronous lesion			0.018
Early gastric cancer	7 (9.6)	5 (4.8)	0.11
Adenoma	7 (9.6)	7 (3.4)	0.163

MSI-H: High microsatellite instability; EGC: Early gastric cancer; WHO: World Health Organization.

0.001), older age (> 70 years) (OR 3.30, 95% CI: 1.57-6.92, $P = 0.002$), a synchronous lesion (OR 2.65, 95% CI: 1.16-6.07, $P = 0.021$), better histologic differentiation (OR 2.35, 95% CI: 1.27-4.34, $P = 0.007$), intestinal type by Lauren classification (OR 2.34, 95% CI: 1.15-4.76, $P = 0.019$), less signet ring cell component (OR 2.44, 95% CI: 1.02-5.86, $P = 0.046$), presence of a mucinous

Table 3 Logistic regression analysis of endoscopic and clinicopathologic features of high microsatellite instability early gastric cancer

	OR	95% CI		P value
		Lower	Upper	
Endoscopic factors				
Elevated gross type (I or II a)	4.38	2.40	8.01	< 0.001
Antral location	3.99	2.12	7.52	< 0.001
Intestinal metaplasia (moderate-to-severe)	1.55	0.87	2.75	0.139
Mucosal atrophy (moderate-to-severe)	1.91	1.05	3.47	0.033
Clinicopathologic factors				
Female sex	2.77	1.53	4.99	< 0.001
Age ≥ 70 years	3.30	1.57	6.92	0.002
Histologic differentiation (well or moderately)	2.35	1.27	4.34	0.007
Lauren classification (intestinal type)	2.34	1.15	4.76	0.019
Submucosal invasion	1.61	0.91	2.87	0.103
Less signet ring cell component	2.44	1.02	5.86	0.046
Mucinous component	5.06	1.27	20.17	0.022
Lymph node metastasis	1.86	0.81	4.25	0.144
Lymphovascular invasion	1.71	0.84	3.47	0.137
Lymphoid stromal reaction (moderate-to-severe)	3.95	1.59	9.80	0.003
Co-existing underlying adenoma	2.66	1.43	4.95	0.002
Synchronous EGC or adenoma	2.65	1.16	6.07	0.021

OR: Odds ratios; CI: Confidence intervals; EGC: Early gastric cancer.

component (OR 5.06, 95% CI: 1.27-20.17, *P* = 0.022), moderate-to-severe lymphoid stromal reaction (OR 3.95, 95% CI: 1.59-9.80, *P* = 0.003), and co-existing underlying adenoma (OR 2.66, 95% CI: 1.43-4.95, *P* = 0.002).

DISCUSSION

Although the worldwide incidence of gastric cancer has been declining steadily, it remains the fourth most common cancer and the second most common cause of cancer death worldwide^[21]. Despite identification of numerous genetic alterations in gastric cancers, their roles in tumorigenesis remain unclear. Across the gastrointestinal tract, the role of MSI-H tumor has been studied more often in colorectal cancer than in gastric cancer, and MSI is one of the clinically significant prognostic factors that is recommended for collection in the 2010 tumor, node, metastasis staging criteria for colorectal cancer^[22,23]. On the other hand, there is no definite consensus about the role of MSI-H in gastric cancer, and up to now, the majority of MSI-H gastric cancer studies have focused on AGC and its prognosis. However, the clinicopathologic features and therapeutic approaches, such as endoscopic submucosal dissection, for EGCs are different from those for AGCs. In the present study, we focused on endoscopic and clinicopathologic features of MSI-H phenotype in EGCs.

Our results revealed that EGC with MSI-H exhibits distinct clinicopathologic features, including distribution of older age and female sex, antral tumor location,

elevated gross morphology, better histologic differentiation, intestinal type by Lauren classification, absence of a signet cell component, presence of a mucinous component, moderate-to-severe lymphoid stromal reaction, co-existing underlying adenoma, and more synchronous lesions including EGC and adenoma. Among the endoscopic features, the correlation between MSI-H and elevated gross morphology was a significant novel finding.

It is reasonable to suspect that elevated gross type of MSI-H EGCs could be explained by cancer progression from co-existing underlying adenoma. And if we take into account the adenoma-carcinoma sequence in carcinogenesis of gastrointestinal tract cancers, our data indicate that MSI-H EGC could more frequently originate from the co-existing underlying adenoma than non-MSI-H EGC. As a consequence, EGC characterized by elevated mass might be preceded by transformation of co-existing underlying adenoma.

A high MSI level has been shown to play an important role in the development of multiple carcinomas in colorectal cancer^[24,25]. Several previous studies, which did not distinguish between AGC and EGC, have also shown that multiple synchronous gastric cancer is frequently associated with MSI-H^[26,27]. In the present study, MSI-H EGCs were more often associated with synchronous epithelial neoplasm including EGC and adenoma than non-MSI-H, although the difference was not significant when the definition of synchronous lesion was limited to carcinoma, but this may be due to small sample size in each group. Nevertheless, our data indicate that clinicians must consider the potential for synchronous lesions including adenomas and carcinomas in cases of EGC with a confirmed MSI-H phenotype.

In our analysis, MSI-H EGCs were more frequently associated with co-existing underlying adenoma than non-MSI-H EGCs, and had a tendency to have synchronous adenoma. Given this association between MSI-H phenotype and adenoma, we cautiously propose that MSI impacts tumorigenesis at an earlier phase than dysplasia. This hypothesis is supported by results from previous studies revealing that MSI might play an important role in the early events of progression from metaplasia or dysplasia to precancerous lesions, and then to gastric cancer^[28,29]. Although there are a limited number of studies on the significance of the main 5 MSI mutations in EGC, a retrospective study from a high risk area of gastric cancer in Italy showed that MSI is part of the spectrum of genetic alterations in gastric non-invasive neoplasia^[30].

Our study was limited by the fact that we only included cases of EGC treated with surgery, which may serve as a bias. We excluded patients with EGCs who underwent endoscopic resection due to uncertainty of lymph node metastasis. Therefore, careful interpretation of our results is needed to avoid bias and additional data from other studies that include all EGCs are required to validate our findings. Also, since this is a retrospective study and there are no actual data to support the temporal relationship between adenoma and progression into EGC, speculation that EGC originated from underlying adenoma may

be overstretching. We used the term “co-existing underlying adenoma” instead of “origin in underlying adenoma” to cope with this limitation.

In conclusion, this study found that MSI-H EGCs more frequently present as protruded (type I or IIa) type in comparison to non-MSI-H EGCs, which is a novel finding; this is likely related to frequent co-existing underlying gastric adenoma. MSI-H EGC was found to have specific clinicopathologic characteristics, including older age, female dominance, antral location, moderate-to-severe atrophy of the surrounding mucosa, better histologic differentiation, intestinal type by Lauren classification, presence of mucinous component, absence of a signet ring cell component, moderate-to-severe lymphoid stromal reaction, underlying adenoma, and synchronous lesions. The finding of more frequent synchronous lesions in MSI-H EGC warrants us to inspect the stomach more thoroughly for other synchronous adenoma or EGC. Altogether, these findings could be used to better serve our understanding of tumorigenesis and progression in MSI-H EGC, and could be used to set up diagnostic and treatment strategies in EGC patients.

COMMENTS

Background

Gastric carcinoma is the fourth most common cancer and the second most common cause of cancer death worldwide. Various genetic alterations have been found in gastric cancers, although their roles in tumorigenesis remain unclear. Detection in the early stage and prevention of progress to advanced cancer are crucial in lowering gastric carcinoma related morbidity and mortality.

Research frontiers

Microsatellite instability (MSI) is a hallmark of a deficiency in the DNA mismatch repair system and is one of the pathways leading to gastric carcinogenesis. However, there are limited data on endoscopic and clinicopathologic characteristics of early gastric cancer (EGC) according to MSI phenotypes.

Innovations and breakthroughs

EGCs with high MSI frequently presented with elevated or protruded pattern, which is a novel finding. In addition, EGCs with high MSI showed more frequent synchronous lesions. Other significant endoscopic and clinicopathologic findings of high MSI (MSI-H) EGCs included moderate-to-severe atrophy in the surrounding mucosa, antral location, predominance of female sex, older age, better histologic differentiation, intestinal type by Lauren classification, absence of a signet ring cell component, presence of mucinous component, moderate-to-severe lymphoid stromal reaction, and co-existing underlying adenoma.

Applications

EGCs with MSI-H seem to originate from underlying adenoma, following an adenoma-carcinoma sequence. Furthermore, more synchronous lesions in MSI-H EGCs indicate that thorough inspection and shorter follow up period is needed for endoscopic surveillance after diagnosis of early gastric carcinoma with MSI-H.

Peer review

This study evaluates relationship of phenotypic characteristics (endoscopic and clinicopathologic) of EGC with MSI-H. Multivariate logistic regression analysis revealed that MSI-H EGCs more frequently presented with elevated pattern (type I or IIa), moderate-to-severe atrophy in the surrounding mucosa, antral location and synchronous lesions than non-MSI-H EGCs.

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Weekend and nighttime effect on the prognosis of peptic ulcer bleeding

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Abstract

AIM: To evaluate whether weekend or nighttime admission affects prognosis of peptic ulcer bleeding despite early endoscopy.

METHODS: Retrospective data collection from four referral centers, all of which had a formal out-of-hours emergency endoscopy service, even at weekends. A total of 388 patients with bleeding peptic ulcers who were admitted via the emergency room between January 2007 and December 2009 were enrolled. Analyzed parameters included time from patients' arrival until endoscopy, mortality, rebleeding, need for surgery and length of hospital stay.

RESULTS: The weekday and weekend admission groups comprised 326 and 62 patients, respectively. There were no significant differences in baseline characteristics between the two groups, except for younger age in the weekend group. Most patients (97%) had undergone early endoscopy, which resulted in a low mortality rate regardless of point of presentation (1.8% overall vs 1.6% on the weekend). The only outcome that was worse in the weekend group was a higher rate of rebleeding (12% vs 21%, $P = 0.030$). However, multivariate analysis revealed nighttime admission and a high Rockall score (≥ 6) as significant independent risk factors for rebleeding, rather than weekend admission.

CONCLUSION: Early endoscopy for peptic ulcer bleeding can prevent the weekend effect, and nighttime admission was identified as a novel risk factor for rebleeding, namely the nighttime effect.

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Key words: Early endoscopy; Nighttime effect; Peptic ulcer bleeding; Rebleeding; Weekend effect

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INTRODUCTION

Upper gastrointestinal bleeding (UGIB) is a common medical emergency and timely endoscopy plays an im-

portant role in hemostasis. UGIB is also a significant healthcare problem in the United States, with an annual hospitalization rate of approximately 150 per 100000 individuals and an overall mortality rate of 6% to 7%^[1-4]. In South Korea, the incidence of UGIB has been reported to be 1 per 1000 individuals with a mortality rate of 5% to 10%, however, the mortality rate has decreased recently due to advances in endoscopy^[5,6].

It has been suggested that patients with UGIB who are admitted on weekends have lower rates of early endoscopy, higher mortality, and more frequently undergo surgery^[7]. Previous studies reported that outcomes are worse on weekends due to lack of availability of staff and services, which is collectively referred to as the “weekend effect”^[8]. Nahon *et al*^[9] suggested that early endoscopy could prevent the weekend effect and reduce mortality. However, Shaheen *et al*^[10] claimed that the weekend effect did not diminish even after adjusting data for the timing of endoscopy. Studies on the prognosis of UGIB on weekends have shown different results from numerous research centers, leaving the weekend effect on UGIB controversial^[7-10].

This issue might have regional and socio-organizational differences, and it is possible that the weekend effect depends on the specific situation and medical environment, such as the availability of emergency endoscopy services on the weekends. The majority of tertiary referral hospitals in South Korea have formal out-of-hours emergency endoscopy for UGIB. We sought to determine whether the weekend effect still influences the outcomes of peptic ulcer bleeding patients in hospitals that offer out-of-hours emergency endoscopy for UGIB, even on weekends. The primary aim of this study was to investigate the difference in prognosis between weekend and weekday admissions for peptic ulcer bleeding at referral hospitals in South Korea. In addition to the weekend effect, we also evaluated whether nighttime admission affected the prognosis of peptic ulcer bleeding.

MATERIALS AND METHODS

Patients

Data was retrospectively collected from an endoscopic database of four referral training hospitals in South Korea. A total of 388 consecutive patients, who were admitted for peptic ulcer bleeding *via* the emergency room (ER) between January 2007 and December 2009, were enrolled. All subjects had endoscopically confirmed peptic ulcer bleeding.

Patient data were collected from medical records, which were reviewed by endoscopists who were blinded to the aim of this study. The Institutional Review Board of Gangnam Severance Hospital approved this study.

We excluded patients with variceal bleeding, Mallory-Weiss tear, lower gastrointestinal bleeding, or bleeding from malignant ulcers. Peptic ulcers without stigma of recent bleeding (Forrest III^[11]) were also excluded due to the obscure source of bleeding.

Endoscopy procedure

All four hospitals that participated in this study were referral training centers in urban areas and have formal out-of-hours emergency endoscopy services. In these centers, at least one endoscopist is scheduled to be on duty for emergency calls for endoscopy, regardless of time and day, even on weekends or at night. Endoscopy is generally conducted as soon as possible in patients with suspected UGIB. However, we do not have a night shift; therefore, one of the day shift endoscopists has to be on duty at night when on emergency call. All on-duty endoscopists can handle the available endoscopic hemostatic procedures. All endoscopic hemostatic procedures were performed using the same protocol set by the guidelines of the Korean Society of Gastroenterology^[12]. Hemostatic procedures were carried out on Forrest Ia to Ib peptic ulcers^[11].

The levels of experience of endoscopists who performed endoscopic hemostasis in UGIB varied slightly among the four centers according to their policies. In one institution, senior instructors were responsible for both daytime and nighttime endoscopic hemostasis, while hospital staff took charge of hemostatic interventions in both daytime and nighttime in the other three institutions. Thus, for a given institution, available expertise remained generally constant day and night.

Definitions

The weekend group was defined as patients who presented to the ER from Friday midnight to Sunday midnight, and the remaining patients were categorized as the weekday group. The nighttime group was defined as patients who presented to the ER between 18:00 and 8:00 the next day. Endoscopy was classified as “early” if the procedure was performed within 24 h^[13]. Active bleeding indicated spurting or oozing, which was based on classification from Ia to Ib according to endoscopic findings^[11]. Rebleeding was defined as bleeding within 2 wk that required secondary hemostasis or was associated with hematemesis; melena with overt decrease in hemoglobin over 2 mg/dL; status requiring blood transfusion; shock (systolic blood pressure < 90 mmHg); or endoscopic findings of recent bleeding, such as spurting, oozing, or adherent clot^[14,15].

Parameters and endpoints

Parameters were chosen only for characteristics representative of bleeding, and were classified into baseline characteristics and treatment outcomes; the following intergroup comparisons were made: weekday *vs* weekend and daytime *vs* nighttime. The baseline parameters were age; disease type; endoscopic findings, including Forrest class; Rockall score^[16,17]; and Charlson score. The Charlson score is a system for the classification of severity that uses recorded data on a patient’s diagnosis to assign a weight to morbidity, thereby predicting a patient’s risk of death. To calculate the Charlson score, we included age factor in the comorbidity score, which is called the Charl-

son age comorbidity index (CACI)^[18]. As treatment outcome parameters, the primary endpoints were mortality rate, rebleeding, length of stay, and the need for surgery or embolization.

Statistical analysis

Data was analyzed using the Statistical Package for the Social Sciences (SPSS, 18.0 Inc, Chicago, United States) software with the assistance of the Yonsei University Statistical Consulting Center. Sensitivity and specificity were calculated with 95% confidence intervals and *P* values less than 0.05 were regarded as significant. According to the 95% confidence interval, we present data as the mean \pm SE. The comparison of categorical variables between the two groups was carried out using the χ^2 test. A logistic regression model was used to analyze the effect of categorical variables and adjust for potential confounders. The comparison of continuous variables between the two groups was carried out by *t*-test. A linear regression model was used to analyze the effect of continuous variables and adjust for potential confounders.

RESULTS

Baseline patient characteristics

The weekday group included 326 patients and the weekend group comprised 62 patients. There were no significant intergroup differences in demographics, except for age (Table 1). The mean age of the weekend group was younger than the weekday group by 5 years (60.7 ± 0.85 years in the weekday group *vs* 55.7 ± 2.10 years in the weekend group, *P* = 0.023). The ages in both groups had normal distributions; the weekday showed skewness and kurtosis values of -0.191 and -0.627, respectively, and the skewness and kurtosis values of the weekend group were -0.83 and -0.760, respectively. All outcome analysis was age-adjusted because age is an important confounding factor for treatment outcomes, including mortality. In order to adjust for age, linear regression was used for continuous variables and logistic regression was used for categorical variables. However, there were no significant differences between the two groups in the patterns and sites of bleeding, Rockall scores, Charlson scores, or comorbidities (Table 1).

Endoscopic procedure

Endoscopic therapy was performed in patients with Forrest I a to II b ulcers, and the following hemostatic tools were frequently used: argon plasma coagulation, hemoclipping, and epinephrine injection as a combination therapy (43%) or monotherapy (57%). However, there was no significant discrepancy between the weekday and weekend groups in hemostatic tool usage, and rebleeding rate did not differ with regard to the applied hemostatic method (from 11% to 19%).

The endoscopists who performed the endoscopic hemostasis could be divided into two groups (instructors and hospital staff) according to the level of experience.

Table 1 Baseline characteristics and treatment outcomes of the weekday and weekend groups *n* (%)

Factors	Weekday	Weekend	<i>P</i> value
Number of patients	326 (84.0)	62 (16.0)	
Age (yr)	60.7 \pm 0.8	55.7 \pm 2.1	0.023
Male	250 (76.7)	47 (75.8)	NS
Gastric ulcer	221 (68.1)	43 (69.4)	NS
Duodenal ulcer	104 (31.9)	19 (30.6)	NS
Active bleeding (Forrest I a, I b)	119 (36.5)	18 (29.0)	NS
Rockall score	5.0 \pm 0.0	4.7 \pm 0.2	NS
Charlson score	3.4 \pm 0.1	3.1 \pm 0.3	NS
Comorbidity	123 (37.7)	26 (41.9)	NS
Endoscopic hemostasis	309 (94.8)	57 (91.9)	NS
Time to endoscopy (min)	338.9 \pm 24.9	306.8 \pm 45.6	NS
Rebleeding	39 (12)	13 (21)	0.030
Angiographic embolization	4 (1.2)	1 (1.6)	NS
Surgery	3 (0.9)	1 (1.6)	NS
Length of stay (d)	8.5 \pm 0.3	6.2 \pm 0.5	0.009
Mortality	6 (1.8)	1 (1.6)	NS

NS: Not significant.

Among a total 388 subjects, 67% (260 patients) of endoscopic hemostases were performed by hospital staff and the other 33% (128 patients) were performed by instructors. However, there was no significant difference in the level of experience between the weekday and weekend group, and between the daytime and nighttime group (proportion of procedures by hospital staff was 68% in the weekday group *vs* 59% in the weekend group, *P* = 0.14; 69% in the daytime group *vs* 58% in the nighttime group, *P* = 0.11). Also the rate of rebleeding was not different between the two groups (instructors and hospital staff).

Most patients (97%) underwent early endoscopy regardless of weekend or nighttime admission. Overall, the mean time interval between presentation to the ER and endoscopy was 333.8 ± 22.2 min. The time to endoscopy was slightly shorter in the weekend group, although the difference was not significant (338.9 ± 24.9 min in the weekday group *vs* 306.8 ± 45.6 min in the weekend group, *P* = 0.56) (Table 1). However, when time to endoscopy was compared between the daytime group and the nighttime group, it was significantly shorter in the nighttime group (352.9 ± 26.3 min in the daytime group *vs* 255.0 ± 30.0 min in the nighttime group, *P* = 0.016) (Table 2).

Treatment outcomes

Mortality: The overall mortality rate was only 1.8% (7/388). The subgroup that had high Rockall scores (≥ 6) included 146 patients, and their mortality rate was 4.1% (6/146). There was no significant difference in mortality between the weekday and weekend groups (1.8% in the weekday group *vs* 1.6% in the weekend group, *P* = 0.902) (Table 1). In addition, the mortality rate did not differ between the daytime and nighttime groups (1.9% in the daytime group *vs* 1.3% in the nighttime group, *P* = 0.757) (Table 2). All deaths occurred in patients with comorbidities, who had significantly higher CACI (7.0 ± 0.7) and Rockall scores (7.6 ± 0.6) than surviving patients (*P* <

Table 2 Baseline characteristics and treatment outcomes of the daytime and nighttime groups *n* (%)

Factors	Daytime	Nighttime	<i>P</i> value
Number of patients	312 (80)	76 (20)	
Age (yr)	60.3 ± 0.9	57.9 ± 1.7	NS
Male	238 (76.2)	59 (77.6)	NS
Gastric ulcer	209 (66.9)	57 (75.0)	NS
Duodenal ulcer	103 (33.0)	19 (25.0)	NS
Active bleeding (Forrest I a, I b)	111 (35.5)	26 (34.2)	NS
Rockall score	4.9 ± 0.1	4.9 ± 0.2	NS
Charlson score	3.5 ± 0.1	3.1 ± 0.3	NS
Comorbidity	119 (38.1)	30 (39.5)	NS
Time to endoscopy (min)	352.9 ± 26.3	255.0 ± 30.0	0.016
Rebleeding	36 (11.5)	16 (21.1)	0.018
Angiographic embolization	3 (0.9)	2 (2.6)	NS
Surgery	4 (1.2)	0 (0.0)	NS
Length of stay (d)	8.3 ± 0.3	7.3 ± 0.5	NS
Mortality	6 (1.9)	1 (1.3)	NS

NS: Not significant.

Table 3 Significant risk factors for mortality

Risk factor	Alive (<i>n</i> = 381)	Dead (<i>n</i> = 7)	<i>P</i> value
Comorbidity rate (%)	37.3	100.0	0.001
Age (yr)	59.7 ± 0.8	72.1 ± 5.3	0.037
Charlson score	3.3 ± 0.1	7.0 ± 0.7	< 0.001
Rockall score	4.9 ± 0.1	7.6 ± 0.6	< 0.001

0.001). The significant risk factors for mortality were age, comorbidity, Rockall score, and Charlson score (Table 3).

Rebleeding: The level of experience did not affect the rate of rebleeding (16% in procedures by instructors *vs* 14% by hospital staff, *P* = 0.39) in our study. Age-adjusted analysis revealed a significantly higher rebleeding rate in the weekend group (12% in the weekday group *vs* 21% in the weekend group, *P* = 0.030) (Table 1). In addition, despite earlier endoscopy, the rate of rebleeding was also higher in the nighttime group, which we called the nighttime effect, than in the daytime group (11.5% in the daytime group *vs* 21.1% in the nighttime group, *P* = 0.018) (Table 2). When the rebleeding rate was compared between the four subgroups that were divided by weekday/weekend and daytime/nighttime, the highest rebleeding rate, which reached up to one third of the patients, was noted in the weekend-nighttime group (11.4% in the weekday daytime, 15.1% in the weekday nighttime, 12.8% in the weekend daytime, and 34.8% in the weekend nighttime, *P* = 0.040) (Table 4).

Multivariate logistic regression was used to analyze potential factors that affected the in-hospital rebleeding rate. The significant independent risk factors for rebleeding were a high Rockall score (≥ 6) and nighttime rather than weekend admission (Table 5). Therefore, statistically, the risk factor associated with rebleeding was not weekend but nighttime admission.

Length of stay: Age-adjusted analysis revealed a shorter

length of stay in the weekend group (8.5 d in weekday *vs* 6.2 d in weekend, *P* = 0.009). However, linear regression analysis revealed that the length of stay was not associated with age or weekend admission, but was strongly associated with the type of comorbidity, which correlated well with the CACI. The length of stay increased in proportion to the increase in the CACI.

DISCUSSION

In this multicenter study, our treatment outcomes were generally favorable. The overall mortality rate due to peptic ulcer bleeding was only 1.8%, which is lower than previous studies that had reported mortality of 6% to 7%^[1-3]. In addition, the need for surgery and angiographic embolization was also low, at 1% and 1.2%, respectively. These outcomes, including mortality, might have been significantly affected by the severity of disease and comorbidities. However, mortality in the subgroup whose Rockall score was above 6 was also only 4.1% in our study (6/146), and mortality was only 0.3% in patients with Rockall scores under 6 (1/388); thus, our low mortality was not due to a milder presentation of bleeding. Such favorable outcomes should result from timely endoscopic hemostasis and appropriate intensive care. We provided early endoscopy within 24 h in most patients (97%), even for weekend and nighttime admissions. The mean time to endoscopy was only 333.8 ± 22.2 min overall. Therefore, we suggest that early intervention could reduce the mortality of peptic ulcer bleeding to 1.8%.

Regarding the so-called weekend effect, our study demonstrated no weekend effect on mortality, need for surgery, angiographic embolization, or length of stay. In fact, the patients in the weekend group showed favorable outcomes comparable to those of the weekday group. We expected to nullify the weekend effect since all four participating hospitals were teaching referral hospitals with well-organized duty systems and formal out-of-hours emergency endoscopy services, which allowed early endoscopy at any time of any day. Contrary to our expectations, the time interval between presentation to the ER and endoscopy was shorter for weekend and nighttime admissions. The reasons for shorter time to endoscopy at night and on weekends might be longer waiting time during weekdays due to previously appointed outpatients, and fewer traffic jams at night and on weekends in urban settings.

However, a higher rebleeding rate was noted in the weekend group, and it is possible that the weekend effect might exist in spite of early endoscopy. In multivariate logistic regression analysis, weekend presentation was not a significant risk factor for rebleeding, but nighttime presentation and a high Rockall score were independent risk factors for rebleeding. Therefore, statistically, the risk factor associated with rebleeding was not weekend but nighttime admission. The rebleeding rate in the weekday-nighttime group was also higher than weekday-daytime group, although the difference was not statistically sig-

Table 4 Subgroup analysis according to daytime and nighttime groups *n* (%)

	Weekday			Weekend		
	Day (<i>n</i> = 273)	Night (<i>n</i> = 53)	<i>P</i> value	Day (<i>n</i> = 39)	Night (<i>n</i> = 23)	<i>P</i> value
Age (yr)	61.0 ± 0.9	58.9 ± 1.9	NS	54.8 ± 2.6	57.1 ± 3.5	NS
Active bleeding (Forrest I a, I b)	99 (36.3)	20 (37.7)	NS	12 (31.0)	6 (26.0)	NS
Rockall score	5.0 ± 0.1	4.9 ± 0.2	NS	4.6 ± 0.3	4.8 ± 0.4	NS
Charlson score	3.5 ± 0.1	2.9 ± 0.3	NS	2.9 ± 0.4	3.6 ± 0.7	NS
Comorbidity	102 (37.4)	21 (39.6)	NS	17 (43.6)	9 (39.1)	NS
Endoscopic hemostasis	259 (94.9)	50 (94.3)	NS	36 (92.3)	21 (91.3)	NS
Time to endoscopy (min)	355.7 ± 28.9	252.3 ± 33.0	0.020	333.7 ± 63.4	261.4 ± 60.4	NS
Rebleeding	31 (11.4)	8 (15.1)	NS	5 (12.8)	8 (34.8)	0.040
Angiographic embolization	3 (1.1)	1 (1.9)	NS	0 (0)	1 (4.3)	NS
Surgery	3 (1.1)	0 (0)	NS	1 (2.6)	0 (0)	NS
Length of stay (d)	8.6 ± 0.4	7.7 ± 0.6	NS	6.1 ± 0.7	6.5 ± 0.9	NS
Mortality	6 (2.2)	0 (0)	NS	0 (0)	1 (4.3)	NS

NS: Not significant.

Table 5 Multivariate analysis for risk factors of rebleeding

Risk factor	Rebleeding rate (%)	<i>P</i> value	Odds ratio	95% confidence interval
Rockall score (≥ 6) ¹	18	0.016	2.083	1.144-3.791
Nighttime	21	0.044	2.012	1.020-3.968
Weekend	21	0.120	1.782	0.861-3.689

¹According to the receiver operating characteristic curve, a Rockall score of 6 points was used as the cutoff value.

nificant (weekday-daytime 11.4% *vs* weekday-nighttime 15.1%, *P* = 0.487). The weekend-daytime group showed a similar rebleeding rate to that of the weekday-daytime group, but the weekend-nighttime group had a significantly higher rebleeding rate (rebleeding rate 34.8% in the weekend nighttime group *vs* 12.8% in the weekend daytime group, *P* = 0.040). However, we cannot completely rule out a weekend effect because the rebleeding rate of the weekend-nighttime group was higher than that of the weekday-nighttime group. Thus, it is reasonable to infer that these findings represent a nighttime effect or a weekend-nighttime effect rather than a weekend effect. Therefore, we concluded that the nighttime effect represented a new risk factor for rebleeding and was more powerful on the weekend through a combination with the weekend effect. Rebleeding after endoscopic hemostasis could also be affected by various endoscopic and clinical factors, such as an active bleeding pattern, gastric location of the peptic ulcer, larger ulcer size^[19-21], comorbidities^[21-23] and even by the level of experience of endoscopists^[24], but our finding of increased rebleeding in the nighttime group, especially the weekend nighttime group, was not associated with these factors. However, we were unable to identify specific factors that accounted for this nighttime effect; potential reasons include fatigue and decreased concentration of endoscopists at night and reduced staffing patterns of physicians, nurses, and other support staff at night. It is necessary to be more alert and particularly careful regarding hemostasis in patients with UGIB who present at night on the weekend.

Rockall scores and Charlson scores were predictors of mortality, rebleeding, and length of stay^[16-18]. The Rockall score was designed to predict mortality and can also be used to predict rebleeding^[16,17,25,26]. To allow for clinical application of the Rockall score and to verify our data (because we observed such a low mortality rate), we used a receiver operating characteristic (ROC) curve to determine the cut-off value predictive of mortality and rebleeding. According to the ROC curve, a Rockall score of 6 points could be chosen as the cut-off value. Rockall scores greater than six were significantly associated with rebleeding [odds ratio (OR) = 2.08] and an increased mortality (OR = 1.77) of 4.1% (6/146). Bessa *et al.*^[27] also reported that the Rockall score indicated a risk of mortality up to 15% if the score was above six. Our data support a Rockall score of 6 points as a critical point.

Contrary to our expectation, length of stay was shorter in the weekend group than in the weekday group. The length of stay increased in proportion to the CACI, and linear regression analysis showed that length of stay was strongly associated with the type of comorbidity rather than weekend presentation, emphasizing the type and severity of illness.

One weak point in our data was that the mean age of the weekend group was 5 years younger than the weekday group. Through a review of the medical records, we observed that there were some weekend patients who postponed visiting the hospital due to a busy work schedule or other circumstances despite having experienced melena for several days. Younger patients are more likely to be employed, thus they are potentially more inclined to present on the weekend rather than on a weekday due to their work. Socio-environmental factors, such as employment, social status, and personal characteristics, might have an influence on visiting the hospital and could be a possible explanation for the younger age of the weekend group compared to the weekday group. However, such factors were not available in the majority of medical records due to the retrospective nature of our study, and we were unable to identify a satisfactory reason in our results. Despite this weakness, we are confident that the

age discrepancy was not a problem because all analyses of outcomes were age-adjusted.

Additional limitations of our study were that the number of deaths was too small to allow for satisfactory analysis and that our study was retrospective; however, all of the endoscopic procedures were based on the same protocol, which should overcome this weakness. In addition, our study was not a nationwide study, but we think that the results of this multicenter study are sufficient to conclude that early endoscopic intervention can lead to favorable outcomes in peptic ulcer bleeding even on weekends and at night.

In conclusion, early endoscopy for peptic ulcer bleeding could reduce mortality to 1.8% and could prevent the weekend effect on the majority of outcomes in patients with peptic ulcer bleeding. However, we identified nighttime presentation as a new risk factor for rebleeding, despite early endoscopy. The Rockall score was also a useful predictor of rebleeding, and we should take this into consideration in the prognosis of peptic ulcer bleeding. Therefore, we need to be more careful and alert at night when dealing with peptic ulcer bleeding, especially in patients who present at nighttime and those with high Rockall scores (≥ 6).

COMMENTS

Background

Upper gastrointestinal bleeding (UGIB) is a common medical emergency and timely endoscopy plays an important role in hemostasis. The "weekend effect" which means a worse outcome in UGIB patients following weekend admission, has been suggested by previous reports, but remains controversial.

Research frontiers

Some authors have suggested a weekend effect on UGIB resulting in higher mortality and more frequent surgery. One report suggests that early endoscopy can prevent the weekend effect and reduce mortality, however, contrary results have also been reported. The issue of the weekend effect on UGIB should be whether early endoscopic hemostasis even at weekends can prevent worse outcomes.

Innovations and breakthroughs

The study analyzed various endoscopic and clinical data from 4 centers which can affect the outcome of UGIB. From their results, they can suggest that early endoscopy for peptic ulcer bleeding, even at weekends, can almost prevent the weekend effect. The most important risk factor for rebleeding was a high Rockall score, however, nighttime admission was also identified as a novel risk factor for rebleeding, namely the nighttime effect.

Applications

Although early endoscopy for peptic ulcer bleeding can prevent the weekend effect in patients with peptic ulcer bleeding, nighttime presentation and high Rockall score were significant risk factors for rebleeding. The authors need to be more careful and alert when dealing with peptic ulcer bleeding, especially in patients who present at nighttime and those with high Rockall score.

Terminology

Weekend effect: The weekend effect has previously been reported as worse outcomes in UGIB patients following weekend admission, such as higher mortality and more frequent surgery; **Nighttime effect:** The nighttime effect is a novel term used in the current study, which means worse outcome due to a higher rebleeding rate in patients with peptic ulcer bleeding following nighttime admission.

Peer review

This is a well-written manuscript of a retrospective multi-center study investigating the weekend and the nighttime effect on the outcomes of patients with upper GIB requiring endoscopic hemostasis. The results were clearly presented and the contents are easily comprehensible.

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Microbial profile and antibiotic sensitivity pattern in bile cultures from endoscopic retrograde cholangiography patients

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Abstract

AIM: To identify the frequency of bacterial growth, the most commonly grown bacteria and their antibiotic susceptibility, and risk factors for bacterial colonization in bile collected from patients with different biliary diseases.

METHODS: This prospective study was conducted between April 2010 and August 2011. Patients with various biliary disorders were included. Bile was aspirated by placing a single-use, 5F, standard sphincterotome catheter into the bile duct before the injection of contrast agent during endoscopic retrograde cholangiopancreatography (ERCP). Bile specimens were transported to the microbiology laboratory in blood culture bottles within an anaerobic transport system. Bacteria were cultured and identified according to the standard protocol used in our clinical microbiology laboratory. The susceptibilities of the organisms recovered were identified using antimicrobial disks, chosen according to

the initial gram stain of the positive cultures.

RESULTS: Ninety-one patients (27% male, mean age 53.7 ± 17.5 years, range: 17-86 years) were included in the study. The main indication for ERCP was benign biliary disease in 79 patients and malignant disease in 12 patients. The bile culture was positive for bacterial growth in 46 out of 91 (50.5%) patients. The most frequently encountered organisms were Gram-negative bacteria including *Escherichia coli* (28.2%), *Pseudomonas* (17.3%) and *Stenotrophomonas maltophilia* (15.2%). There were no significant differences between patients with malignant and benign disease (58% vs 49%, $P = 0.474$), patients with acute cholangitis and without acute cholangitis (52.9% vs 50%, $P = 0.827$), patients who were empirically administered antibiotics before intervention and not administered (51.4% vs 60.7%, $P = 0.384$), with regard to the bacteriobilia. We observed a large covering spectrum or low resistance to meropenem, amikacin and imipenem.

CONCLUSION: We did not find a significant risk factor for bacteriobilia in patients with biliary obstruction. A bile sample for microbiological analysis may become a valuable diagnostic tool as it leads to more accurate selection of antibiotics for the treatment of cholangitis.

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Key words: Cholangitis; Endoscopic retrograde cholangiopancreatography; Bacteriobilia; Bile culture

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INTRODUCTION

Usually the bile ducts are sterile. However, the presence of gallstones within either the gallbladder or biliary tree is associated with the bacterial colonization of the bile^[1]. In patients without stone disease, previous biliary intervention is associated with high rates of bacteriobilia^[2,3]. Under conditions of normal bile flow, bacteria in the biliary system are of no clinical significance. Upon bile duct obstruction, bacteria proliferate within the stagnant bile while biliary pressure increases. Eventually, the bacteria presumably translocate into the circulation causing a systemic infection. Acute cholangitis spans a continuous clinical spectrum and can progress from a local biliary infection to advanced disease with sepsis and multiple organ dysfunction syndrome^[4]. Even recent studies have reported mortality rates of up to 10%^[5,6]. Acute cholangitis is mainly a result of microbial infection caused by bacteria and fungi^[7].

Blood cultures provide an opportunity to detect the causative organism but, even in febrile patients with cholangitis, blood cultures remain negative in more than half of the cases^[8]. Although definitive management of cholangitis involves the relief of bile stasis, effective empiric antibiotic therapy is an indispensable part of the treatment^[4,9]. Antimicrobial therapy recommendations state that antibiotics should be administered as soon as the diagnosis of acute cholangitis is suspected or established. Empirically administered antibiotics should be selected on the basis of antimicrobial activity against the causative bacteria, the severity of the cholangitis, the presence or absence of renal and hepatic failure, a recent (one year) history of antimicrobial therapy, local susceptibility pattern (antibiogram) and the biliary penetration of the antimicrobial agent^[4]. Previous studies have shown that Gram-negative bacteria, in particular *Escherichia coli*, are the most common pathogens isolated from infected bile^[9,10]. Because of the rapid development of multi-drug-resistant Gram-negative organisms, the choice of appropriate empiric antimicrobial therapy has become more complicated. Whenever any empirical antimicrobial agents are used, they should be switched for the best available narrower-spectrum agents to avoid superinfection or the emergence of antimicrobial resistance as a cause of treatment failure^[4]. Thus, knowledge of the common etiologic agents and their local susceptibility profile is essential to ensure the appropriate choice and timely administration of empiric antimicrobial therapy.

The aim of this study was to identify the most common bacteria grown in the bile and their antibiotic susceptibility. Furthermore, we investigated risk factors for microbiological colonization of the bile in patients with different biliary diseases.

MATERIALS AND METHODS

Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical

Association. This study was approved ethically by Dicle University, School of Medicine. All patients provided informed written consent.

Patients

This prospective study was conducted between April 2010 and August 2011 in the Department of Gastroenterology, Dicle University Hospital. Patients with various biliary disorders were included. Written informed consent was obtained from all patients and the trial was approved by the Local Ethical Committee. Exclusion criteria were age under 16 years, incomplete clinical and laboratory data or the absence of written, informed consent before the procedure.

Methods

All duodenoscopes (Olympus-TJF-260V, 2601168) were disinfected according to the guidelines and contamination was excluded by regular smear tests. The diagnosis of cholangitis was made when a patient had a fever (higher than 38 °C), abnormalities in liver function test results, and the exacerbation of jaundice (if any), with and without right upper quadrant pain, provided that other septic complications were ruled out. Bile was aspirated by placing a single-use, 5F, standard sphincterotome catheter (after guide-wire cannulation) into the bile duct before the injection of contrast agent for endoscopic retrograde cholangiopancreatography procedure. Approximately 2 to 8 mL of bile (mean of 4 mL) was collected and transferred in a sterile tube.

Bile specimens were transported to the microbiology laboratory in blood culture bottles and in an anaerobic transport system (BacT/Alert 3D Culture Media bioMérieux SA; Marcy l'Etoile, France). Bacteria were cultured and identified according to the standard protocol used in our clinical microbiology laboratory. The susceptibilities of the organisms recovered were identified using antimicrobial disks, chosen according to the initial Gram stain of the positive cultures. Microorganisms in concentrations of > 10 000 per mL were considered as an infection; lower concentrations were accepted as contamination or colonization.

Statistical analysis

Data from each group (cholangitis *vs* no cholangitis, malignant *vs* benign biliary disease, antibiotic administered *vs* not administered before procedure) were compared using the χ^2 test or the Fisher's exact test. All *P*-values were based on two-tailed tests. Analysis was performed with SPSS for Windows (SPSS Inc., Chicago, IL). A *P* value < 0.05 was considered significant.

RESULTS

Patients and general microbiological characteristics

Initially 125 patients were included in the study. Seventeen patients were excluded because of inadequate bile aspiration and 17 patients were excluded because of missing bile culture data. Finally, a total of 91 patients (27%

male, mean age 53.7 ± 17.5 years, range: 17-86 years) were analyzed. The main indications for cholangiographic interventions (Table 1) were benign biliary disease in 79 patients and malignant disease in 12 patients. Two patients with choledocholithiasis also had plastic stents and choledochojejunostomy was performed in one patient with choledocholithiasis. Seventeen (21.5%) patients with benign biliary disease had acute cholangitis before cholangiography. Thirty-five out of 91 (38.4%) patients were administered antibiotics empirically prior to the cholangiography (at least a single dose). All patients with acute cholangitis were administered antibiotics before cholangiography.

Bile culture was positive for bacterial growth in 46 out of 91 (50.5%) patients including 39 patients with benign disease and 7 with malignant disease. There were no patients in whom the concentration of microorganisms was lower than 10 000 per mL of bile. Table 2 shows the frequency of different organisms in positive bile cultures. A total of 48 organisms were isolated, comprising 15 different species. The most frequently encountered organisms were Gram-negative bacteria including *Escherichia coli* (28.2%), *Pseudomonas* (17.3%) and *Stenotrophomonas maltophilia* (15.2%).

Of 39 patients with benign biliary disease and positive bile cultures, there were aerobic bacteria grown in bile culture of 34 patients (87%), anaerobic bacteria in 3 (8%) and both aerobic and anaerobic bacteria grown in bile culture of 2 (5%) patients. Of 7 patients with malignant disease and positive bile cultures, there were aerobic bacteria grown in 6 bile cultures (86%) and anaerobic bacteria grown in 1 bile culture (13%). Monomicrobial growth was more frequent (96%) in comparison with polymicrobial cultures (4%). There was bacterial growth in bile culture of 9 out of 17 (52.9%) patients with acute cholangitis and in bile culture of 37 out of 74 (50%) patients without acute cholangitis. There was bacterial growth in bile culture of 17 out of 35 (48.6%) patients who were administered antibiotics and in bile culture of 34 out of 56 (60.7%) patients who were not administered antibiotics.

There were no significant differences between patients with malignant and benign disease (58% *vs* 49%, $P = 0.474$), patients with acute cholangitis and without acute cholangitis (52.9% *vs* 50%, $P = 0.827$), patients who were administered antibiotics empirically before the intervention and patients who were not administered them (51.4% *vs* 60.7%, $P = 0.384$), as regards the bacterial growth in bile culture.

Antibiotic susceptibility testing and resistance profiling

Antibiotics administered were ceftriaxone in 6 patients, ceftriaxone + ciprofloxacin in 1, ceftriaxone + ornidazole in 6, ciprofloxacin + sulbactam/amoxicillin in 5, ciprofloxacin in 10, cefuroxime axetil in 1, amoxicillin/clavulanic acid in 1, cefoperazone/sulbactam in 2, meropenem in 2 and cefazolin in 1 patient. Antibiotic susceptibility was measured for at least 11 types of antimicrobial substances. Table 3 shows the antibiotic susceptibility rate

Table 1 Main indications for cholangiographic intervention in the study population *n* (%)

Diagnosis	<i>n</i> (%)
Choledocholithiasis	75 (83)
Pancreatic cancer	6 (7)
Papilla cancer	4 (4)
Cholangiocellular cancer	2 (2)
Chronic pancreatitis	1 (1)
Bile leakage	1 (1)
Benign stricture	1 (1)
Fasciola hepatica	1 (1)

Table 2 Distribution of different organisms in positive bile cultures *n* (%)

Bacteria	<i>n</i> (%)
<i>Escherichia coli</i>	13 (28.2)
<i>Pseudomonas</i>	8 (17.3)
<i>Stenotrophomonas maltophilia</i>	7 (15.2)
<i>Enterococcus faecium</i>	4 (8.6)
<i>Enterobacter cloacae</i>	3 (6.5)
<i>Enterobacter aerogenes</i>	1 (2.1)
<i>Citrobacter freundii</i>	2 (4.2)
<i>Staphylococcus aureus</i>	1 (2.1)
<i>Streptococcus acidominimus</i>	1 (2.1)
<i>Achromobacter</i> species	1 (2.1)
<i>Pentoniphilicus asaccharolyticus</i>	1 (2.1)
<i>Lactobacillus gasseri</i>	1 (2.1)
<i>Bifidobacterium</i>	1 (2.1)
<i>Provotella disiens</i>	1 (2.1)
<i>Chryseobacterium meningosepticum</i>	1 (2.1)

Table 3 Results of antibiotic susceptibility tests

Antibiotic	Susceptibility (%)
Meropenem	86
Amikacin	86
Imipenem	79
Piperacillin/tazobactam	61
Gentamicin	53
Ciprofloxacin	52
Levofloxacin	51
Ceftazidime	46
Ampicillin	21
Cefotaxime	14
Ampicillin/sulbactam	11

of the identified bacteria in the bile culture. We observed a broad spectrum or low resistance to meropenem, amikacin and imipenem. There was high resistance for gentamicin (47%), ciprofloxacin (48%), levofloxacin (49%), ceftazidime (54%), ampicillin (79%), cefotaxime (86%) and ampicillin/sulbactam (89%). The most common bacterial organisms growing outside the covering antibiotic spectrum or with high resistance profile were *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecium*.

DISCUSSION

The traditional presentation of cholangitis includes the

triad of jaundice, fever and right upper quadrant pain^[11], though the actual presentation can be quite diverse, ranging from mild abdominal discomfort to life-threatening septic shock. Both biliary obstruction and bacteria in the bile are required for the development of cholangitis. In a healthy person, the biliary tree is normally sterile, but biliary pathologies are often associated with secondary bacterial colonization^[1]. Other studies have detected bacteriobilia in 16% to 85% of patients, depending on different disease groups^[8,10,12-15]. Bacterial bile culture positive rates are 58% to 76% in patients with choledocholithiasis + cholangitis, 29% to 54% in patients with acute cholecystitis, 13% to 32% in patients with cholelithiasis, and 70% to 93.9% in patients with hepatolithiasis + cholangitis^[4]. There is an increased incidence of positive bile culture in patients with acute cholangitis as compared to without cholangitis (67% *vs* 33%, $P = 0.012$), which has been reported by Salvador *et al*^[9]. Risk factors for bacteriobilia are orthotopic liver transplantation (OLT), steroid treatment, biliary stenting and repeated biliary intervention^[10]. Although some studies have reported that malignant biliary strictures and biliary stents increase risk of fungal colonization^[10], others have not reported this association^[16]. In our study, bacteriobilia was found in 50.5% of all patients. There were not any patients with OLT, steroid treatment or repeated biliary intervention and only two patients had biliary stenting. We did not find significant differences between patients with malignant and benign biliary disease, with cholangitis and without cholangitis and in patients who have been administered and not administered antibiotics before the intervention, as regards bacterial growth in the bile culture. These results may suggest that bacterial colonization in bile is dependent on multiple factors. The ineffectiveness of antibiotic administration before cholangiography on the bacterial growth in the bile culture may be related to high bacterial resistance against the antibiotics used. Although we identified bacterial growth in half of all patients, there were clinical findings of acute cholangitis in only 9 out of 46 patients with positive bile culture. We also found that 8 patients had all signs of acute cholangitis, but the bile culture was negative. Therefore, we suggest that all bacteriobilia can not cause obvious clinical symptoms of acute cholangitis and some patients with acute cholangitis may have negative bile culture. However, we cannot totally exclude contamination of the endoscope when it is passed through the upper gastrointestinal tract. To avoid contamination *via* cross-transmission between different patients, the duodenoscope was vigilantly disinfected. In all our patients with positive bile cultures, the concentration of microorganisms was higher than 10 000 per mL of bile. Therefore, we conclude that all patients with positive bile culture had actual bacterial growth that originated from aspirated bile.

It has been reported that microbial organisms contained in the bile from various biliary diseases are of intestinal origin. Aerobic bacteria such as *Escherichia coli*, *Klebsiella*, *Enterococcus* and *Enterobacter* are most frequently isolated, whereas *Streptococcus spp*, *Pseudomonas* and *Proteus*

are less frequently isolated^[17-19]. Although anaerobic bacteria such as *Clostridium* and *Bacteroides* are often isolated, most of these patients have polymicrobial infections with aerobic bacteria^[20-22]. Salvador *et al*^[9] have found that Gram-negative bacilli (or *Enterobacteriaceae*) are the most commonly isolated bacteria in the bile culture of both patients with (94%) and without (95%) cholangitis. In a large study involving 243 consecutive patients in Germany, polymicrobial growth in bile culture has been found more frequently (67%) in comparison with monomicrobial growth (33%). In this study, Gram-negative bacteria were found in 43% of patients, Gram-positive bacteria in 40%, *Candida* species in 10% and strict anaerobes in 7% of patients. The most frequently encountered organisms were *Enterococcus* species (31%), *Escherichia coli* (10%) and *Klebsiella* species (9%)^[10]. In our study, Gram-negative bacteria were the most common bile culture isolates for patients with and without acute cholangitis and almost all patients (95%) had monomicrobial growth in bile culture. *Escherichia coli* and *Pseudomonas* were the most frequently isolated bacteria. The results of our study may suggest that most of the bacteria in bile originate from the intestinal tract.

The management of cholangitis depends primarily on interventional biliary drainage and supplementation with antibiotic treatment. The combination of ampicillin and an aminoglycoside was regarded as a standard regimen for cholangitis in the 1980s^[4] and most randomized, controlled trials have concluded that recently developed antimicrobial drugs are effective and useful equivalents to that of ampicillin and aminoglycosides^[23,24]. Therefore, according to the clinical trials available so far, piperacillin, ampicillin and an aminoglycoside, and several cephalosporins, are recommended for the treatment of acute cholangitis^[4]. In the Tokyo Guidelines, the selection of antimicrobial agents is based on the severity of acute cholangitis. It has been recommended that antimicrobial agents administered empirically should be changed in favor of more appropriate agents, according to the identified causative microorganisms and their sensitivity to antimicrobials^[4]. In our patients, the most common empirically administered antibiotics were ceftriaxone, ciprofloxacin and sulbactam/amoxicillin. We did not identify a bacterial susceptibility test for ceftriaxone, but there was high resistance to other routines using antibiotics for cholangitis in our clinic. This high resistance may be related to commonly inappropriate use of these antibiotics in our region. A bile sample collected during cholangiography for microbiological analysis and antibiotic susceptibility tests may be valuable in the selection of appropriate antibiotics for the treatment of cholangitis.

In conclusion, our results indicate that about half of patients with biliary obstruction had bacteriobilia in their bile culture and the most commonly isolated bacteria were Gram-negative bacteria including *Escherichia coli* and *Pseudomonas*. When antibiotics were administered before cholangiography, the presence of cholangitis and the etiology of biliary obstruction had no significant effect on the incidence of bacteriobilia. There was a high resistance

against routinely used antibiotics, such as ciprofloxacin, and ampicillin and ampicillin/sulbactam. A bile sample for microbiological analysis may become a valuable diagnostic tool as it leads to more accurate selection of antibiotics for the treatment of cholangitis.

COMMENTS

Background

Acute cholangitis spans a continuous clinical spectrum and can progress from a local biliary infection to advanced disease with sepsis and multiple organ dysfunction syndrome. Knowledge of the common etiologic agents of cholangitis and their local susceptibility profile is essential to ensuring the appropriate choice and timely administration of empiric antimicrobial therapy.

Research frontiers

Blood cultures provide an opportunity to detect the causative organism but, even in febrile patients with cholangitis, blood cultures remain negative in more than half of the cases. Gram-negative bacteria, in particular *Escherichia coli*, are the most common pathogens isolated from infected bile. Because of the rapid development of multi-drug-resistant gram-negative organisms, the choice of appropriate empiric antimicrobial therapy has become more complicated.

Innovations and breakthroughs

Other studies have detected bacteriobilia in 16% to 85% of patients, depending on different disease groups. In this study, bacteriobilia was found in 50.5% of all patients. There was a large covering spectrum or low resistance to meropenem, amikacin and imipenem. The authors did not find significant risk factors for bacterial growth in the bile culture. The findings show that all bacteriobilia can not cause obvious clinical symptoms of acute cholangitis and some patients with acute cholangitis may have negative bile culture.

Applications

A bile sample for microbiological analysis may become a valuable diagnostic tool as it leads to more accurate selection of antibiotics for the treatment of cholangitis.

Terminology

Acute cholangitis is bile duct microbial infection caused by bacteria and fungi. Its main clinical findings are right upper quadrant pain, fever and jaundice.

Peer review

The authors have investigated the frequency of bacterial growth in the bile, the most commonly grown bacteria, their antibiotic susceptibility and risk factors for the bacterial colonization of the bile. They show that a bile sample for microbiological analysis during cholangiography may become a valuable diagnostic tool as it leads to more accurate selection of antibiotics for the treatment of cholangitis.

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Gender preference and implications for screening colonoscopy: Impact of endoscopy nurses

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Abstract

AIM: To assess the gender preferences, specifically the gender of the nursing staff (endoscopy assistants) and the impact on acceptance for screening colonoscopy (SC).

METHODS: Patients or relatives attending the clinics or health care workers working in a tertiary center were invited to participate in this questionnaire study. The questionnaire enquired on the general demographics (1) age, gender, ethnicity, education level, and employment status, previous history of colonoscopy, family or personal history of colonic pathologies, personal and family history of any cancers; (2) subjects were asked if they would go for an SC if they had appropriate indications (age over 50 years, family history of colorectal cancer (CRC), fecal occult blood positive, anemia especially iron deficiency anemia, bleeding per rectum with or without loss of appetite, weight loss and abdominal pain) with and without symptoms attributable to CRC; and (3) preferences for the gender of the endoscopists and assistants and whether they would still undergo SC even if their preferences were not met.

RESULTS: Eighty-four point seven percent (470/550) completed questionnaire were analysed. More female

subjects expressed gender preferences for the endoscopists [overall 70%; female (67.7%) and male (2.3%)] compared to male subjects [overall 62.8%; male (56%) and female (6.8%), $P = 0.102$]. Similarly, more female subjects expressed gender preferences for the assistants [overall 74.5%; female (73.4%) and male (1.1%)] compared to male subjects [overall 58%, male (49.3%) and female (8.7%), $P < 0.001$]. Overall, a third would decline an SC, despite having appropriate indications, if their preferences were not met. On univariate analysis, male gender, non-Malay ethnicity (Chinese and others) and previous colonoscopy experience were more likely to undergo an SC, even if their preferences were not met (all $P < 0.05$). Gender and previous experience [odds ratio (OR) 1.68, 95% confidence interval (CI) 1.00-2.82, $P < 0.05$] with colonoscopy (OR 4.70, 95% CI 1.41-15.66, $P < 0.05$) remained significant on multivariate analysis.

CONCLUSION: Genders preference for the endoscopy nurses/assistants is more common than for the endoscopist among women and has implications for the success of a screening colonoscopy program.

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Key words: Colorectal cancer; Screening colonoscopy; Gender preference; Patient satisfaction; Endoscopy

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in both men and women and remains an important cause of morbidity and mortality^[1,2]. The incidence rates are reported to be increasing, especially in the developing nations, but are stable or are leveling off in the more developed nations, such as the United States and Canada^[1,2].

Currently, colonoscopy is the recommended procedure of choice for CRC screening^[3-5]. Despite the effectiveness of CRC screening programs, with reduction in subsequent development of CRC and related mortality^[6,7], the uptake rates are still low. Ethnicity, socioeconomic status, public knowledge of CRC and availability of services are some of the factors that can affect the uptake of screening programs. Gender disparities among health care providers are evident in certain specialties^[8-10]. A similar situation exists in the gastroenterology fraternity^[11-14]. Several studies have shown that women prefer women endoscopists^[15-21]. However, none of these studies have looked at the gender of the endoscopists' assistants. This study assesses preferences with regard to the genders of the endoscopists and the assistants and their implications for screening colonoscopy (SC) uptake.

MATERIALS AND METHODS

Setting

This study was conducted (March to June 2008) in a referral center (RIPAS Hospital) in a country with a predominant Malay population. The study was conducted in accordance with the standard set out in the Declaration of the Helsinki and ethics approval was obtained from the Institutional Medical Health and Research Ethic Committee of the Hospital, Ministry of Health.

Subjects

Subjects included patients attending the various clinics or accompanying relatives and health care workers working in the center. Subjects were randomly approached and invited to participate in this self-administered questionnaire study. Verbal and detailed written explanations were given prior to handing out the questionnaires. Consents were also obtained. Only subjects 18 years old or above were included in the study.

Questionnaire

Development: The questionnaire was provided in two versions, English and Malay. The initial questionnaire was developed in English and forward translations to Malay were carried out by two persons fluent in both languages. Inconsistencies were amended based on consensus. This process was repeated until the two versions were consistent. The questionnaire was tested ($n = 20$) to assess for any problem before starting the survey.

Data collection: The questionnaire consisted of three parts; (1) demographic data (gender, age, ethnic group,

education level, and employment status, previous colonoscopy, family or personal history of colonic pathologies (polyps and cancer), personal and family history of any cancers (specifically CRC, other gastrointestinal cancers, pulmonary, obstetric and gynecology and breast cancers); (2) in the second section, subjects were asked if they would go for an SC assuming they had appropriate indications (age over 50 years, family history of CRC, fecal occult blood positive, anemia especially iron deficiency anemia, bleeding per rectum with or without loss of appetite, weight loss and abdominal pain)^[3-5]. They were also asked if they would go for an SC if they had appropriate indications but without any gastrointestinal symptoms attributable to a CRC. All these questions required only a "yes" or "no" response; and (3) subjects were asked for their preferences for the gender of the endoscopists and assistants (nursing staff involved with the procedure). These two questions required one out of three responses (same gender, opposite gender or no preferences). The final question inquired if they would still go for an SC if they had definite indications, but without their preferences being met. Subjects were informed that colonoscopies are carried out following the practice of the unit using conscious sedation (titrated intravenous midazolam and fentanyl).

Statistical analysis

The data were entered into the Statistical Program for the Social Sciences (SPSS, Version 10.0, Chicago IL, United States) for analysis. Univariate analysis (χ^2 test) was used to compare the categorical variables and only those variables that were found to be significant ($P < 0.10$) were entered into multivariate analysis. The level of significance was set at $P < 0.05$.

RESULTS

There were 470 completed questionnaire out of total 550 distributed, giving a response rate of 84.5%. The mean age of the sample was 39.4 ± 12.8 years old, with a male to female ratio of 44:56. Family history of any cancer was reported at 19.8% and of these, 7.4% were CRC. The demographic data is shown in Table 1.

In the presence of appropriate indications, including symptoms attributable to CRC, 90.9% would agree to undergo an SC. However, in the absence of any symptoms, only 59.4% would agree to undergo SC ($P < 0.05$).

With regard to the genders of the endoscopists, more female subjects expressed a gender preference (overall 70%; 67.7% for a female endoscopist and 2.3% for a male endoscopist), with 30% having no gender preference, compared to male subjects, of whom 62.8% ($P = 0.102$) expressed a gender preference, (56% for male endoscopists and 6.8% for female endoscopists) and 37.2% had no preference.

With regard to the gender of the assistants, significantly more female subjects expressed a gender preference [overall 74.5%; female (73.4%), male (1.1%) and no

Table 1 Demographic and previous medical history of subjects

Demographic data	n (%)
Gender	
Male	207 (44)
Female	263 (56)
Race	
Malays	370 (78.7)
Chinese	75 (16.0)
Others	25 (5.3)
Educations levels	
Higher	169 (36.0)
Lower/none	301 (64.0)
Employments status	
Working	356 (75.7)
Not working	114 (24.3)
Marital status	
Married	325 (69.1)
Single	129 (27.4)
Others	16 (3.4)
Previous colonoscopy	65 (13.8)
Family history of cancer	93 (19.8)
Gastric	15 (3.2)
Colorectal	35 (7.4)
Breast	17 (3.6)
Obstetric and gynecology	8 (1.7)
Pulmonary	12 (2.6)

Table 3 Multivariate analysis showing willingness to undergo screening colonoscopy despite preferences not being met

Variables	OR	95% CI	P value
Gender			
Male vs female	1.68	1.00-2.82	0.049
Race			
Malays vs non-malays	1.94	0.910-4.130	0.086
Previous colonoscopy			
Yes vs no	4.70	1.41-15.66	0.012

OR: Odd ratio; CI: Confidence interval.

preference (25.5%)] compared to male subjects [overall 58%, male (49.3%), female (8.7%) and no preference (42%) ($P < 0.001$).

Overall, a third would decline an SC if their preferences were not met (for either or both endoscopists and assistants), even if there were indications for an SC. Factors that were associated with likelihood of agreeing to undergo SC even when their preferences were not met included male gender, non-Malay ethnicity and previous experience of colonoscopy. Education level, marital status, employment status and family history of cancers, including CRC, were not predictive (Table 2).

On multivariate analysis, male gender and previous experience of colonoscopy remained significant predictive factors for agreeing to undergo an SC, despite preferences not being met (Table 3).

DISCUSSION

To date, there have been several studies that had looked at gender preferences during colonoscopy^[15-21]. However,

Table 2 Factors associated with likelihood for subjects to agree to undergo screening colonoscopy despite their preferences not being met

Parameters	Will undergo SC	P value
Recommended age		
Yes	102 (86.4)	0.222
No	287 (81.5)	
Gender		
Male	180 (87.0)	0.033
Female	209 (79.5)	
Race		
Malays	308 (81.1)	0.043
Non-malays	81 (90.0)	
Education		
Higher	134 (79.3)	0.135
Lower/none	255 (84.7)	
Marital status		
Single	102 (79.1)	0.192
Married/widowed	287 (84.2)	
Employment		
Yes	294 (82.6)	0.854
No	95 (83.3)	
Previous colonoscopy		
Yes	62 (95.4)	0.004
No	327 (80.7)	
Family history of cancer		
Yes	76 (81.7)	0.766
No	313 (83.0)	
Family history of CRC		
Yes	28 (80.0)	0.652
No	361 (83.0)	

SC: Screening colonoscopy; CRC: Colorectal cancer.

they only assessed the preference for the gender of the endoscopists and not that of the endoscopy nurses or assistants.

Of these studies, most looked at mainly Caucasian populations^[15-18,20] and one study from the States concentrated on a Hispanic population^[19]. To date, there is only one study from the East, which assessed the preferences of Korean women^[18]. Two studies specifically looked at female subjects^[17,18]. Most of the studies recruited subjects from clinics^[17-21] and two studies looked at patients who were scheduled for colonoscopy^[15,16]. All these studies showed similar findings, with women expressing more preferences for female endoscopists, even among healthcare professionals^[21]. The present study showed the highest preference rates reported for both women (70%) and men (62.8%). The findings of these studies are summarized in Table 4.

Generally, when patients express a preference, they usually prefer an endoscopist of the same gender. Several factors have been reported to be important and not just for endoscopic procedures^[22]. Factors reported to be important include ethnicity, female gender, younger age, low income, history of abuse (either physical or emotional), being cared for by a female primary care physician, being employed, and being single^[17-21]. However, not all the studies reported consistently similar findings. One of the reasons that may account for the finding in the current study is cultural differences. Asian populations are gener-

Table 4 Comparison of gender preference during colonoscopy

Authors (yr)	Subjects	Setting	Genders	Population	Gender preference
Fidler <i>et al</i> ^[14] , 2000	Patients undergoing colonoscopy	Endoscopy	Both	United Kingdom	Women (48%) and men (0%)
Varadarajulu <i>et al</i> ^[15] , 2002	Patients undergoing colonoscopy	Endoscopy	Both	United States	Overall (26%): Women (45%) and men (4.3%). No difference post procedure
Menees <i>et al</i> ^[16] , 2005	Subjects not scheduled	Clinics	Female	United States	44.4% expressed preference [endoscopist: Women (43%) and men (1.4%)]
Lee <i>et al</i> ^[17] , 2008	Subjects not scheduled for colonoscopy	Clinics	Female	South Korea	45.5% expressed preference [endoscopist: Women (32.1%) and men (13.4%)]
Schneider <i>et al</i> ^[18] , 2009	Patients undergoing colonoscopy	Endoscopy	Both	United States	Women (42%) and men (24%)
Zapatier <i>et al</i> ^[19] , 2011	Patients not scheduled for colonoscopy	Clinics	Both	United States	Overall (25.7%): Women (30.8%) and men (20.4%); women: Hispanic (35%) and Caucasian; men (20.4%): Hispanic men
Shah <i>et al</i> ^[20] , 2011	Patients not scheduled for colonoscopy	Primary clinics	Both	United States	Patients: Women (53%) and men (27.8%); Health care professionals: Women (43.1%) and men (26.1%)
Present study, 2012	Subjects not scheduled for colonoscopy	Clinics	Both	Southeast Asia	Endoscopists: Women (70%; women 67.7% and men 2.3%); men (62.8%; women 6.8% and 56% men) Assistants: Women (74.5%; women 73.4% and men 1.1%); men (58%; women 8.7% and 49.3% men)

ally more reserved, especially women, and are more likely to prefer to deal with health care providers of the same gender.

When the preference for the gender of the assistants was assessed, similar findings were seen. However, the preference rate for women subjects was higher compared to the rate expressed for the endoscopist. In contrast, it was slightly lower for men. This indicates that, the gender of the assistants is more important for female patients than for male patients. The reasons for this are probably similar to those found in studies looking at gender preferences among endoscopists^[15-21]. Colonoscopy can be considered as invasive, uncomfortable, and embarrassing and patients usually have more interactions with the endoscopists' assistants or nurses. For women, embarrassment, feeling at ease talking to, and being examined by, female health care providers are some of the important factors. Most were willing to wait or pay extra for their preferences to be met.

When subjects' preferences were not a problem, the uptake of SC was good in the presence of symptoms attributable to colonic pathology, but not in the absence of symptoms. However, when their preferences were not met, a third would decline an SC even if an SC was indicated. On univariate analysis, male gender, non-Malay ethnicity and previous experience of colonoscopy were significant predictive factors for agreeing to undergo an SC, even if preferences were not met. Only gender and previous experience of colonoscopy remained significant on multivariate analysis. None of the previous studies assessed the uptake of colonoscopy if subjects' preferences were not met^[15-21]. The finding of the present study are not unexpected. Previous studies have already shown that women expressed more preferences. Interestingly, one study showed the rates of SC did not increase when women were offered a female endoscopist in a health promotion outreach program^[23], suggesting other factors are involved^[24,25]. Surprisingly, socioeconomic status and family history of cancer, including gastrointestinal cancers, did

not influence subjects' decisions for SC.

There are several limitations with the present study. First, it was a single center study and the result may not be generalizable to other populations. However, the findings are consistent with findings of previous studies. Second, the mean age of the studied population was much younger than the other studies and the recommended screening age. However, as with all screening programs, all subjects are potential screening subjects and all will eventually become eligible with time.

In conclusion, the gender of the endoscopy nurse or assistant is more important than the gender of the endoscopist among female subjects. Importantly, a third of the subjects, particularly women, would decline an SC if their preferences were not met. Male gender and previous experience of colonoscopy were predictive factors for agreeing to undergo an SC, even when their preferences were not met. Therefore, addressing the gender disparities of health care providers, not just of the endoscopists, but also of the endoscopists' assistants is important to improve the uptake rate of any SC programs.

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COMMENTS

Background

Colorectal cancer (CRC) is the third most common cancer and remains an important cause of cancer related mortality. Screening colonoscopy (SC) has been shown to reduce the incidence of CRC and prevent CRC related death. However, uptake of SC remains low for various reasons, including both patient and non-patient factors. Gender preference for the healthcare service providers is common and may impact on uptake rates of service delivery.

Research frontiers

Several studies based on patients scheduled for lower gastrointestinal endoscopy and clinics have shown that gender preference for a same gender en-

doscopist is more common among women than men. This is true even among health care professionals. Most of those who expressed a gender preference were willing to wait or pay for their preferences to be met.

Innovations and breakthroughs

Previous studies have only assessed subjects' preferences for the gender of the endoscopist. This study also assessed the subjects' preferences for the gender of the assistants and their impact on uptake of SC.

Applications

This study showed that the gender of the endoscopist's assistants or staff nurse involved with the procedure is also important. It also showed that not meeting the preference of patients, especially women, would affect the uptake of SC if indicated. In this study of a Southeast Asian population, a third of subjects would decline an SC if their preferences were not met.

Terminology

CRC is a malignant neoplasm of the colon and rectum and commonly develops through the adenoma-dysplasia-carcinoma sequence, which usually takes many years. SC is now the recommended screening procedure of choice for CRC, as it allows detection and removal of early neoplasms (adenoma with or without carcinoma *in situ*).

Peer review

This study shows that gender of not just the endoscopist but also of the endoscopy nurse or assistants need to be considered in the delivery of colonoscopy and that this may impact on the uptake rate of an SC program.

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Sedation-associated hiccups in adults undergoing gastrointestinal endoscopy and colonoscopy

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Abstract

AIM: To investigate whether the incidence of hiccups in patients undergoing esophagogastroduodenoscopy (EGD) or same-day bidirectional endoscopy (EGD and colonoscopy; BDE) with sedation is different from those without sedation in terms of quantity, duration and typical onset time.

METHODS: Consecutive patients scheduled for elective EGD or same-day BDE at the gastrointestinal endoscopy unit or the health examination center were allocated to two groups: EGD without sedation (Group A) and BDE with sedation (Group B). The use of sedation was based on the patients' request. Anesthesiologists participated in this study by administering sedative drugs as usual. A single experienced gastroenterologist performed both the EGD and the colonoscopic examinations for all the patients. The incidence, duration and onset time of hiccups were measured in both groups. In addition, the association between clinical variables and hiccups were analyzed.

RESULTS: A total of 435 patients were enrolled in the study. The incidences of hiccups in the patients with and without sedation were significantly different (20.5% and 5.1%, respectively). The use of sedation for patients undergoing endoscopy was still significantly associated with an increased risk of hiccups (adjusted odds ratio: 8.79, $P < 0.001$) after adjustment. The incidence of hiccups in males under sedation was high (67.4%). The sedated patients who received 2 mg midazolam developed hiccups more frequently compared to those receiving 1 mg midazolam ($P = 0.0028$). The patients with the diagnosis of gastroesophageal reflux disease (GERD) were prone to develop hiccups ($P = 0.018$).

CONCLUSION: Male patients undergoing EGD or BDE with sedation are significantly more likely to suffer from hiccups compared to those without sedation. Midazolam was significantly associated with an increased risk of hiccups. Furthermore, patients with GERD are prone to develop hiccups.

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Key words: Anesthesia; Midazolam; Hiccup; Gastroesophageal reflux disease; Esophagogastroduodenoscopy; Bidirectional endoscopy

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INTRODUCTION

Esophagogastroduodenoscopy (EGD) and colonoscopy are important diagnostic tools for evaluating gastrointestinal diseases or for the early detection of malignant lesions. Although same-day bidirectional endoscopy (EGD and colonoscopy; BDE) has many advantages, including shorter hospital stays and expedited decision making for patient care^[1], BDE remains a difficult and longer endoscopic procedure. Sedation is usually used to alleviate patient discomfort. Several regimens are available for inducing moderate sedation, but the combination of propofol with an opioid^[2-4] and/or a benzodiazepine^[5,6] is most commonly used. The combined regimens could improve the safety profile of propofol by reducing its dose-dependent potential to induce deep sedation^[7], in which patients may develop inadequate spontaneous ventilation and may require assistance to maintain a patent airway. In addition to hemodynamic or respiratory depression, the other frequently encountered clinical situation during endoscopic procedures with sedation is hiccups.

Hiccups are an involuntary spasmodic contraction of the diaphragm and accessory muscles followed by the sudden closure of the glottis^[8]. Hiccups during an endoscopic procedure are commonly acute and temporary, but the intermittent involuntary contraction of the diaphragm may interfere with spontaneous breathing and hamper endoscopic investigations and interventions^[9]. In addition, this powerful reflex can result in markedly negatively intrathoracic pressures and increased abdominal pressures^[8]. Accompanied by gastric distension due to the air inflation that is required during EGD, hiccups can induce reflux of gastric contents into the esophagus and can cause regurgitation and aspiration, particularly in sedated patients^[10,11].

Although their exact mechanism remains unknown, hiccups may be induced by stimulating the postulated hiccup reflex arc, which is a complex interaction mainly composed of the phrenic and vagus nerves, the sympathetic chain and accessory nerves connecting to the glottis and the inspiratory intercostal muscles^[12-14]. Vagus and phrenic nerve irritation, central nervous system disorders, toxic-metabolic disorders, and psychogenic factors are 4 possible mechanisms leading to hiccups^[12,14,15]. As described above, gastric distention during endoscopy stimulates the phrenic nerve and causes hiccups. Drug-induced hiccups are uncommon, but anesthetics and sedative agents, such as benzodiazepines, opioids, and intravenous general anesthesia drugs, are suspected agents in cases of drug-induced hiccups^[13]. On the other hand, the sedation of patients to relieve anxiety and discomfort appears to decrease the incidence of hiccups during endoscopic procedures^[12]. Therefore, whether sedation can protect the patients undergoing endoscopic procedures against the onset of hiccups is unknown and requires further investigation.

Gastroesophageal reflux disease (GERD) is a common outpatient gastroenterology problem^[16,17]. Patients with GERD may present a variety of symptoms, ranging

from typical symptoms, such as heartburn or regurgitation, to atypical symptoms, such as chest pain, asthma, laryngitis, or chronic cough^[16]. Hiccups represent an atypical symptom of GERD, with a prevalence of 4.5% to 9.5% in GERD patients^[18,19]. The major pathophysiology of GERD includes increased episodes of transient lower esophageal sphincter (LES) relaxations, ineffective esophageal motility, and reduced LES tone^[20-22]. Interestingly, hiccups could also induce the transient decrease of LES tone^[8,20] and even the loss of LES tone^[23]. Therefore, the increased probability of hiccups in GERD patients during sedation requires further investigation.

The goals of this study were to determine the incidence of hiccups in patients undergoing EGD or BDE with or without sedation, the possible causes of hiccups in sedated patients and the association of hiccups with GERD in sedated patients.

MATERIALS AND METHODS

Patient selection

This prospective study was approved by the Institutional Review Board of the E-DA hospital (EMRP-098-125). Consecutive adult (18 years or older) patients scheduled for outpatient EGD or same-day BDE at the gastrointestinal endoscopy unit or the health examination center were included. The exclusion criteria included history of esophageal, gastric or colorectal cancer; history of esophageal or gastric surgery; esophageal or gastric varices; allergy to propofol or eggs; and American Society of Anesthesiology risk Class 3 or higher.

The participating patients were allocated into 2 groups: EGD without sedation (Group A) and BDE with sedation (Group B). Sedation used for endoscopic examination was based on the patient's explicit request. The sample size was calculated from the results of our pilot study of patients who underwent EGD without sedation. We assumed that the patients with sedation would show a different (approximately 15%) prevalence of hiccups than the nonsedated patients. Therefore, 195 patients per group were required [where $\alpha = 0.05$ (two-tailed), $\beta = 0.2$]. At least 400 patients were studied to allow for any loss of follow-up.

Study design

After consent was obtained, demographic, medical and drug history data were recorded for each patient. On arrival in the endoscopy room, each patient was routinely given supplemental oxygen (4 L/min) thorough a nasal cannula. In addition, an intravenous route was set up in patients of Group B, and no intravenous fluids were given before the initiation of sedation. Routine patient monitoring included electrocardiography, noninvasive arterial blood pressure measurement, and pulse oximetry.

In all the participating patients (Group A and Group B), 20 mg hyoscine *N*-butyl bromide (Buscopan) was administered intravenously as premedication except in those with glaucoma, obstructive uropathy, coronary heart diseases, or a history of allergies to anticholiner-

gic drugs. Anesthesiologists participated in this study by administering sedative drugs. Fifty microgram of fentanyl plus midazolam were given, followed by the administration of propofol approximately 1 to 2 min later. However, the choice of whether to use midazolam as an adjuvant and the administration dosage were determined by the anesthesiologists without further input from the researchers. To avoid any pain from injecting propofol, lidocaine was mixed with propofol before administration (1 mL of 2% lidocaine in every 20 mL of 1% propofol). An initial bolus of 1.5 mg/kg of propofol was given and then titrated in 10- to 20-mg increments to achieve an adequate level of sedation.

A single experienced gastroenterologist performed both the EGD and the colonoscopic examinations for all the patients. The EGD started when an adequate level of sedation (in which the patient was asleep, not responding verbally but ventilating spontaneously) was achieved. Once the EGD was finished, the subsequently colonoscopy was preformed immediately. During the endoscopic procedure, the need to add more propofol was estimated by the patient's pain response (i.e., moans, grimaces, gag reflex, and movements).

Predefined complications (hypotension, bradycardia, airway obstruction, hypoventilation, and hypoxia) were managed according to the hospital's routine protocol. At the end of the procedure, the patients were transferred to the recovery room. The time when the patient was ready for discharge according to hospital criteria (oxygen saturation > 95% on air, heart rate > 60 beats/min, systolic blood pressure \pm 30 mmHg from preoperative values, orientated, pain score < 4/10, and no nausea or vomiting) was recorded.

Measurements

Demographic data were recorded, including each patient's age, gender and body weight. Medical data were collected, including diabetes, hypertension, cigarette smoking/alcohol consumption, cerebrovascular diseases, gastrointestinal disorders, GERD-like syndromes, liver diseases, psychological disorders, and history of intractable hiccups. The patients' drug histories regarding agents suspected of causing hiccups [i.e., non-steroidal anti-inflammatory drugs (NSAIDs), benzodiazepines and aminophylline] was also recorded. Sedative regimens, midazolam and propofol dose titrated to an adequate level of sedation for EGD insertion and total propofol dose were recorded. Oxygen saturation, heart rate and arterial blood pressure were recorded every 5 min during sedation. All the times were recorded from a continuously running stopwatch. Endoscopy time was defined as the time from the insertion and until withdrawal of the colonoscope from the anus. If hiccups occurred during the procedure, the time hiccups occurred, the time hiccups subsided and the administered doses of propofol were recorded. After the endoscopic examinations, the patients with a diagnosis of GERD and other gastrointestinal disease revealed by the EGD were collected.

Statistical analysis

All the continuous data were tested for normality. Normally distributed continuous data were expressed as the mean \pm SD, and *t* tests were used to compare the means of continuous data. Skewed data were expressed with medians and ranges and were compared using Wilcoxon's ranked sum test. Categorical data were analyzed using the Pearson χ^2 test with Yates' correction or Fisher's exact probability test. When there were more than two groups, normally distributed data were compared using analysis of variance, and skewed data were compared using the Kruskal-Wallis test.

In all tests, a 2-tailed *P* < 0.05 was considered statistically significant. The relative magnitudes of the associations between individual (categorical and continuous) variables and the likelihood of the occurrence of hiccups was compared using crude odds ratios (ORs). The precision of the estimated ORs was assessed by examining the 95% confidence intervals (CIs). A multivariate logistic regression model containing all the candidate variables was used to examine the independent contribution made by each variable, while controlling for all the variables. This resulted in an adjusted OR and a calculated 95% CI. The statistical analyses were performed with SPSS for Mac version 19.0 (SPSS Inc., Chicago, IL).

RESULTS

A total of 435 patients were invited to participate in our study. Ten patients were excluded from the analysis because of incomplete data collection. Therefore, a total of 425 data sets were eligible for analysis: 215 data sets were from the non-sedation group, and 210 were from the sedation group. Table 1 summarizes the baseline characteristics of the patients in this study. There was no significant difference between the 2 groups regarding gender, body weight, hypertension, psychological disorders, or aminophylline intake history. However, there were more patients in Group A who took NSAIDs and benzodiazepines and had histories of diabetes, cigarette smoking/alcohol consumption, cerebrovascular diseases, gastrointestinal disorders, GERD-like syndromes and liver diseases than in Group B.

Though the indications for endoscopic examination in our patients were including epigastralgia, abdominal fullness, dysphagia, change in bowel habit, positive fecal occult blood test, and bloody stool *etc.*, the indication in most patients was for health check-up. Hence, these significant differences in baseline characteristics were because most Group B participants undergoing endoscopic examination were for health check-up.

Assessment for the association between sedation and hiccups

The incidences of hiccups in Group B (under sedation) and Group A (without sedation) were 20.5% and 5.1%, respectively. The ORs and 95% CI for the occurrence of hiccups induced by sedation were calculated. The use of sedation for patients undergoing endoscopy was

Table 1 Baseline characteristics of patients in the study *n* (%)

	Group A (<i>n</i> = 215)	Group B (<i>n</i> = 210)	<i>P</i> value
Gender (male)	118 (54.9)	111 (52.9)	0.675
Body weight (kg)	63.6 ± 12.4	64.3 ± 17.6	0.675
Age (yr)	53.1 ± 15.3	48.9 ± 11.6	0.002
Diabetes	28 (13.0)	12 (5.7)	0.012
Hypertension	47 (21.9)	33 (15.7)	0.109
Cigarette smoking/ alcohol consumption	72 (33.5)	36 (17.1)	< 0.001
Cerebrovascular diseases	18 (8.4)	6 (2.9)	0.019
Gastrointestinal diseases	130 (60.5)	30 (14.3)	< 0.001
GERD-like syndromes	103 (47.9)	17 (8.1)	< 0.001
Liver diseases	48 (22.3)	22 (10.5)	0.001
Psychological disorders	2 (0.9)	4 (1.9)	0.445
Drug history			
NSAIDs	36 (16.7)	6 (2.9)	< 0.001
Benzodiazepines	22 (10.2)	7 (3.3)	0.006
Aminophylline	7 (3.3)	2 (1)	0.175

The data are presented as the mean ± SD (normally distributed data), or *n* (%) (categorical data). Group A: Esophagogastroduodenoscopy (EGD) without sedation; Group B: Bidirectional endoscopy (BDE) with sedation; NSAIDs: Non-steroid anti-inflammatory drugs; GERD: Gastroesophageal reflux disease.

significantly associated with an increased risk of hiccups (OR, 4.78; 95% CI: 2.39-9.56). This association remained significant and independent after adjustment for age, diabetes, cigarette smoking/alcohol consumption, cerebrovascular diseases, gastrointestinal disorders, GERD, liver diseases, and the use of NSAIDs or benzodiazepines (adjusted OR, 8.79; 95% CI: 3.27-23.60).

Comparison of the contributing factors to hiccups

Table 2 summarizes the characteristics of the patients who developed hiccups and those who did not during sedation. Gender was the only significant difference between these groups (*P* < 0.05). The majority of the patients who developed hiccups during sedation were male (67.4%).

Comparison of the effect of sedative drugs on hiccups

The onset time (counted from the insertion of EGD) of hiccups was 6 min (1-21 min) [median (range)], and their duration was 1 min (1-19 min) [median (range)]. All the hiccups occurred before the end of the EGD in sedated patients, except in 3 patients who suffered from hiccups after the insertion of the colonoscope (onset time was 21, 17 and 16 min). Two patients suffered from hiccups that lasted for 19 and 15 min.

In the pharmaceutical characteristics of the development of hiccups, the effect of the midazolam dose was observed (Table 3). The patients who received 2 mg midazolam (1-2 mg) (range) developed hiccups more frequently compared with patients who received 1 mg midazolam (range: 0-2.5 mg, *P* = 0.028).

Assessment for the association between diagnostic findings of endoscopy and hiccups

Diagnostic findings of endoscopy were listed in Table 3.

Table 2 Baseline characteristics of patients in the sedation group (Group B) *n* (%)

	Hiccups (<i>n</i> = 43)	No hiccups (<i>n</i> = 167)	<i>P</i> value
Gender (male)	29 (67.4)	82 (49.1)	0.032
Body weight (kg)	66.0 ± 10.1	63.9 ± 19.0	0.524
Age (yr)	47.3 ± 11.5	49.3 ± 11.5	0.306
Diabetes	4 (9.3)	8 (4.8)	0.256
Hypertension	7 (16.3)	26 (15.6)	0.909
Cigarette smoking/ alcohol consumption	6 (14.0)	30 (18.0)	0.534
Cerebrovascular diseases	2 (4.7)	4 (2.4)	0.428
Gastrointestinal diseases	7 (16.3)	23 (13.8)	0.675
GERD-like syndromes	5 (11.6)	12 (7.2)	0.341
Liver diseases	4 (9.3)	18 (10.8)	0.778
Psychological disorders	1 (2.3)	3 (1.8)	0.821
Drugs history			
NSAIDs	3 (7.0)	3 (1.8)	0.069
Benzodiazepines	1 (2.3)	6 (3.6)	0.680
Aminophylline	0 (0)	2 (1.2)	0.471
Premedication			
Buscopan	37 (86.0)	121 (76.5)	0.066

The data are presented as the mean ± SD (normally distributed data) or *n* (%) (categorical data). NSAIDs: Non-steroid anti-inflammatory drugs; GERD: Gastroesophageal reflux disease.

The patients with diagnosed GERD were more prone to develop hiccups (*P* = 0.018).

DISCUSSION

To the best of our knowledge, this is the first study to examine the prevalence of hiccups in patients undergoing EGD or BDE. In this prospective study, sedation was associated with a significantly increased occurrence of hiccups. Midazolam, which was used in our combined sedation regimens, was associated with the onset of hiccups. In addition, the patients diagnosed with GERD revealed by EGD were more prone to develop hiccups while undergoing endoscopic procedures with sedation.

Clinically, most hiccup episodes begin with an acute onset, are benign, and are self-limited, typically ceasing within minutes^[24]. However, the sudden onset of hiccups may become a safety hazard while patients are sedated. Hiccup-associated acute negative intrathoracic pressure may occur, resulting in hypotension and bradycardia^[24]. This effect is attributed to decreased vascular resistance resulting from increased dilation and volume of the thoracic aorta^[25]. In our study, no statistically significant changes occurred in any hemodynamic measure following the onset of hiccups. Whether hiccup-associated systolic hypotension is deleterious to cardiovascular function in adults is unclear, but it remains a plausible etiology of pathological hemodynamic changes in those patients with underlying heart disease^[26].

Hiccups could influence the respiration of the patient during anesthesia. Unexpected pulmonary aspiration has been diagnosed in positron emission tomography (PET) screening following panendoscopy under conscious sedation^[27]. In our patients, we observed no respiratory

Table 3 Intra- and post-procedure study parameters of patients in the sedation group (Group B) *n* (%)

	Hiccups (<i>n</i> = 43)	No hiccups (<i>n</i> = 167)	<i>P</i> value
Endoscopy time (min)	16 (5-40)	15 (0-35)	0.186
Sedation regimens, mean (range)			
Fentanyl (μg)	50	50	-
Midazolam (mg)	2 (0-2)	1 (0-2.5)	0.028
2% Lidocaine (mg)	40 (0-60)	40 (0-60)	0.453
Propofol (mg)	70 (50-150)	80 (40-200)	0.955
Diagnostic findings of endoscopy, <i>n</i> (%)			
Superficial gastritis	41 (95.3)	163 (97.6)	0.428
Gastric ulcer	3 (7.0)	18 (10.8)	0.459
Duodenal ulcer	4 (9.3)	14 (8.4)	0.848
Gastric polyp	3 (4.7)	10 (6.0)	0.736
Esophageal diverticulum	0 (0.0)	2 (1.2)	0.471
GERD	11 (25.6)	19 (11.4)	0.018
Internal hemorrhoid	20 (46.5)	68 (40.7)	0.492
Colon polyp	4 (9.3)	7 (4.2)	0.180
Rectal polyp	4 (9.3)	7 (4.2)	0.180
Cecal diverticulosis	1 (2.3)	3 (1.8)	0.821

The data are presented as median (range) (skewed data) or *n* (%) (categorical data). GERD: Gastroesophageal reflux disease.

distress associated with cyanosis or desaturation on pulse oximetry in patients with hiccups during the endoscopic examinations or in the recovery room. Although aspiration during sedation may be silent and uneventful, hiccups in sedated patients do carry clinical risks^[10,28].

Acute gastric distention due to air inflation during endoscopy might result in hiccups by stimulating gastric vagal afferent activity^[24]. To avoid any influence of the gas inflation that is required during a colonoscopy, we conducted EGD before colonoscopy in the patients of Group B. Furthermore, to avoid inter-observer variation, a single physician performed all the EGD and colonoscopy procedures. In the sedation group, the hiccups all occurred during the EGD, and almost all of the hiccups ceased before the initiation of colonoscopy. Further, the incidence was significantly greater than in the nonsedated group (Group A). Therefore, causes other than this mechanical factor could have induced hiccups. Psychogenic factors (e.g., anxiety, stress, and excitement) are other possible causes of hiccups^[12]. Therefore, sedation for EGD or BDE relieves patient anxiety and seems to decrease the incidence of hiccups. However, Group B did not show a lesser incidence of hiccups than Group A. In fact, the use of sedative drugs during endoscopic procedures favored the onset of hiccups.

Although there was insufficient evidence in Thompson's^[15] review to conclude that any specific drug induces drug-related hiccups, corticosteroids and benzodiazepines are the most frequently suspected agents in cases of drug-induced hiccups^[15,29]. Midazolam is a benzodiazepine with rapid onset, brief duration of action and an amnestic effect^[30], and it is commonly administered in combination with an opiate and/or propofol to achieve adequate sedation for an EGD or colonoscopy. In addi-

tion, midazolam has gained popularity for use in procedural sedation and anxiolysis in pediatric patients. Midazolam is water-soluble and can be safely administered in a variety of ways. Marhofer *et al*^[31] had investigated that the incidence of hiccups in pediatric patients who were pre-medicated with two different doses of rectally administered midazolam for minor surgery. Their patients in group A received 0.5 mg/kg of midazolam, while their patients in group B were treated with 1 mg/kg. Twenty-four percent of the children developed hiccups, but no statistically significant difference was noted between the two doses (22% in group A *vs* 26% in group B). Hiccups were more common among younger children (i.e., those aged 5-6 mo *vs* 20 mo). In our sedated patients, the total incidence of hiccups was similar, but age was not a statistically significant variable. In addition, the development of hiccups was associated with higher doses of midazolam.

Interestingly, drug-induced hiccups are reported more commonly in men than women^[29]. In our study, the majority of patients who developed hiccups during sedation were male. This finding is consistent with recent studies revealing a significant male predominance of hiccups in patients receiving cytotoxic chemotherapy^[32,33]. However, the mechanisms for the male predominance of hiccups and the midazolam-induced hiccups are still unknown. Hiccups might be induced by the influence of midazolam on a supraspinal hiccup center localized in the brain stem^[34]. Another factor causing midazolam-induced hiccups could be a direct stimulation of the inspiratory muscles, specifically on diaphragm contractility^[35]. By contrast, Fujii *et al*^[36] indicated that midazolam decreases the contractility of the diaphragm in a dose-dependent manner in an animal study. Furthermore, intravenously administered midazolam has been successfully used in patients with terminal hiccups^[37]. The discrepancies among these results perhaps reflect the complicated etiology of hiccups; further studies are required to improve our understanding of their etiology. Nevertheless, our study provides further evidence for the association of midazolam administration with the onset of hiccups.

Graham^[23] showed that there is no detectable LES tone during a hiccup attack. Hiccups add tension to the phrenoesophageal ligament and thus have the same sphincter-dilating effect. Therefore, hiccups could induce episodes of transient decrease of LES tone^[8,20] that result in reflux both in normal subjects and in esophagitis patients^[38]. Furthermore, LES function can be overcome by the transient peritoneal-pleural gradient created during hiccups^[39]. In the study of Werlin *et al*^[40], 27% transient increases in intra-abdominal pressure, such as would be caused by a hiccup, were associated with reflux. According to Vanner^[11], approximately 40% of patients who hiccup after induction of anesthesia develop detectable gastroesophageal reflux.

The most important aspect of the pathophysiology of GERD is the competency of the anti-reflux barriers^[22]. Factors contributing to their integrity include the

LES pressure, the presence or absence of a hiatal hernia, and the occurrence of transient LES relaxation^[22,41]. Transient LES relaxation is considered the most common pathophysiologic event at the time of a reflux episode^[22,41]. Hence, the fact that patients with GERD are prone to develop hiccups during sedation might be due to previous abnormalities of LES.

This study had several weaknesses. Because of the use of sedation is a self-pay service and based on the patients' request, it is difficult to perform a randomized controlled trial to detect the incidence of hiccups in patients undergoing EGD or BDE with or without sedation. Though there is potential for bias in our prospective and cross sectional study, the study design could examine the association among the variables (e.g., sedation *vs* hiccups in our study)^[42]. In addition, the fact that the choice of midazolam as an adjuvant was not random limits the ability to draw conclusions about its hiccup-inducing effect, but it raises interesting hypotheses and provides pilot data for testing in the future.

In conclusion, sedation was associated with the occurrence of hiccups in patients undergoing gastrointestinal endoscopic procedures, and hiccups occurred primarily in males. Midazolam, a sedative drug, was significantly associated with an increased risk of hiccups. Furthermore, patients with GERD were prone to develop hiccups.

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COMMENTS

Background

During esophagogastroduodenoscopy (EGD) and colonoscopy procedure, sedation is usually used to alleviate patient discomfort. In addition to hemodynamic and respiratory depression, hiccups are the other frequently encountered clinical situation during endoscopic procedures with sedation. Hiccups, though commonly acute and temporary, may interfere with spontaneous breathing and hamper endoscopic investigations and interventions. However, the exact mechanism of hiccups remains unknown.

Research frontiers

There are 4 possible mechanisms leading to hiccups, including vagus and phrenic nerve irritation, central nervous system disorders, toxic-metabolic disorders, and psychogenic factors. Anesthetics and sedative agents are suspected agents in cases of drug-induced hiccups. On the other hand, the sedation of patients to relieve anxiety and discomfort appears to decrease the incidence of hiccups during endoscopic procedures. Therefore, whether sedation can protect the patients undergoing endoscopic procedures against the onset of hiccups is unknown and requires further investigation. In addition, hiccups represent an atypical symptom of gastroesophageal reflux disease (GERD), the increased probability of hiccups in GERD patients during sedation also requires further investigation.

Innovations and breakthroughs

To the best of our knowledge, this is the first study to examine the prevalence of hiccups in patients undergoing EGD or colonoscopy. Sedation for endoscopic procedures relieves patient anxiety and seems to decrease the incidence of hiccups. However, in this prospective study, the use of sedative drugs during endoscopic procedures favored the onset of hiccups. Although there was insufficient evidence to conclude that any specific drug induces drug-related hiccups, benzodiazepines are the most frequently suspected agents in cases of drug-induced hiccups. Midazolam, a benzodiazepine with rapid onset and brief

duration of action, was associated with the development of hiccups, especially in higher doses. In addition, the study also shows that patients with GERD are prone to develop hiccups during sedation.

Applications

The study results suggest that the development of hiccups was associated with higher doses of midazolam. Therefore, avoid using midazolam or decreasing the midazolam dosage in combined sedation regimen for endoscopic procedure can decrease the incidence of onset of hiccups.

Terminology

Hiccups: Hiccups are an involuntary spasmodic contraction of the diaphragm and accessory muscles followed by the sudden closure of the glottis; GERD: Patients with GERD may present a variety of symptoms, ranging from typical symptoms, such as heartburn or regurgitation, to atypical symptoms, such as chest pain, asthma, laryngitis, chronic cough, or hiccups. The major pathophysiology of GERD includes increased episodes of transient lower esophageal sphincter (LES) relaxations, ineffective esophageal motility, and reduced LES tone.

Peer review

The results of the study are interesting and it was well written. Nevertheless, there are some major points of concern.

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Factors predicting survival in patients with proximal gastric carcinoma involving the esophagus

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Abstract

AIM: To investigate the clinicopathologic features which

predict surgical overall survival in patients with proximal gastric carcinoma involving the esophagus (PGCE).

METHODS: Electronic pathology database established in the Department of Pathology of the Nanjing Drum Tower Hospital was searched for consecutive resection cases of proximal gastric carcinoma over the period from May 2004 through July 2009. Each retrieved pathology report was reviewed and the cases with tumors crossing the gastroesophageal junction line were selected as PGCE. Each tumor was re-staged, following the guidelines on esophageal adenocarcinoma, according to the 7th edition of the American Joint Commission on Cancer Staging Manual. All histology slides were studied along with the pathology report for a retrospective analysis of 13 clinicopathologic features, i.e., age, gender, *Helicobacter pylori* (*H. pylori*) infection, surgical modality, Siewert type, tumor Bormann's type, size, differentiation, histology type, surgical margin, lymphovascular and perineural invasion, and pathologic stage in relation to survival after surgical resection. Prognostic factors for overall survival were assessed with uni- and multi-variate analyses.

RESULTS: Patients' mean age was 65 years (range: 47-90 years). The male: female ratio was 3.3. The 1-, 3- and 5-year overall survival rates were 87%, 61% and 32%, respectively. By univariate analysis, age, male gender, *H. pylori*, tumor Bormann's type, size, histology type, surgical modality, positive surgical margin, lymphovascular invasion, and pT stage were not predictive for overall survival; in contrast, perineural invasion ($P = 0.003$), poor differentiation ($P = 0.0003$), > 15 total lymph nodes retrieved ($P = 0.008$), positive lymph nodes ($P = 0.001$), and distant metastasis ($P = 0.005$) predicted poor post-operative overall survival. Celiac axis nodal metastasis was associated with significantly worse overall survival ($P = 0.007$). By multivariate analysis, ≥ 16 positive nodes ($P = 0.018$), lymph node ratio > 0.2 ($P = 0.003$), and overall pathologic stage (P

= 0.002) were independent predictors for poor overall survival after resection.

CONCLUSION: Patients with PGCE showed worse overall survival in elderly, high nodal burden and advanced pathologic stage. This cancer may be more accurately staged as gastric, than esophageal, cancer.

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Key words: Cancer; Esophagus; Gastroesophageal junction; Staging; Stomach

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INTRODUCTION

Carcinoma in the gastroesophageal junction (GEJ) region can be sub-grouped by the Siewert classification system into 3 types on the basis of the distance between tumor epicenter and the GEJ line^[1,2]. Type I carcinomas are centered in the distal esophagus, 1-5 cm above the GEJ. They arise largely from Barrett's esophagus (BE), may or may not invade the GEJ, and are commonly reported as Barrett's adenocarcinoma. Type II tumors straddle the GEJ line and are believed to be true GEJ cancers with epicenter within 1 cm above and 2 cm below the GEJ. Type III tumors are sub-cardial gastric cancers with epicenter 2-5 cm below the GEJ that is crossed as they grow proximally.

Although the Siewert classification system has been widely used internationally, its prognostic value has been challenged^[3-5]. In Asian countries, type I GEJ cancer is rare and types II and III carcinomas behave similarly^[5,6]. In China, almost all GEJ carcinomas arise in the proximal stomach with a stable or slightly increased incidence in recent years^[7-10]. In our most recent study comparing clinicopathologic features of GEJ cancer between Chinese patients treated in Nanjing, China, and American patients treated in Boston, the United States, we showed that almost all GEJ cancers in Chinese patients were Siewert types II and III tumors^[11], unlike those seen in American patients in which the distribution of these three types of tumors was almost evenly^[12]. Their tumors were not BE-related, but associated with proximal gastritis with *Helicobacter pylori* (*H. pylori*) infection and a better overall survival rate, despite a larger tumor size and more advanced pathologic stages at diagnosis^[11]. Surprisingly, the

studies on factors predicting post-operative overall survival in Chinese patients with proximal gastric carcinoma involving the esophagus (PGCE) are scarce. The purpose of the present study was to investigate clinicopathologic features that may predict overall survival after surgical resection in Chinese patients with PGCE who were treated at a single high-volume tertiary medical center in Nanjing, China.

MATERIALS AND METHODS

Selection of patients

A total of 177 consecutive resection cases of histopathologically confirmed proximal gastric carcinoma were identified through a search of the computerized pathology database established in the Department of Pathology of the Nanjing Drum Tower Hospital in Nanjing, China, over the period from May 2004 through July 2009. Each pathology report was reviewed (by Huang Q) for cases with tumors crossing the GEJ line. Inclusion criteria were: (1) a tumor with epicenter in the proximal stomach within 5 cm below the GEJ and invading into the distal esophagus, which corresponded to Siewert types II and III tumors; (2) no chemotherapy or radiation therapy before the surgical resection; and (3) the availability of follow-up information through telephone interviews (by Feng AN) to the patient or family members. Exclusion criteria consisted of: (1) the tumor not crossing the GEJ; and (2) the patient lost to follow-up. Following a standard comprehensive surgical pathology processing protocol, all resection specimens were evaluated for the Bormann's gross type and surgical margins. The GEJ line, defined by the proximal end of gastric longitudinal mucosal folds, was evaluated in each case. The tumor epicenter location and its distance from the GEJ were recorded. The number of overall survival months after surgery was calculated until May 2010, based on whether the patient was alive or had died of any cause. For all selected patients, medical records and pathology reports were re-evaluated for demographic and clinicopathologic information, tumor stage, surgical approach (total or partial gastrectomy or Ivor-Lewis procedure), completeness of resection, and histopathology of the tumor. The study protocol was approved by the Medical Ethics Committee of the Nanjing Drum Tower Hospital in Nanjing, China.

Tumor staging

All tumors were staged with the esophageal cancer staging criteria, according to the 7th edition of the American Joint Commission on Cancer Staging Manual (AJCC 7)^[13]. The status of regional involved lymph nodes in the para-distal esophageal, para-cardial and peri-gastric regions was determined microscopically. The lymph nodes in the celiac axis region, including the left gastric artery, celiac artery, hepatic and splenic hila, *etc.*, were identified by the surgeon during the operation, submitted as separate specimens and examined microscopically. Lymph node metastasis was determined by the routine histological examination. In this study, since PGCE was classified as GEJ cancer

and staged as esophageal cancer, metastases to celiac axis regional nodes were considered as distant metastasis^[13,14].

The proximal, distal and radial margins of resection were routinely inked for microscopic examination and classified as negative or positive if there was histological evidence of carcinoma present at, or within 1 mm of the inked resection edge. Lymphovascular and peri-neural invasion was assessed microscopically on routine histology sections. Suspected distant tumor metastasis in the liver or other organs detected and biopsied intraoperatively was confirmed microscopically.

Statistical analysis

All patients' demographic and tumor gross characteristics were considered categorical variables except for age, overall survival month, number of lymph nodes retrieved and number of lymph nodes involved, which were classified as continuous variables. All statistical analysis was carried out (by Shi J) using the SPSS software (SPSS Inc., version 15.0, Chicago, IL, United States). Specific comparison between groups was performed using χ^2 and Student *t* tests. Patient overall survival rates after surgical resection were estimated with the Kaplan-Meier method and the log rank test. The patients who were alive at the last follow-up were censored for calculation of the overall survival rates. Cox multivariate proportional hazards regression models were used to assess the overall survival power of these parameters. The *P* value of < 0.05 was considered statistically significant.

RESULTS

Clinicopathologic characteristics

A total of 142 cases were eligible for this study. The patients' demographics, clinicopathologic characteristics, and the results of univariate overall survival analysis were shown in Table 1. Most (82%) tumors were type II of the Siewert classification, while there was no type I tumor in this cohort. The mean patient age was 65 years (range: 47-90). The male-female ratio was 3.3. The number of months of patient follow-up after surgical resection was 29 ± 17 mo (mean \pm SD, range: 1-70 mo). By the time of the last follow-up, 58 (41%) patients had died and the remaining survivors were censored. Overall, the 1-, 3- and 5-year overall survival rates were 87%, 61% and 36%, respectively. Among the demographic and pathologic variables, perineural invasion and poor tumor differentiation were associated with worse overall survival (Table 1). None of the surgical modalities were found significant for overall survival prediction. The status of *H. pylori* infection, tumor gross type, gender, age, tumor size, lymphovascular invasion, and even positive surgical margins were not significant for overall survival prediction by univariate analysis.

Pathologic staging

Following the AJCC 7 staging guidelines, the vast majorities (88%) of tumors were skewed to pT3 and only a few staged at pT1 (*n* = 4), pT2 (*n* = 12) and pT4 (*n* = 1). The

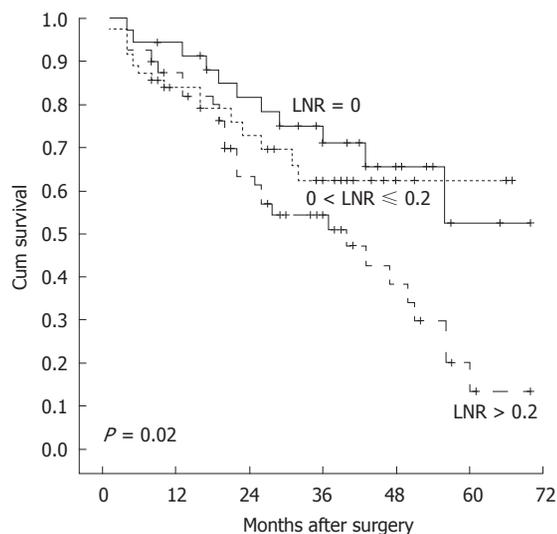


Figure 1 Kaplan-Meier overall survival curves of patients stratified by various lymph node ratios. The difference in overall survival among lymph node ratio (LNR) groups was statistically significant.

pT stage was found not to be a relevant factor for overall survival prediction. In contrast, positive lymph node metastasis were detected in 106 (75%) cases and significantly associated with worse overall survival (Table 2). Twelve of 142 patients (8%) had distant metastasis at the time of operation and showed significantly worse overall survival.

Lymph node status with overall survival

The mean number of lymph nodes retrieved per case was 21 (range: 4-66). The presence of lymph node metastasis was found in 106 (75%) cases. There were 12 patients (8%) with cancer metastasis in celiac axis lymph nodes, which significantly predicted worse overall survival by univariate analysis (Table 2). There was a statistically significant overall survival difference between patients with a total number of retrieved lymph nodes ≤ 15 and ≥ 16 (Table 2). The numbers of positive lymph nodes more than 7 and 16 were worse overall survival predictors. The ratio of the number of positive nodes to the total number of nodes retrieved, i.e., the lymph node ratio, was significantly associated with worse overall survival (Tables 2 and 3, Figure 1).

Significance of the lymph node ratio on overall survival

Among 3 different lymph node ratio groups, the lymph node ratios were significantly associated with the worse overall survival (Tables 2 and 3). The overall survival status was better illustrated on a Kaplan-Meier plot among groups with lymph node ratios > 0.2 , compared to that with the ratio ≤ 0.2 (Figure 1). We found that the relative risk for poor overall survival was 37-fold when the lymph node ratio was over 0.4 and 75-fold when over 0.5, compared with the ratio at 0.

Independent factors predicting overall survival retained at multivariate analysis

By multivariate analysis, over 16 positive lymph nodes per

Table 1 Demographic, clinical, and pathological features predicting overall survival in patients with proximal gastric carcinoma involving esophagus

Characteristics	Number of patients (%)	Months after surgery (mean ± SD)	P value	1-yr survival %	3-yr survival %	5-yr survival %
Age (yr)			0.108			
< 70	97 (68.3)	30 ± 17		91.1	65.3	40.1
≥ 70	45 (31.7)	27 ± 19		77.0	49.3	17.3
Gender			0.594			
Male	109 (76.8)	28 ± 17		71.1	61.1	23.1
Female	33 (23.2)	30 ± 18		88.3	60.1	48.1
<i>Helicobacter pylori</i> infection			0.400			
Negative	80 (56.3)	30 ± 18		87.3	67.6	29.4
Positive	62 (43.7)	27 ± 17		87.1	50.8	32.3
Surgical modality			0.950			
Partial gastrectomy	112 (78.9)	29 ± 18		85.7	68.6	35.1
Total gastrectomy	21 (14.8)	28 ± 18		76.2	65.3	0.0
Ivor-Lewis procedure	9 (6.3)	29 ± 17		89.2	60.6	34.3
Siewert type			0.444			
Type II	116 (81.7)	29 ± 17		86.8	63.8	27.2
Type III	26 (18.3)	33 ± 19		88.5	50.2	40.2
Bormann's type			0.380			
Polypoid	2 (1.4)	27 ± 26		100.0	50.1	50.1
Fungating	17 (12.0)	34 ± 20		83.3	70.9	53.2
Ulcerated	96 (67.6)	28 ± 17		88.0	56.3	26.8
Flat	27 (19.0)	26 ± 18		86.5	66.6	29.6
Tumor size (cm)			0.087			
< 3	19 (13.4)	30 ± 17		87.0	72.1	17.2
3.1-7.9	112 (78.9)	30 ± 17		89.7	61.5	38.5
> 8	11 (7.7)	18 ± 15		63.6	26.5	0.0
Surgical margin			0.165			
Negative	126 (88.7)	30 ± 17		88.8	60.6	30.1
Positive	16 (11.3)	23 ± 19		74.5	66.2	33.1
Lymphovascular invasion			0.050			
Negative	56 (39.4)	33 ± 18		90.9	65.6	31.2
Positive	86 (60.6)	27 ± 17		84.0	57.6	31.7
Perineural invasion			0.003			
Negative	55 (38.7)	35 ± 18		92.7	63.4	41.3
Positive	87 (61.3)	26 ± 17		83.6	59.4	19.3
Tumor differentiation			0.0003			
Well	1 (0.7)	57		100.0	100.0	100.0
Moderately	70 (49.3)	34 ± 17		95.2	69.3	44.2
Poorly	70 (49.3)	24 ± 16		81.3	52.7	11.3
Undifferentiated	1 (0.7)	4		0.0	0.0	0.0
Tumor histopathology			0.548			
Adenocarcinoma (nitric oxide synthase)	112 (78.9)	31 ± 17		89.6	71.0	34.2
Adenocarcinoma with micropapillary feature	24 (16.9)	24 ± 10		100	0.0	0.0
Adenosquamous carcinoma	9 (6.3)	28 ± 15		88.9	33.3	0.0
Mucinous carcinoma	9 (6.3)	24 ± 14		88.9	30.5	30.5
Signet ring cell carcinoma	23 (16.2)	23 ± 19		69.3	58.5	39.0
Carcinoma with neuroendocrine features	7 (4.9)	28 ± 17		100.0	50.0	25.0
Carcinoma with mixed types	37 (26.1)	24 ± 13		86.3	50.1	50.1
Pathologic T stage			0.400			
1A	2	38 ± 27		100.0	50.0	50.0
1B	2	47 ± 3		100.0	100.0	100.0
2	12	27 ± 17		83.3	72.9	54.7
3	125	29 ± 17		87.0	59.8	29.2
4A	0	0		-	-	-
4B	1	18		100.0	0.0	0.0
Pathologic N stage			0.001			
0	36	35 ± 17		94.4	72.0	53.5
1	22	32 ± 17		91.3	70.1	70.1
2	35	30 ± 17		85.3	64.7	15.9
3A	35	25 ± 17		85.3	57.4	14.4
3B	14	15 ± 9		70.7	41.3	10.3
Pathologic M stage			0.005			
M0	130	28 ± 18		88.3	64.5	32.5
M1	12	12 ± 4		75.0	13.1	13.1
Overall stage			0.012			
1A	4	42 ± 16		100.0	75.0	75.0
1B	7	30 ± 15		100.0	100.0	100.0
2A	26	34 ± 19		88.5	63.0	42.0
2B	26	32 ± 17		84.6	66.8	60.7
3A	29	31 ± 17		93.1	72.6	19.8
3B	29	27 ± 18		86.2	62.9	15.3
3C	9	18 ± 10		76.2	15.2	15.2
4	12	16 ± 9		75.0	13.1	13.1

Table 2 Univariate analysis of overall survival in patients with lymph node metastasis

Characteristic	No. of patients (%)	Overall survival			
		HR	95% CI ¹		P value
Celiac axis lymph node					
Negative	130 (92)	1.00			
Positive	12 (8)	3.33	1.40	7.95	0.007
Perineural invasion					
Negative	55 (38.7)	1.00			
Positive	87 (61.3)	1.48	0.54	4.11	0.447
Number of nodes retrieved/case					
10	21 (15)	1.00			
≥ 11	121 (85)	1.48	0.72	3.06	0.290
≤ 15	46 (32)	1.00			
≥ 16	96 (68)	2.29	1.24	4.22	0.008
≤ 23	100 (70)	1.00			
≥ 24	42 (30)	1.15	0.65	2.03	0.627
Number of positive nodes/case					
≤ 6	58 (41)	1.00			
≥ 7	48 (34)	1.72	1.20	2.46	0.003
≤ 15	92 (65)	1.00			
≥ 16	14 (10)	2.30	1.40	3.77	0.001
Lymph node ratio					
0	36 (25)	1.00			
≤ 0.2	40 (28)	1.34	0.61	2.97	0.467
> 0.2	66 (46)	2.28	1.15	4.51	0.018

¹Hazards ratios (HR), 95% confidence interval (CI) and *P* values for post-operative time to recurrence and overall survival were adjusted according to important clinical characteristics. Survival time was defined as the period from the surgical treatment to endpoint of follow-up.

case, lymph node ratio > 0.2, and the overall pathologic stage were found to be independent factors for predicting worse surgical overall survival (Table 3).

DISCUSSION

In this study, the factors predicting overall surgical survival in Chinese patients with PGCE are similar to those of gastric cancers but different from those of GEJ cancers reported in patients from Western countries^[15-18]. We show that in Chinese patients, type I GEJ carcinomas remain vanishingly rare and PGCE tumors are mostly as type II and some as type III GEJ cancers. The factors predicting surgical overall survival are comparable to those reported in Japan^[6] and China Taiwan^[5]. Importantly, nodal burden in PGCE correlates highly with post-operative overall survival, as seen in gastric cancer. Nodal metastasis in the celiac axis region is a significant predictor of worse overall survival. Finally, the independent risk factors for worse overall survival in our patients include tumor metastasis in more than 16 nodes, the lymph node ratio > 0.2, distant metastasis, and overall pathologic stage; in contrast, the prognostic factors, such as > 70 years, BE, the male gender, tumor histology type, the surgical resection method, and even positive resection margin, *etc.*, are not significant for predicting overall survival in Chinese patients with PGCE, which are predictive in type I GEJ carcinomas in Western patients^[15,19], probably

Table 3 Independent overall survival predictors retained by multivariate analysis

Factor	No. of patients	Multivariate analysis		
		HR	95% CI	P value
Celiac axis lymph node				
Compared to pN0				
Negative	130			
Positive	12	1.76	0.68	4.57
Number of positive nodes/case				
Compared to pN0				
LN ⁺ ≥ 7	48	1.34	0.6	2.96
LN ⁺ ≥ 16	14	2.77	1.15	4.51
Lymph node ratio				
Compared to pN0				
≤ 0.2	40	3.8	1.28	11.26
> 0.2	66	7.79	2.05	29.57
Overall stage pIV				
Compared to pI				
	12	18.43	2.27	145.62
Tumor differentiation				
Compared to well differentiated				
poorly differentiated	70	1.42	0.79	2.55

LN: Lymph node; HR: Hazards ratio; CI: Confidence interval.

because of the rarity of the type I GEJ carcinomas in Chinese patients. Our results would impact upon surgical management of Chinese patients with PGCE, if confirmed in a larger prospective trial(s).

Accurate GEJ cancer staging is difficult. The AJCC 7 staging system requires the use of the esophageal scheme for pathologic staging of this group of cancers, regardless of the location of tumor epicenters. This new mandate is controversial. In a recent study, we showed that PGCE staged with the gastric cancer staging rules was better stratified, especially for the pN and pIII stages, compared to the use of the esophageal scheme that showed an erroneously better overall survival in the patients staged at pIII A than those at p I A and p II B^[20]. The results shown in this study further substantiate the above conclusion and lent support to the contention that the current AJCC 7 cancer staging system for GEJ cancer needs to be modified when applied to Chinese patients with PGCE.

Lymph node metastasis in gastric cancer has been shown to be more significant in predicting overall survival than tumor invasion depth. This concept was confirmed in this study. For instance, we found no significant differences in overall survival among patients with different pT stages by either univariate or multivariate analysis. In contrast, the number of positive lymph nodes dictated patient overall survival^[16]. Furthermore, the total number of nodes retrieved (> 15) had a significant overall survival predictive value, as suggested by others^[17]. This may have resulted in a more precise evaluation of positive nodes and thus more accurate pN staging. In the current series, the overall survival was significantly worse in the patients with considerable nodal burden such as more than 7 positive nodes and higher lymph node ratios. Apparently, a rich lymphatic network in the GEJ region, and/or protein lytic enzymes secreted by neoplastic cells, may facilitate the lymphatic dissemination of neoplastic cells to intra-abdominal nodes^[16,21]. It was reported that even for pT1b GEJ cancers, 30% of cases with positive

nodes had a 5-year overall survival rate of only 33%^[18]. Moreover, patients with primary tumors in the middle to lower esophagus and the proximal stomach were found to have nodal diseases within the abdomen at rates of as high as 45% to 93%^[22,23]. Taken together, our data emphasize the important overall survival predictive value for a thorough abdominal nodal dissection in Chinese patients with PGCE^[21].

The significance of the ratio between involved and retrieved lymph nodes for overall survival prediction has not been described in patients with PGCE. Our results add into a growing body of evidence for the value of a lymph node ratio in predicting overall survival of patients with PGCE. In the late 1990s, Siewert *et al.*^[24] published their findings of prognostic significance of the lymph node ratio in gastric cancer in a large German cohort of 1654 cases. Their conclusion was repeatedly confirmed by the studies in gastric cancer patients in Japan^[25,26], China Taiwan^[5], mainland China^[27,28], South Korea^[29], Spain^[30] and Italy^[31]. The advantage of the use of this parameter in patients with PGCE lies upon its ease to use, irrespective of the surgical methods used by different surgeons and various types of resection specimens from different patients^[24]. In reality, the number of retrieved lymph nodes is largely influenced by the extent of lymph node dissection by different surgeons, whose nodal dissection skills vary^[32,33]. It was reported that patients with a lymph node ratio smaller than 0.2 had a better overall survival rate^[33,34], which is also our experience. As the lymph node ratio increases, so is the increased relative risk of poor overall survival. Our data show that in patients with PGCE, the lymph node ratio could be used clinically as a powerful overall survival predictor.

The significance of metastatic nodal disease in the celiac axis region in patients with GEJ cancers for overall survival prediction remains obscure due to limited studies in the literature^[18,21]. When nodal metastasis is discovered in this region, some studies classify it as a pM1a disease of distal esophageal or GEJ cancers^[14], according to the 6th AJCC cancer staging system. The overall survival predictive value of positive celiac lymph nodes in proximal gastric cancer stays controversial^[18,35-37]. It was reported that the patients with undetected celiac nodal disease at the time of surgical resection were subsequently found to have celiac nodal involvement with overall survival similar to that of patients with stage III disease^[21]. In our previous^[28] and current studies on PGCE, nodal disease in the celiac axis region was also an important overall survival predictor by univariate analysis, which, however, did not reach a statistically significant level by multivariate analysis, probably due to the small sample size. Nevertheless, it appears that the site of nodal disease may be as important as the number of nodal metastasis in predicting overall survival for patients with PGCE.

The major limitations of this study are several. First, the sample size of the current cohort was relatively small and most cancer cases were advanced and staged at pIII. There were only a few cases staged at pI or pIV, which might have contributed to the lack of significance for the

tumor pT stage in overall survival prediction. Second, the patient follow-up was carried out by telephone interview only. This might have invited inaccurate and biased results. At present, an accurate electronic patient medical record system has not been established in China and the government death record of citizens is not available to the public. Therefore, telephone interview has been the primary tool to collect the overall survival information. Finally, because of the retrospective nature of the study, the methods for surgical resection, lymph node retrieval, and specimen dissection were not standardized, which might have caused inconsistent nodal retrieval results. However, in over 75% of cases in this study, the number of lymph nodes retrieved per case was over 21. Therefore, the overall data quality should be reasonably solid and reliable.

In conclusions, PGCE, like gastric cancer, has similar overall survival after resection in patients with no or minimal nodal burden in this cohort of consecutively treated Chinese patients. However, the elderly patients over 70 years and those with considerable nodal diseases including celiac nodal metastasis, distant metastasis, and advanced summary pathologic stage fare worse in overall survival after resection. Application of the AJCC 7 esophageal staging scheme to PGCE may be less accurate in predicting overall survival than applying the gastric staging scheme^[20]. Because of the small sample size and a single institution experience, further larger, prospective studies are required to validate our findings in the Chinese patient population.

COMMENTS

Background

In China, almost all gastroesophageal junction (GEJ) carcinomas arise in the proximal stomach with a stable or slightly increased incidence in recent years. In our most recent study comparing clinicopathologic features of GEJ cancer between Chinese patients treated in Nanjing, China, and American patients treated in Boston, the United States, the authors showed that GEJ cancers in Chinese patients were unlike those seen in American patients. However, the studies on factors predicting post-operative overall survival in Chinese patients with proximal gastric carcinoma involving the esophagus (PGCE) are scarce.

Research frontiers

The purpose of the present study was to investigate clinicopathologic features that may predict overall survival after surgical resection in Chinese patients with PGCE who were treated at a single high-volume tertiary medical center in Nanjing, China.

Innovations and breakthroughs

In this study, the factors predicting overall surgical survival in Chinese patients with PGCE are similar to those of gastric cancers but different from those of GEJ cancers reported in patients from Western countries. The authors show that in Chinese patients, type I GEJ carcinomas remain vanishingly rare and PGCE tumors are mostly as Siewert type II and some as type III GEJ cancers. The factors predicting surgical overall survival are comparable to those reported in Japan and Taiwan. Importantly, nodal burden in PGCE correlates highly with post-operative overall survival, as seen in gastric cancer. Nodal metastasis in the celiac axis region is a significant predictor of worse overall survival. Finally, the independent risk factors for worse overall survival in our patients include tumor metastasis in more than 16 nodes, the lymph node ratio > 0.2, distant metastasis and overall pathologic stage; in contrast, the prognostic factors, such as > 70 years, Barrett's esophagus, the male gender, tumor histology type, the surgical resection method, and even positive resection margin, *etc.*, are not significant for predicting overall survival in Chinese patients

with PGCE, which are predictive in Siewert type I GEJ carcinomas in Western patients, probably because of the rarity of the Siewert type I GEJ carcinomas in Chinese patients.

Applications

Their results would impact upon surgical management of Chinese patients with PGCE, if confirmed in a larger prospective trial(s). The authors suggest that the GEJ cancer in Chinese patients be treated as gastric cancer.

Peer review

This study was designed as the next step to examine the clinicopathologic features that may predict survival after surgical resection of proximal gastric carcinoma, keeping in mind that this tumor type is rare in Chinese patients. The manuscript is well written and methodology is accurately described. By multivariate analysis, > 16 positive nodes, lymph node ratio > 0.2, distant metastasis and summary pathologic stage were found to be independent predictors for poor survival. The authors stress that the lymph node ratio could be used as a reliable survival predictor, as this parameter is not influenced by the surgical methods and the number of retrieved lymph nodes.

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Impact of lymphatic and/or blood vessel invasion in stage II gastric cancer

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Abstract

AIM: To determine the prognostic value of lymphatic and/or blood vessel invasion (LBVI) in patients with stage II gastric cancer.

METHODS: From January 2001 to December 2006, 487 patients with histologically confirmed primary gastric adenocarcinoma were diagnosed with stage II gastric cancer according to the new 7th edition American Joint Committee on Cancer stage classification at the Department of Gastric Cancer and Soft Tissue Surgery, Fudan University Shanghai Cancer Center. All patients underwent curative gastrectomy with standard lymph node (LN) dissection. Fifty-one patients who died in the postoperative period, due to various complications or other conditions, were excluded. Clinicopathological

findings and clinical outcomes were analyzed. Patients were subdivided into four groups according to the status of LBVI and LN metastases. These four patient groups were characterized with regard to age, sex, tumor site, pT category, tumor grading and surgical procedure (subtotal resection *vs* total resection), and compared for 5-year overall survival by univariate and multivariate analysis.

RESULTS: The study was composed of 320 men and 116 women aged 58.9 ± 11.5 years (range: 23-88 years). The 5-year overall survival rates were 50.7% and the median survival time was 62 mo. Stage II a cancer was observed in 334 patients, including 268 T3N0, 63 T2N1, and three T1N2, and stage II b was observed in 102 patients, including 49 patients T3N1, 51 T2N2, one T1N3, and one T4aN0. The incidence of LBVI was 28.0% in stage II gastric cancer with 19.0% (51/269) and 42.5% (71/167) in LN-negative and LN-positive patients, respectively. In 218 patients (50.0%), there was neither a histopathologically detectable LBVI nor LN metastases (LBVI⁻/LN⁻, group I); in 51 patients (11.7%), LBVI with no evidence of LN metastases was detected (LBVI⁺/LN⁻, group II). In 167 patients (38.3%), LN metastases were found. Among those patients, LBVI was not determined in 96 patients (22.0%) (LBVI⁻/LN⁺, group III), and was determined in 71 patients (16.3%) (LBVI⁺/LN⁺, group IV). Correlation analysis showed that N category and the number of positive LNs were significantly associated with the presence of LBVI ($P < 0.001$). The overall 5-year survival was significantly longer in LN-negative patients compared with LN-positive patients (56.1% *vs* 42.3%, $P = 0.015$). There was a significant difference in the overall 5-year survival between LBVI-positive and LBVI-negative tumors (39.6% *vs* 54.8%, $P = 0.006$). Overall 5-year survival rates in each group were 58.8% (I), 45.8% (II), 45.7% (III) and 36.9% (IV), and there was a significant difference in overall survival between the four groups ($P = 0.009$). Multivariate analysis in stage II gastric cancer patients revealed that LBVI independently affected patient prognosis in LN-negative

patients ($P = 0.018$) but not in LN-positive patients ($P = 0.508$).

CONCLUSION: In LN-negative stage II gastric cancer patients, LBVI is an additional independent prognostic marker, and may provide useful information to identify patients with poorer prognosis.

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Key words: Stage II cancer; Gastric cancer; Lymphatic invasion; Blood vessel invasion; Prognosis

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INTRODUCTION

Gastric cancer remains a major public health issue as the fourth most common cancer and second leading cause of cancer-related death worldwide despite a decrease in its incidence level^[1-3]. Surgery, including gastrectomy in combination with systemic lymph node (LN) dissection, is the current treatment of choice for gastric cancer^[4-6]. A correct definition of poor prognostic factors may help to guide more aggressive adjuvant treatment protocols. Therefore, it is urgently needed to identify new biological or pathological markers related to survival in addition to well-known prognostic factors such as the tumor-node-metastasis (TNM) staging classification and clinical stage.

According to the new American Joint Committee on Cancer (AJCC) TNM staging classification (7th edition), staging of gastric cancer by TNM classification is composed of nine groups^[7]. Stage II gastric cancer is an intermediate stage between stage I and stage III. Stage II gastric cancer is defined as tumor that invades into or through the muscular wall of the stomach, but not into nearby local structures, or has regional LN involvement with any extent of primary cancer, but no invasion of local structures. Surgical resection with regional lymphadenectomy is the treatment of choice for patients with stage II gastric cancer. However, preoperative and intraoperative staging to confirm stage II disease is difficult. Failure to distinguish stage II from stage I disease may lead to under- or over-treatment. Therefore, the identification of additional prognostic factors, which is timely and cost-efficient as well as available, would help in detecting those patients with poorer prognosis among the

different groups of patients with stage II gastric cancer. It might be of clinical significance to select candidates for treatment considerations, such as the extent of LN dissection and further adjuvant and neoadjuvant chemotherapy. Comparisons of survival in subgroups of stage II have been reported^[8]. However, to date there is no study on the subgroups of stage II gastric cancer classified by the 7th edition of the AJCC TNM, and the prognostic factors for each subgroup.

As the cancer stage advances, tumor cells invade blood vessels and lymphatic vessels near the tumor; lymphatic and/or blood vessel tumor invasion (LBVI) is the critical step of tumor cell dissemination and metastasis in various types of cancer^[4-6]. The prognostic significance of LBVI in gastric cancer has been previously investigated in a few studies, without reaching a consensus^[7-11]. To date, LBVI has not been studied in patients with stage II disease alone.

In this study, we investigated the value of LBVI as a prognostic factor in patients with stage II gastric cancer who underwent curative resection. The association of LBVI with the clinicopathological factors and the effect of LBVI on survival were analyzed.

MATERIALS AND METHODS

Patients

From January 2001 to December 2006, a total of 487 patients with histologically confirmed primary gastric adenocarcinoma were diagnosed with stage II gastric cancer according to new 7th edition TNM stage classification. All 487 patients underwent curative gastrectomy with standard LN dissection at the Department of Gastric Cancer and Soft Tissue Surgery, Fudan University Shanghai Cancer Center. Fifty-one patients who died in the postoperative period, due to various complications, or other conditions, were excluded from the study. Data were retrieved from operative and pathology reports, with follow-up data being obtained from the outpatient clinical database. All subjects were preoperatively diagnosed with gastric adenocarcinoma by analysis of endoscopic biopsy specimens. Standardized operative procedures were performed, such as total or subtotal gastrectomy, depending on the location of the gastric cancer, and D2 LN dissection according to the rules of the Japanese Research Society for Gastric Cancer. All chemotherapy for the enrolled patients was postoperative, and no patient with stage II gastric cancer underwent neoadjuvant chemotherapy. Patient survival was evaluated from information collected by using mail, telephone, or outpatient records.

All of the resected primary tumors and regional LNs were examined. The final diagnosis for each patient was decided by 2 pathologists to avoid misdiagnosis. Hematoxylin and eosin (HE) staining is commonly used for pathological examination. When it was difficult to identify the lymphatics or the venous structures by HE staining, either Elastica staining or Victoria-blue HE staining was performed. LBVI was defined according to

the Japanese Classification of Gastric Carcinoma (absence *vs* presence). LBVI presence is defined as lymphovascular invasion and is detected in lymphatics or small veins in a mounted specimen containing the deepest portion of the tumor on a glass slide. The histology was grossly divided into the differentiated type (papillary and tubular adenocarcinoma) and the undifferentiated type (poorly differentiated adenocarcinoma, signet-ring cell carcinoma, mucinous carcinoma, and miscellaneous).

Gastric carcinoma was classified according to the new AJCC TNM staging criteria (7th edition). The clinical and pathological parameters evaluated included sex, age, depth of the tumor (T category), involvement of the LNs, and lymphatic and vascular invasion.

For the prognostic evaluation of isolated LBVI, the patients enrolled according to the criteria mentioned above were further subdivided into four prognostic groups: Group I: no detection of LBVI or any LN metastases (LBVI⁻/LN⁻); Group II: detection of LBVI but no LN metastases (LBVI⁺/LN⁻); Group III: detection of LN metastases but no detection of LBVI (LBVI⁻/LN⁺); Group IV: detection of LN metastases and LBVI (LBVI⁺/LN⁺). These four patient groups were characterized with regard to age, sex, tumor site, pT category, tumor grading, and surgical procedure (subtotal resection *vs* total resection) and compared with regard to the 5-year overall survival by univariate and multivariate analysis.

Statistical analysis

Statistical analysis was performed using SPSS 11.0 software. Survival analysis and curves were established according to the Kaplan-Meier method and compared by the log-rank test. Survival time was calculated from the month of surgery until the time of death or confirmation of survival, and survival rate was represented by the percentage of survivals at the end of the observed interval (in years and months). Multivariate analysis with Cox proportional hazard model was used to assess the role of LBVI and the other clinicopathological features as prognostic factors. $P < 0.05$ was considered significant.

RESULTS

Patients' clinicopathological characteristics

The study was composed of 320 men and 116 women aged 58.9 ± 11.5 years (mean \pm SD, range: 23-88 years). According to the new AJCC 7th edition TNM stage classification among those with stage II gastric cancers, stage II a was observed in 334 patients, including 268 T3N0, 63 T2N1, and three T1N2, and stage II b was observed in 102 patients, including 49 T3N1, 51 T2N2, one T1N3, and one T4aN0. Histological grade was reported as differentiated in 177 (40.6%) and undifferentiated in 259 (59.4%) cases. Total gastrectomy was performed in 113 (25.9%) cases, and subtotal gastrectomy in 323 (71.4%) cases. Overall follow-up ranged from 11 to 99 mo (median: 39 mo). The 5-year overall survival rates were 50.7% and the median survival time was 62 mo.

Table 1 Clinicopathological factors according to lymphatic and/or blood vessel invasion *n* (%)

Clinicopathological factors	Tumor without lymphovascular invasion	Tumor with lymphovascular invasion	<i>P</i> value
Patients	314	122	
Age (yr)	58.95 ± 11.88	58.84 ± 10.66	0.927
Sex			
Male	235 (74.8)	85 (69.7)	0.279
Female	79 (25.2)	37 (30.3)	
Tumor location			
Upper	110 (35.0)	42 (34.4)	0.733
Middle	57 (18.2)	20 (16.4)	
Lower	140 (44.6)	55 (45.1)	
Whole	7 (2.2)	5 (4.1)	
Resection type			
Subtotal	236 (75.2)	87 (71.3)	0.465
Total	78 (24.8)	35 (28.7)	
Tumor size (cm)			
< 5 cm	206 (65.6)	82 (67.2)	0.822
\geq 5 cm	108 (34.4)	40 (32.8)	
Histological type			
Differentiated	123 (39.2)	54 (44.3)	0.331
Undifferentiated	191 (60.8)	68 (55.7)	
No. of retrieved LNs	21.5 ± 5.5	22.3 ± 7.0	0.248
LN			
Positive	96 (30.6)	51 (41.8)	0.032
Negative	218 (69.4)	71 (58.2)	
Stage			< 0.001
II a	264 (84.1)	70 (57.4)	
II b	50 (15.9)	52 (42.6)	
Survival at 5 yr (%)	39.6	54.8	0.006

LN: Lymph node.

Comparison of clinicopathological features

LBVI was identified in 122 (28.0%) and absent in 314 (72.0%) cases. When the patients were classified into two groups according to LBVI, the LBVI group had a higher incidence of LN metastasis (30.6% *vs* 58.2%, $P = 0.032$) and higher tumor stage (39.6% *vs* 54.8%, $P < 0.001$). However, other clinicopathological variables, such as age, sex, tumor stage, tumor size, operative methods, and histological type showed no significant differences between the two groups (Table 1).

The incidence of LBVI was 19.0% (51/269) and 42.5% (71/167) in LN-negative patients and LN-positive patients, respectively. In 218 patients (50.0%), there was neither a histopathologically detectable LBVI nor LN metastases (LBVI⁻/LN⁻, Group I); in 51 patients (11.7%), LBVI with no evidence of LN metastases was detected (LBVI⁺/LN⁻, Group II). In 167 patients (38.3%), LN metastases were found. Among these patients, LBVI was not determined in 96 patients (22.0%) (LBVI⁻/LN⁺, Group III), and was determined in 71 patients (16.3%) (LBVI⁺/LN⁺, Group IV). An overview on the patient groups is shown in Table 2. There were no significant differences between the four patient groups with regard to age ($P = 0.367$), sex ratio ($P = 0.160$), distribution of tumor sites ($P = 0.959$), surgical procedures ($P = 0.328$), total number of retrieved LNs ($P = 0.413$) and tumor size ($P = 0.929$) (Table 2).

Table 2 Patient and tumor characteristics of Groups I-IV ($n = 436$) n (%)

	Group I (LBVI/LN ⁻)	Group II (LBVI ⁺ /LN ⁻)	Group III (LBVI/LN ⁺)	Group IV (LBVI ⁺ /LN ⁺)	<i>P</i> value
Patients	218	51	96	71	
Age (yr)	59.7 ± 11.5	59.4 ± 9.1	57.3 ± 12.6	58.4 ± 11.7	0.367
Male:female	3.36	1.68	2.31	2.94	0.160
Tumor location					
Upper	79 (36.2)	17 (33.3)	31 (32.3)	25 (35.2)	0.959
Middle	39 (17.9)	8 (15.7)	18 (18.8)	12 (16.9)	
Lower	96 (44.0)	23 (45.1)	44 (45.8)	32 (45.1)	
Whole	4 (1.8)	3 (5.9)	3 (3.1)	2 (2.8)	
Tumor size					
≤ 5 cm	141 (64.7)	35 (68.6)	65 (67.7)	47 (66.2)	0.929
> 5 cm	77 (35.3)	16 (31.4)	31 (32.3)	24 (33.8)	
No. of retrieved LNs	21.2 ± 5.7	22.1 ± 8.0	22.0 ± 5.0	22.45 ± 6.2	0.413
Histological type					
Differentiated	85 (39.0)	22 (43.1)	38 (39.6)	32 (45.1)	0.801
Undifferentiated	133 (61.0)	29 (56.9)	58 (60.4)	39 (59.4)	
Resection type					
Subtotal	167 (76.6)	33 (64.7)	69 (71.9)	54 (76.1)	0.328
Total	51 (23.4)	18 (35.3)	27 (28.1)	17 (23.9)	
5-year overall survival (%)	58.8	45.8	45.7	36.9	0.009

LBVI: Lymphatic and/or blood vessel invasion; LN: Lymph node.

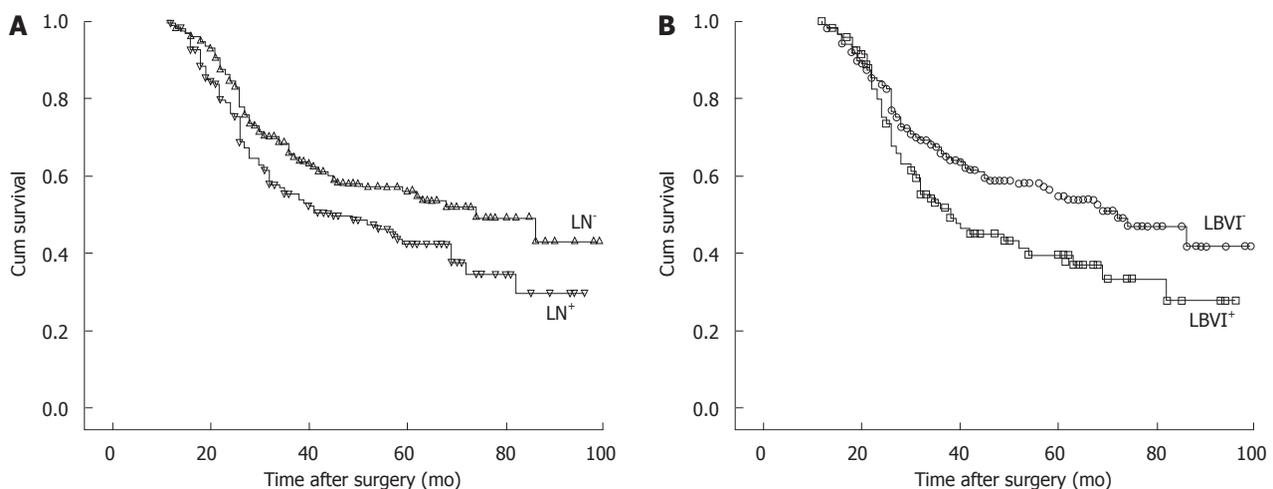


Figure 1 Survival curves of patients with stage II gastric cancer according to lymph node metastasis and lymphatic and/or blood vessel invasion. A: Overall survival curve of the lymph node (LN)-positive patients (LN⁺) was significantly worse than that of the LN-negative patients (LN⁻) ($P = 0.015$); B: Overall survival curve of the lymphatic and/or blood vessel invasion (LBVI)-positive (LBVI⁺) patients was significantly worse than that of the LBVI-negative (LBVI⁻) patients ($P = 0.006$).

Correlation analysis

Correlation analysis with the Spearman correlation coefficient showed that LBVI and LN category or the number of positive LNs were significantly correlated (correlation coefficient 0.255, 0.317; $P < 0.001$). There was a significantly greater incidence and number of positive LNs when LBVI was present than when LBVI was absent. Of the 122 patients with LBVI⁺ tumors, 71 (58.2%) had positive nodes compared with 96 (30.6%) of the 314 patients with LBVI⁻ tumors ($P < 0.001$). The average number of positive nodes in patients with LBVI⁺ tumors was 1.59 compared with 0.62 in patients with LBVI⁻ tumors ($P < 0.001$).

Survival

The 5-year overall survival was significantly longer in LN-

negative patients compared with LN-positive patients (56.1% *vs* 42.3%, $P = 0.015$; Figure 1A). There was a significant difference in the 5-year overall survival between LBVI-positive and LBVI-negative tumors (39.6% *vs* 54.8%, $P = 0.006$; Figure 1B). Five-year overall survival rates in each group were 58.8% (I), 45.8% (II), 45.7% (III) and 36.9% (IV), and there was a significant difference in overall survival between the four groups ($P = 0.009$; Figure 2).

Comparison of survival time in LN-negative gastric cancer patients according to LBVI: The 5-year overall survival rate was 58.8% in LN-negative gastric cancer patients with no LBVI, and 45.8% in LN-negative patients with LBVI. There was a significant difference in

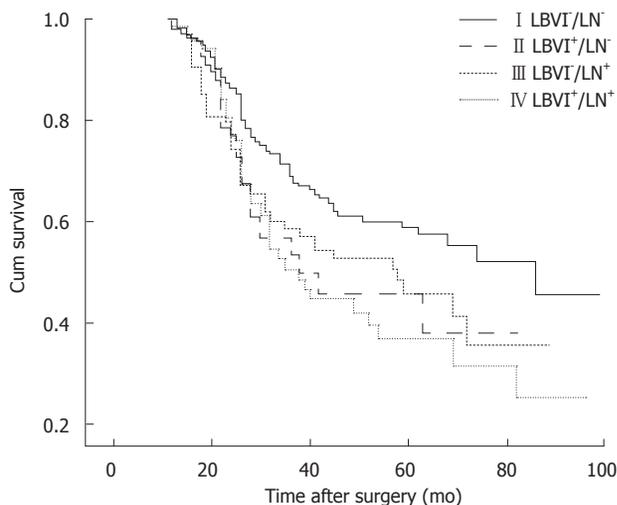


Figure 2 Cumulative survival of Groups I-IV according to the status of lymph node metastasis and lymphatic and/or blood vessel invasion. There was a significant difference in overall survival between the four groups ($P = 0.009$). Moreover, there was a significant difference in overall survival between Groups I and II ($P = 0.022$). However, the comparison of Group III with IV revealed no significant difference ($P = 0.482$). LBVI: Lymphatic and/or blood vessel invasion; LN: Lymph node.

overall survival between Groups I and II ($P = 0.022$). The 5-year overall survival rate was 42.3% in LN-positive gastric cancer patients and 45.7% and 36.9% in Groups III and IV, respectively. By the paired log rank test, the comparison of Groups III and IV revealed no significant difference (log rank test, $P = 0.482$).

Comparison of survival time between LN-negative stage II gastric cancer patients with LBVI and LN-positive stage II gastric cancer patients: No difference was observed in survival between group II and LN-positive patients ($P = 0.924$) or between each group ($P = 0.753$).

Cox regression analysis

To assess the independent prognostic relevance of several parameters such as LBVI, in particular, with regard to 5-year overall survival rate, the Cox proportional hazard analysis, including lymphatic vessel invasion (LBVI *vs* LBVI⁺), T category (T1-T4a), metastatic tumor growth within the LNs (LN⁻ *vs* LN⁺), tumor histological type, tumor location, type of surgical procedure (subtotal *vs* total), sex (male *vs* female), age, and tumor stage (IIa *vs* IIb) were determined. Multivariate analysis in all stage II gastric cancer patients revealed that no factor influenced patient prognosis independently. Multivariate analysis in LN-negative patients showed that LBVI and age independently affected patient prognosis ($P = 0.018$, $P = 0.036$). However, the multivariate analysis did not detect any independent impact of LBVI on 5-year overall survival in LN-positive patients ($P = 0.512$).

DISCUSSION

In the present retrospective study, the effect of LBVI on

survival in patients with stage II gastric carcinoma who underwent D2 curative gastrectomy was investigated. According to the study of del Casar *et al.*^[12], LBVI was defined as either the presence of neoplastic cells with fibrin clots, erythrocytes, or both within an endothelium-lined space without erythrocyte extravasation into the surrounding tissue, or by the presence of neoplastic cells within a smooth-muscle-cell-lined space. We found that 122 of the 436 patients (28%) were LBVI-positive. Multivariate analysis LN-negative patients showed that LBVI independently affected patient prognosis. However, multivariate analysis did not detect any independent impact of LBVI on 5-year overall survival in LN-positive patients. We also found that survival outcomes between LN-negative stage II gastric cancer patients with LBVI and LN-positive stage II gastric cancer patients were similar, suggesting that it may be necessary to treat these two groups as having the same malignant potential.

The incidence of LBVI in gastric cancer varies from 5.4% to 86%, with the lowest incidence of 20%-26.8% reported in patients with node-negative tumors, using routine pathological examinations with HE staining or different staining methods with endothelial markers^[12-17]. This could be caused, in part, by the different patient populations included in the different studies. In our analysis, 28% of stage II gastric patients who underwent curative resection were found to have evidence of LBVI. The incidence of LBVI was 19.0% (51/269) and 42.5% (71/167) in LN-negative and LN-positive patients, respectively.

In our study, the impact of LBVI on survival in patients with stage II gastric carcinoma who underwent D2 curative gastrectomy was investigated, and we found that there was a significant difference with respect to survival between LBVI-positive and LBVI-negative patients. By proportional hazards analysis, LBVI was an independent prognostic factor for survival. The mean and median overall survival interval for patients with LBVI-positive tumors was 52.9 and 38 mo, respectively, which was significantly worse than that of the patients with LBVI-negative tumors at 64.7 and 72 mo. The presence and prognostic importance of LBVI have been investigated previously in patients with gastric cancer by only a few studies and a consensus was not reached^[12-19]. Several studies have shown that LBVI can be a useful marker to predict cancer recurrence and prognosis in gastric cancer patients^[12,15,17]. In contrast, the presence of LBVI showed no prognostic value as an independent factor in the overall group of patients with curatively resected gastric cancer^[18,19]. A possible explanation for the different results in assessing the prognostic impact of LBVI could be the methodological discrepancies in the various analyses.

In the current study, LBVI was not also an independent prognostic factor in LN-positive stage II gastric cancer. Liu *et al.*^[20] found that, in patients with T1N1M0 gastric cancer, the survival rate was not significantly different between those with and those without lymphatic vessel invasion. Therefore, LBVI may not be a prognos-

tic factor in LN-positive gastric cancer. In contrast, our results showed that LBVI independently influenced prognosis in LN-negative stage II cancer patients, and were in agreement with previous studies examining node-negative gastric cancer, further supporting LBVI as a potential marker of biological behavior. This observation was subsequently supported in a study by Lee *et al.*^[21], which showed that LBVI was an adverse prognostic indicator, independent of clinicopathological factors in node-negative gastric cancer. The above-mentioned study concluded that LBVI may provide useful information for prognosis and clinical management in the subset of patients with node-negative gastric cancer. More recently, Kooby *et al.*^[22] showed that vascular invasion in node-negative patients was an independent predictor of poor outcome, and identified more aggressive lesions independent of tumor size and depth of invasion. This finding was consistent with our results, in which a subgroup analysis demonstrated that LBVI was independently associated with poor outcome in patients with node-negative gastric cancer. We also found that survival outcomes between stage T3N0 gastric cancer patients with LBVI and stage II gastric cancer patients with positive LN metastasis were similar, suggesting that it may be necessary to treat these two populations as having the same malignant potential.

According to our results, a possible indication to investigate LBVI after radical gastric cancer resection might be the case if there were no LN metastases detectable in conventional pathohistological investigation. However, analysis of various studies have shown that in 10.0%-30.1% of tumor-cell-negative LNs, revealed by conventional histopathological investigation, micrometastases were detectable with immunohistochemistry, which have been described as a negative prognostic factor^[23-27]. Therefore, it is recommended to investigate LNs in a more subtle manner in patients with positive LBVI, so that detection of micrometastases is made more cost-efficient and time-saving, with selective immunohistochemical and gene-amplifying investigations of the prepared LNs in patients who have been previously classified as pN0.

However, there were some limitations in the present study due to its retrospective nature, despite our efforts to make a clinically and scientifically sound experiment design. First, due to the inherent limitations of a retrospective study and the small sample size of this study, the results require further investigation to reach a firm conclusion. Second, the presence of LVBI was identified by routine histological HE staining. The accuracy of the identification of LBVI is affected by various parameters. A crucial factor is the number of reference slices obtained from the primary tumor lesion and the level of serious attention for LBVI in the histopathological routine examination. Currently, the presence of LBVI is detected by a routine HE staining method. How novel immunohistochemical lymphangio-markers may improve the sensitivity and specificity of detection needs to be proved in further studies. The third limitation was the heterogene-

ity in clinical decision-making, surgical intervention and pathological evaluation. Radical gastric cancer resection was performed by several surgeons, and the specimens were evaluated by several pathologists. These doctors were trained in academic centers with experience in gastrectomy and the data are probably valid. Another limitation of our study was the choice of overall survival as the end point. In some ways this can be regarded as a powerful end point, given that overall survival is a concrete end point that we were able to ascertain reliably. Although time to recurrence would be an interesting end point to analyze, patients were not on a predefined follow-up schedule and were observed at the discretion of the treating physician. Therefore, although we tried to collect recurrence data for these patients, there were limitations to the validity of investigating this end point due to the lack of a predefined surveillance plan and schedule.

In conclusion, patients with LBVI⁺ tumors had a significantly increased incidence and number of positive nodes and a shorter 5-year overall survival. On multivariate analysis, LBVI was an independent prognostic factor in stage II gastric cancer patients with negative LNs. This suggests that the presence of lymphovascular invasion in stage II gastric cancer patients may provide valuable information to determine which patients would benefit from radical surgery, adjuvant chemotherapy or radiotherapy after surgery.

COMMENTS

Background

Surgical resection with regional lymphadenectomy is the treatment of choice for patients with stage II gastric cancer. However, preoperative and intraoperative staging to confirm stage II disease is difficult. Failure to distinguish stage II from stage I disease may lead to under- or over-treatment. Therefore, the identification of additional prognostic factors, which is timely and cost-efficient as well as available, would help in detecting those patients with poorer prognosis among the different groups of patients with stage II gastric cancer.

Research frontiers

A retrospective study was conducted, attempting to clarify the risk factors and to investigate the effect of lymphatic and/or blood vessel invasion (LBVI) on survival in patients with stage II gastric cancer who underwent D2 curative gastrectomy.

Innovations and breakthroughs

The study revealed that LBVI independently affected patient prognosis in lymph node (LN)-negative patients but not in LN-positive patients with stage II gastric cancer.

Applications

In LN-negative stage II gastric cancer patients, LBVI is an additional independent prognostic marker, and may provide useful information to identify patients with poorer prognosis. This suggests that the presence of LBVI in stage II gastric cancer patients may provide valuable information to determine which patients would benefit from radical surgery, adjuvant chemotherapy or radiotherapy after surgery.

Terminology

LBVI is the critical step of tumor cell dissemination and metastasis in various types of cancer. As the cancer stage advances, tumor cells invade blood vessels and lymphatic vessels near the tumor.

Peer review

This is a retrospective clinicopathological study on the implication of LBVI in the patients who received surgical therapy for gastric cancer. The results are important and applicable to clinical practice and studies.

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Effect of interferon- γ and tumor necrosis factor- α on hepatitis B virus following lamivudine treatment

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Abstract

AIM: To evaluate anti-hepatitis B virus (HBV) activity and cytotoxicity of interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) following lamivudine treatment of HepG2.2.15 cells.

METHODS: HepG2.2.15 cells were treated with 2 μ mol/L lamivudine for 16 d (lamivudine group), cultured for 10 d, followed by 5 ng/mL TNF- α and 1000 U/mL IFN- γ for 6 d (cytokine group), or treated with 2 μ mol/L lamivudine for 10 d followed by 5 ng/mL TNF- α and 1000 U/mL IFN- γ for 6 d (sequential group), or cultured without additions for 16 d (control group). Intracellular DNA was extracted from 3×10^5 HepG2.2.15 cells from each group. The extracted DNA was further purified with mung bean nuclease to remove HBV relaxed circular DNA that may have remained. Both HBV covalently closed circular DNA (cccDNA) and HBV DNA were examined with real-time polymerase chain reaction. The titers of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) were quantified with enzyme-linked

immunosorbent assay. Cell viability was measured with the cell counting kit-8 assay.

RESULTS: Compared to lamivudine alone ($22.63\% \pm 0.12\%$), both sequential ($51.50\% \pm 0.17\%$, $P = 0.034$) and cytokine treatment ($49.66\% \pm 0.06\%$, $P = 0.041$) showed a stronger inhibition of HBV cccDNA; the difference between the sequential and cytokine groups was not statistically significant ($51.50\% \pm 0.17\%$ vs $49.66\% \pm 0.06\%$, $P = 0.88$). The sequential group showed less inhibition of HBV DNA replication than the lamivudine group ($67.47\% \pm 0.02\%$ vs $82.48\% \pm 0.05\%$, $P = 0.014$); the difference between the sequential and cytokine groups was not statistically significant ($67.47\% \pm 0.02\%$ vs $57.45\% \pm 0.07\%$, $P = 0.071$). The levels of HBsAg and HBeAg were significantly decreased in the sequential treatment group compared to the other groups [HBsAg: 3.48 ± 0.04 (control), 3.09 ± 0.08 (lamivudine), 2.55 ± 0.13 (cytokine), 2.32 ± 0.08 (sequential), $P = 0.042$ for each between-group comparison; HBeAg: 3.48 ± 0.01 (control), 3.08 ± 0.08 (lamivudine), 2.57 ± 0.15 (cytokine), 2.34 ± 0.12 (sequential), $P = 0.048$ for each between-group comparison]. Cell viability in the cytokine group was reduced to $58.03\% \pm 8.03\%$ compared with control cells ($58.03\% \pm 8.03\%$ vs 100% , $P = 0.000$). Lamivudine pretreatment significantly reduced IFN- γ + TNF- α -mediated toxicity of HepG2.2.15 cells [$85.82\% \pm 5.43\%$ (sequential) vs $58.03\% \pm 8.03\%$ (cytokine), $P = 0.002$].

CONCLUSION: Sequential treatment overcame the lower ability of lamivudine alone to inhibit cccDNA and precluded the aggressive cytotoxicity involving IFN- γ and TNF- α by decreasing the viral load.

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Key words: Hepatitis B virus; Covalently closed circular DNA; Interferon- γ ; Tumor necrosis factor- α ; Lamivudine

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INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem, and more than 350 million people are chronically infected with this virus worldwide^[1]. Chronic hepatitis B infection is associated with an increased risk of cirrhosis, hepatic decompensation, and hepatocellular carcinoma^[2]. Seven drugs are currently approved for the treatment for chronic hepatitis B, including conventional interferon (IFN)- α , pegylated IFN- α , and the nucleos(t)ide analogs lamivudine, adefovir, entecavir, telbivudine and tenofovir. However, none of the currently available drugs can eliminate viral covalently closed circular DNA (cccDNA) from the nucleus of infected hepatocytes^[3,4].

During HBV infection, HBV cccDNA accumulates in cell nuclei where it persists as a stable episome and acts as a template for transcription of viral genes^[5]. The elimination of cccDNA is a prerequisite for curing HBV infection^[6]. Current knowledge suggests that clearance of HBV cccDNA occurs mainly through two pathways. The first is long-term and potent antiviral therapy, which effectively depletes the mature cytoplasmic nucleocapsid pool available for conversion into cccDNA. Based on mathematical models, the period needed to achieve complete clearance of intrahepatic cccDNA is 14.5 years^[7]. Short-term antiviral therapy cannot completely exhaust the viral pool, which is stable and constitutes the source of renewal of viral production after cessation of therapy^[8,9]. Nevertheless, long-term antiviral therapy can result in the development of antiviral resistance, and it is very expensive^[10,11]. The second mechanism of cccDNA clearance involves two immune mechanisms: A cytotoxic T lymphocyte (CTL)-dependent cytolytic mechanism by which infected cells are eliminated and replaced with non-infected cells^[12] and a non-cytolytic cytokine-dependent mechanism^[13]. In addition to killing HBV-positive hepatocytes, HBV-specific CTLs can downregulate hepatocellular HBV gene expression and replication via a non-cytopathic, cytokine-induced process. These processes are mediated by inflammatory cytokines such as IFN- γ and tumor necrosis factor- α (TNF- α), which are secreted by CTLs following antigen recognition in the liver^[14,15].

We previously reported that IFN- γ and TNF- α play a role in cell death of HBV-expressing HepG2.2.15 cells, a human hepatoblastoma cell line. Lamivudine treatment significantly reduces killing of HepG2.2.15 cells that is mediated by IFN- γ and TNF- α ^[16]. Lamivudine is the first

oral nucleoside analog to be approved for the treatment of chronic hepatitis B patients, and it has been shown to suppress HBV replication by interfering with HBV DNA polymerase and disease activity, reducing the incidence of hepatocellular carcinoma and prolonging survival^[17,18]. Lamivudine is potent and well tolerated, but its use is limited by the development of resistance. Viral breakthrough, which is defined as an abrupt increase in serum HBV DNA levels after a period of persistent suppression, may occur during lamivudine therapy^[19]. Persistence of HBV cccDNA in hepatocytes plays a key role in viral persistence, reactivation of viral replication after cessation of antiviral therapy, and resistance to therapy^[20]. To achieve effective suppression of HBV replication and elimination of HBV cccDNA and to avoid aggressive immune-mediated hepatitis and liver damage, we combined the above two established strategies of HBV cccDNA clearance. HBV-expressing HepG2.2.15 cells were initially given lamivudine to inhibit viral replication and reduce the level of HBV, and then the cells were given IFN- γ and TNF- α , two important immune mediators. We evaluated the antiviral potential of sequential treatment with lamivudine followed by IFN- γ and TNF- α in HepG2.2.15 cells, especially the potential to inhibit cccDNA amplification and eliminate its persistence.

MATERIALS AND METHODS

Cell culture and treatment

HepG2.2.15 cells, which were derived from the stable transfection of HepG2 cells with a plasmid containing two head-to-tail dimers of the HBV genome, were used in this study. The HepG2.2.15 line supports persistent replication of HBV and produces intact HBV particles^[21]. HBV cccDNA is detectable in the culture medium and intracellularly in HepG2.2.15 cells^[22,23]. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 2 mmol/L L-glutamine, 50 IU/mL penicillin, 50 mg/L streptomycin, 500 mg/L G418, 5% (vol/vol) fetal bovine serum in 5 mL/L CO₂ at 37 °C.

HepG2.2.15 cells were treated as follows: (1) with medium alone (control group); (2) 2 μ mol/L lamivudine (GSK, London, United Kingdom) for 16 d (lamivudine group); (3) culture medium for 10 d, followed by 5 ng/mL recombinant human TNF- α (Invitrogen, CA, United State) and 1000 U/mL recombinant human IFN- γ (R and D Systems China, Shanghai, China) for 6 d (cytokine group); or (4) 2 μ mol/L lamivudine for 10 d followed by 5 μ g/L TNF- α and 1000 U/mL IFN- γ for 6 d (sequential group). The supernatant was replaced with fresh medium (with or without lamivudine and cytokines as per treatment protocols) every 2 d.

Detection of HBV cccDNA

Intracellular DNA was extracted from 3×10^5 HepG2.2.15 cells from each group with the QIAamp Mini DNA kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The extracted product was further purified with mung bean nuclease (Gibco, Camarillo, CA,

United State) to remove HBV relaxed circular DNA that may have remained. The purification reaction was carried out in a 50- μ L volume containing 44 μ L extracted DNA solution, 1 μ L mung bean nuclease (10 000 U/mL), and 5 μ L 10 \times mung bean nuclease buffer at 37 $^{\circ}$ C for 30 min. EGTA (2 μ L; 100 mmol/L, pH 7.4) was then added to stop the reaction. HBV cccDNA was quantified with real-time polymerase chain reaction (PCR) using the ABI 7500 Real-Time PCR System (ABI, Foster City, CA, United States). Amplification was performed in a 20- μ L reaction containing 2 μ L isolated DNA and the Premix Ex Taq (Perfect Real-Time) kit (Takara, Dalian, China). The PCR primers were: forward 5'-TGAATCCYGC-GGACGACC-3' (nucleotides 1444-1461) and reverse 5'-CAGCTTGGAGGCTTGAACAG-3' (nucleotides 1862-1881) (Y = C/T). The TaqMan probe was: 5'-FAM-CCTAATCATCTCWTGTTTCATGTC-MGB-3' (nucleotides 1836-1858) (W = A/T). For HBV cccDNA amplification, the cycling conditions were an initial incubation of 30 s at 95 $^{\circ}$ C followed by 40 cycles of 5 s at 95 $^{\circ}$ C and 34 s at 60 $^{\circ}$ C. The inhibition ratio of cccDNA was calculated as: (control-treatment)/control \times 100%.

Quantification of HBV DNA using real-time PCR

Intracellular DNA was extracted from 3×10^5 HepG2.2.15 cells from each group with the QIAamp Mini DNA kit. HBV DNA was quantified with real-time PCR using SYBR Premix Ex Taq (Perfect Real Time) (Takara) with the ABI 7500 Real-Time PCR System. The PCR primers were forward 5'-CCTCTTCATCCTGCTGCT-3' and reverse 5'-AACTGAAAGCCAAACAGTG-3'. The PCR cycling program consisted of an initial denaturation step at 95 $^{\circ}$ C for 10 s, followed by 40 amplification cycles of 95 $^{\circ}$ C for 10 s, 60 $^{\circ}$ C for 10 s, 72 $^{\circ}$ C for 10 s, and 79 $^{\circ}$ C for 35 s, and then one cycle of 95 $^{\circ}$ C for 15 s, 60 $^{\circ}$ C for 60 s, and 95 $^{\circ}$ C for 15 s. The inhibition ratio of HBV DNA was calculated as: (control-treatment)/control \times 100%.

HBV antigen detection

HepG2.2.15 cell culture supernatants at days 12, 14 and 16 from each experimental group were collected and centrifuged at $118 \times g$ for 10 min to remove cellular debris and then transferred to clean tubes and stored at -20 $^{\circ}$ C until antigen measurement. The titers of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Kehua, Shanghai, China) according to the manufacturer's instructions.

Cell counting kit-8 assay

After treatment for 10 d according to the protocols, cells were seeded at 3×10^4 /well in 100 μ L medium in 96-well plates and incubated overnight to allow cell adherence. Cells were then exposed to lamivudine and/or TNF- α + IFN- γ for 6 d. Ten microliters of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt (Dojindo, Kumamoto, Japan) was added to each well, and the culture plate was incubated at 37 $^{\circ}$ C

for 1 h. Absorbance was measured at 450 nm. The percent cell viability was calculated as: (value after treatment-blank)/(control-blank) \times 100.

Statistical analysis

All data are expressed as the mean \pm SD from three different experiments. Statistical analysis was performed with analysis of variance using SPSS 17.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

Sequential treatment has a stronger suppressive effect on HBV cccDNA replication than treatment with lamivudine alone

The level of HBV cccDNA is an important parameter for determining the outcome of anti-HBV therapy. Both sequential treatment (51.50% \pm 0.17%) and cytokine treatment (49.66% \pm 0.06%) showed a stronger inhibition of HBV cccDNA compared to lamivudine alone (22.63% \pm 0.12%) (51.50% \pm 0.17% *vs* 22.63% \pm 0.12%, $P = 0.034$; 49.66% \pm 0.06% *vs* 22.63% \pm 0.12%, $P = 0.041$). The difference between the sequential and cytokine groups was not statistically significant ($P = 0.88$) (Figure 1A).

Sequential treatment has a weaker inhibitory effect on HBV DNA replication than treatment with lamivudine alone

The effect of the three different treatments on HBV DNA was investigated with real-time PCR. The sequential group (67.47% \pm 0.02%) showed lower inhibition of HBV DNA than the lamivudine group (82.48% \pm 0.05%) ($P = 0.014$). The inhibitory effect on HBV DNA between the sequential (67.47% \pm 0.02%) and cytokine groups (57.45% \pm 0.07%) was not significantly different ($P = 0.071$) (Figure 1B).

Sequential treatment causes the greatest reduction in HBsAg and HBeAg

Secretion of HBsAg and HBeAg into cell media at days 12, 14 and 16 during the treatment period was detected with a commercial ELISA kit. After 16 d of treatment, we observed statistically significant decreases in the levels of HBsAg and HBeAg with sequential treatment compared to the other groups [HBsAg: 3.48 \pm 0.04 (control), 3.09 \pm 0.08 (lamivudine), 2.55 \pm 0.13 (cytokine), 2.32 \pm 0.08 (sequential), $P = 0.042$ for each between-group comparison; HBeAg: 3.48 \pm 0.01 (control), 3.08 \pm 0.08 (lamivudine), 2.57 \pm 0.15 (cytokine), 2.34 \pm 0.12 (sequential), $P = 0.048$ for each between-group comparison] (Figure 2).

Sequential treatment suppresses TNF- α + IFN- γ -mediated cellular toxicity

The effect of each treatment on HepG2.2.15 cell viability was quantified with a CCK-8 assay. The viability in the cytokine group was reduced to 58.03% \pm 8.03% compared with control cells (100%, $P = 0.000$). Lamivudine pretreatment significantly reduced TNF- α + IFN- γ -mediated toxicity of HepG2.2.15 cells [85.82% \pm 5.43% (sequential) *vs* 58.03% \pm 8.03% (cytokine), $P = 0.002$] (Figure 3).

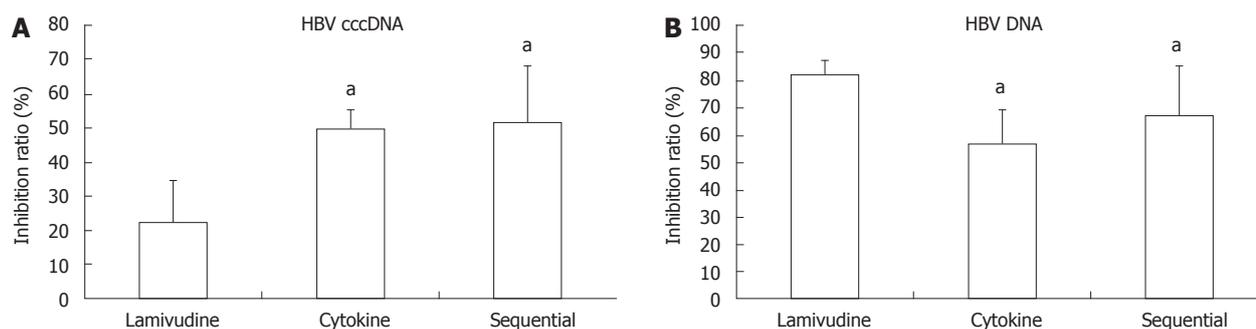


Figure 1 Inhibition ratio of hepatitis B virus covalently closed circular DNA (A) and DNA (B) following different treatments of HepG2.2.15 cells. A: Hepatitis B virus covalently closed circular DNA (HBV cccDNA) was quantified with real-time polymerase chain reaction; B: Hepatitis B virus DNA (HBV DNA) was quantified with real-time polymerase chain reaction. Data are expressed as the mean \pm SD from three individual experiments. ^a $P < 0.05$ vs lamivudine group.

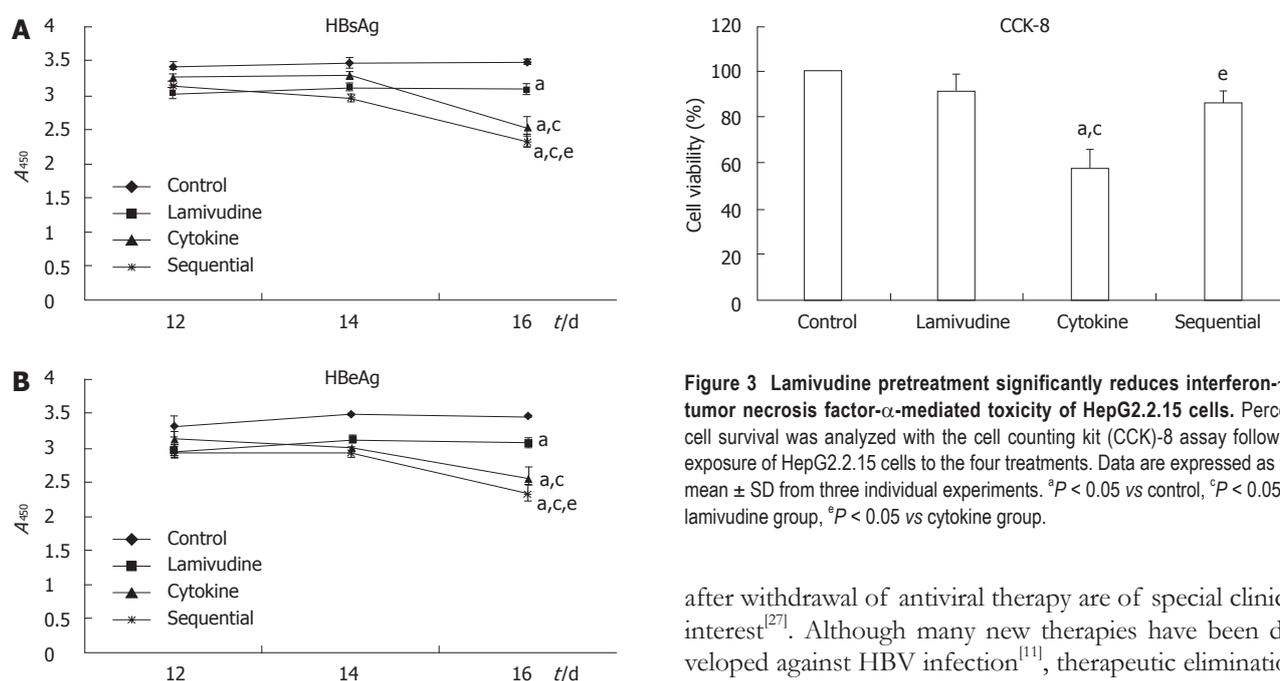


Figure 2 Levels of hepatitis B surface antigen and hepatitis B e antigen following different treatments of HepG2.2.15 cells. Sequential treatment reduced the levels of hepatitis B surface antigen (HBsAg) (A) and hepatitis B e antigen (HBeAg) (B) most strongly at 16 d in the treatment period. ^a $P < 0.05$ vs control group, ^c $P < 0.05$ vs lamivudine group, ^e $P < 0.05$ vs cytokine group.

Figure 3 Lamivudine pretreatment significantly reduces interferon- γ + tumor necrosis factor- α -mediated toxicity of HepG2.2.15 cells. Percent cell survival was analyzed with the cell counting kit (CCK)-8 assay following exposure of HepG2.2.15 cells to the four treatments. Data are expressed as the mean \pm SD from three individual experiments. ^a $P < 0.05$ vs control, ^c $P < 0.05$ vs lamivudine group, ^e $P < 0.05$ vs cytokine group.

DISCUSSION

HBV infection affects about 350 million people globally and is a leading cause of end-stage liver disease, hepatocellular carcinoma, and mortality^[24]. HBV primarily infects hepatocytes by a single pathway, although the exact mechanism remains poorly understood. After endocytosis, nucleocapsids are released into the cytoplasm, and the relaxed circular DNA genome is transported to the nucleus where it is converted into cccDNA^[25]. Persistence of HBV cccDNA in hepatocytes plays a key role in viral persistence, reactivation of viral replication after cessation of antiviral therapy, and resistance to therapy^[20,26]. Thus, prevention of the formation of cccDNA and elimination of cccDNA to prevent reactivation of viral replication

after withdrawal of antiviral therapy are of special clinical interest^[27]. Although many new therapies have been developed against HBV infection^[11], therapeutic elimination of HBV cccDNA remains a major challenge in curing chronic HBV infections. Novel approaches to improving current therapy for HBV infection are demanding but remain a global health priority.

IFN- γ and TNF- α are important immune mediators in host defense against HBV infection^[28,29]. Synergistic antiviral activity of murine IFN- γ and murine TNF- α on HBV gene expression was previously demonstrated in an HBV-Met transgenic hepatocyte cell line^[30]. IFN- γ and TNF- α abolished HBV gene expression and replication without killing the hepatocytes^[31,32]. Nevertheless, IFN- γ and TNF- α also play a role in apoptotic cell death. IFN- γ inhibits large HBV surface protein storage disease and the ground-glass hepatocyte appearance, but it exacerbates inflammation and apoptosis in HBV surface protein-accumulating transgenic livers^[33]. IFN- γ has been proposed to act as a pro-apoptotic regulator, triggering death receptors and other mediators^[34]. HBV X protein (HBx) sensitizes cells to apoptosis by TNF- α ^[35]. We previously reported that IFN- γ and TNF- α play a role in cell death of HBV-expressing HepG2.2.15 cells. Expression of HBV sensitizes the cells to IFN- γ + TNF- α -mediated

apoptosis. Increased expression of genes encoding interferon regulatory factor 1, c-myc and caspase 7 may be responsible for the synergistic induction of apoptosis by IFN- γ and TNF- α ^[16]. Cell death mediated by both IFN- γ and TNF- α may help eradicate the virus by eliminating residually infected cells. In patients with high levels of HBV DNA, however, aggressive immune-mediated processes involving IFN- γ and TNF- α may contribute to HBV-associated fulminant hepatic failure^[16].

To achieve effective suppression of HBV replication and elimination of HBV cccDNA and also avoid aggressive immune-mediated hepatitis and liver damage, we combined the two established strategies of clearance of HBV cccDNA. We found that sequential treatment with lamivudine followed by IFN- γ and TNF- α had a stronger suppressive effect on HBV cccDNA replication and antigen expression compared to the lamivudine-only group. Although the differences between the sequential and cytokine groups in the inhibition ratio of both HBV cccDNA and HBV DNA were not statistically significant, sequential treatment showed stronger inhibition of antigen expression than cytokines only. More importantly, lamivudine pretreatment significantly reduced IFN- γ + TNF- α -induced cytotoxicity of HepG2.2.15 cells.

Our *in vitro* findings suggest that sequential treatment with IFN- γ and TNF- α following lamivudine not only overcame the lower ability of lamivudine alone to inhibit HBV cccDNA by cytokine treatment but also precluded the aggressive immune-mediated cytotoxicity involving IFN- γ and TNF- α by decreasing the viral load with lamivudine pretreatment. This novel treatment suggests a new strategy for treating HBV infection and may shorten the course of antiviral therapy, minimize the emergence of drug-resistant mutants, and reduce the financial burden of patients. Because our present work was carried out *in vitro*, and HBV cccDNA was not eliminated in the relative short study period, further *in vivo* studies are warranted to evaluate the sequential treatment protocol to combat HBV infection.

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COMMENTS

Background

Hepatitis B virus (HBV) infection affects about 350 million people globally and is a leading cause of end-stage liver disease, hepatocellular carcinoma, and mortality. Although many new therapies have been developed against HBV infection, therapeutic elimination of HBV covalently closed circular DNA (cccDNA) remains a major challenge in curing chronic HBV infections. Novel approaches to improve current therapy for HBV infection are demanding but remain a global health priority.

Research frontiers

Current knowledge suggests that clearance of HBV cccDNA occurs mainly through two pathways: The first is long-term and potent antiviral therapy, which effectively depletes the mature cytoplasmic nucleocapsid pool available for

conversion into cccDNA. The second involves two immune mechanisms: A cytotoxic T lymphocyte (CTL)-dependent cytolytic mechanism by which infected cells are eliminated and replaced with non-infected cells and a non-cytolytic cytokine-dependent mechanism. These processes are mediated by inflammatory cytokines such as interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α), which are secreted by CTLs following antigen recognition in the liver. Nevertheless, IFN- γ and TNF- α have been reported to play a role in cell death of HBV-expressing HepG2.2.15 cells. Increased expression of genes encoding interferon regulatory factor 1, c-myc, and caspase 7 may be responsible for the synergistic induction of apoptosis by IFN- γ and TNF- α .

Innovations and breakthroughs

To achieve effective suppression of HBV replication and elimination of HBV cccDNA and also to avoid aggressive immune-mediated hepatitis and liver damage, the authors combined the two established strategies of clearance of HBV cccDNA. HBV-expressing HepG2.2.15 cells were initially treated with lamivudine to inhibit viral replication and reduce HBV levels, and then the cells were treated with IFN- γ and TNF- α , two important immune mediators, to achieve a synergistic effect on elimination of HBV. Based on the results, the authors found that sequential treatment with lamivudine followed by IFN- γ and TNF- α not only overcame the lower ability of lamivudine alone to inhibit HBV cccDNA by cytokine treatment but also precluded the aggressive immune-mediated cytotoxicity involving IFN- γ and TNF- α by decreasing the viral load with lamivudine pretreatment.

Applications

This novel treatment suggests a new strategy for treating HBV infection and may shorten the course of antiviral therapy, minimize the emergence of drug-resistant mutants, and reduce the financial burden of patients. Further *in vivo* studies are warranted to evaluate the sequential treatment protocol for combating HBV infection.

Terminology

HBV cccDNA: During HBV infection, HBV cccDNA accumulates in cell nuclei where it persists as a stable episome and acts as a template for the transcription of viral genes. Persistence of HBV cccDNA in hepatocytes plays a key role in viral persistence, reactivation of viral replication after cessation of antiviral therapy, and resistance to therapy. The elimination of cccDNA is necessary for curing HBV infection.

Peer review

The authors assessed the effect of sequential treatment with lamivudine followed by IFN- γ and TNF- α on HBV in HepG2.2.15 cells. It was observed that sequential treatment not only overcame the lower ability of lamivudine alone to inhibit HBV cccDNA but also precluded the aggressive immune-mediated cytotoxicity involving IFN- γ and TNF- α . The methodology is sound and the results obtained in cell culture studies have useful potential to be used in humans.

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Difficulty in differentiating two cases of sigmoid stenosis by diverticulitis from cancer

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Abstract

The incidence of colonic diverticulosis with or without diverticulitis has increased in the Japanese population due to the modernization of food and aging. The rate of diverticulitis in colon diverticulosis ranges from 8.1% to 9.6%. However, few cases of stenosis due to diverticulitis have been reported. These reports suggest that the differentiation between sigmoid diverticulitis and colon cancer is difficult. This report describes two cases of colon stenosis due to diverticulitis that were difficult to differentiate from colon cancer. Case 1 was a 70-year-old woman with narrowed stools for 1 month who underwent colonofiberscopy (CFS). CFS revealed a diverticulum and circumferential stenosis in the sigmoid colon. Barium enema revealed a marked, hourglass-shaped, 2-cm circumferential stenosis in the sigmoid colon. Fluorodeoxyglucose (FDG)-positron emission tomography computed tomography (CT) revealed an increased FDG uptake at the affected portion of the sigmoid colon. Sigmoid colon cancer was suspected,

and laparoscopic sigmoidectomy was performed. Pathological examination demonstrated active inflammation with no evidence of malignancy. Case 2 was a 50-year-old man who presented to a nearby clinic with reduced stool output despite the urge to defecate. CFS detected severe stenosis in the sigmoid colon approximately 25 cm from the dentate line. Contrast-enhanced abdominal CT revealed multiple diverticula, wall thickening, and swelling of the lymph nodes around the peritoneal aorta and the inferior mesenteric artery. A partial sigmoidectomy was performed. Pathological examination of the resected specimen revealed no changes in the mucosal epithelial surface, but a marked infiltration of inflammatory cells was observed.

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Key words: Diverticulosis; Colon cancer; Colon stenosis; Positron emission tomography-computed tomography; Magnetic resonance imaging

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INTRODUCTION

The incidence of colonic diverticulosis with or without diverticulitis has increased in the Japanese population due to the modernization of food and aging^[1].

The rate of diverticulitis in colon diverticulosis ranges from 8.1% to 9.6%. However, few cases of stenosis due

to diverticulitis have been reported. These reports suggest that the differentiation between sigmoid diverticulitis and colon cancer is difficult.

No causal relationship between colonic diverticulosis and colon cancer has been established^[2], but a significant correlation between these two conditions has been identified^[3]. Barium enema^[4], positron emission tomography-computed tomography (PET-CT), and magnetic resonance imaging-diffusion weighted imaging (MRI-DWI) are useful for the differential diagnosis between inflammation and cancer in the large intestine. This report describes and discusses two cases of sigmoid stenosis due to sigmoid diverticulitis that were difficult to differentiate from colon cancer using colonofiberscopy (CFS), barium enema, and PET-CT.

CASE REPORT

Case 1 was a 70-year-old woman with narrowed stools for 1 month who underwent CFS of the lower gastrointestinal tract at a nearby clinic. CFS revealed a diverticulum and circumferential stenosis in the sigmoid colon. The mucosa at the stenotic site was discolored, and the scope could not be advanced beyond the stenosis (Figure 1A). Biopsy showed no evidence of malignancy, and laboratory tests revealed no abnormal findings, including tumor markers [carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9)]. Contrast-enhanced abdominal CT revealed a marked thickening in the sigmoid colon wall. Barium enema revealed a marked, hourglass-shaped, 2-cm circumferential stenosis in the sigmoid colon (Figure 1B). Fluorodeoxyglucose [¹⁸F] (FDG)-PET CT revealed an increased FDG uptake at the affected portion of the sigmoid colon (Figure 2). Sigmoid colon cancer was suspected, and laparoscopic sigmoidectomy was performed. Pathological examination of the resected specimen revealed active inflammation characterized by a marked thickening of the muscularis propria, fibrous thickening of interstitial tissue, and a marked infiltration of inflammatory cells with no evidence of malignancy (Figure 3). The patient was diagnosed with inflammatory thickening and stenosis of the sigmoid colon due to sigmoid diverticulitis. The patient had a favorable postoperative course, and she was discharged from the hospital.

Case 2 was a 50-year-old man who presented to a nearby clinic with reduced stool output despite the urge to defecate. Abdominal X-ray revealed mild gas accumulation from the ascending colon to the transverse colon but no evidence of ileus. The patient was placed under observation; however, six months later, he was admitted to our hospital with a lack of bowel movement and lower abdominal pain with increased body temperature. Laboratory tests after admission indicated severe inflammation, including a white blood cell count of 13 520 cells/ μ L and C-reactive protein of 13.5 mg/dL but no abnormal tumor markers (CEA and CA 19-9). Barium enema revealed a segmental stenosis of approximately 10 cm, mucosal wall thickening, and cobblestone-like mucosa in the sigmoid colon (Figure 4). CFS detected severe stenosis in

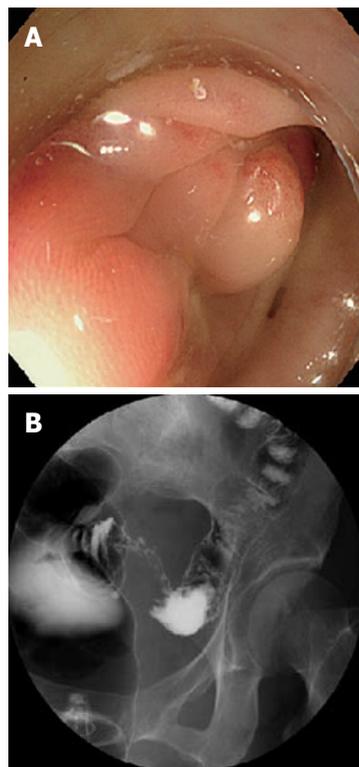


Figure 1 Colonofiberscopy and Barium enema revealed a marked, hourglass-shaped, 2-cm circumferential stenosis in the sigmoid colon. A: Colonofiberscopy; B: Barium enema.

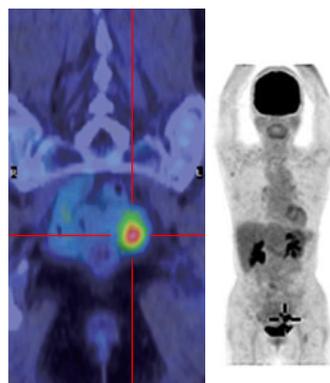


Figure 2 Fluorodeoxyglucose-positron emission tomography computed tomography imaging. A fluorodeoxyglucose-positron emission tomography computed tomography scan showing increased fluorodeoxyglucose (FDG) uptake in the affected portion of the sigmoid colon (maximum standardized uptake value: 5.6 in the early phase and 7.4 in the late phase), but no increase in FDG uptake was observed in the surrounding lymph nodes or distant organs.

the sigmoid colon approximately 25 cm from the dentate line, and the scope could not be advanced further. Mucosal surfaces in the visible range were edematous and red with a partial polyposis-like appearance (Figure 5). Biopsy suggested the presence of inflammation only. Contrast-enhanced abdominal CT revealed multiple diverticula, wall thickening, an increase in surrounding fat tissue, and swelling of the lymph nodes around the peritoneal aorta and the inferior mesenteric artery. FDG-PET also revealed wall thickening, lumen narrowing, and abnormal

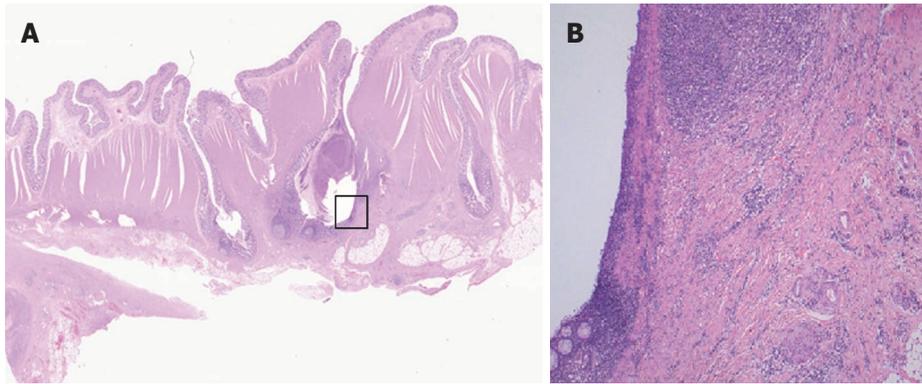


Figure 3 Magnified pathological image of resected specimen. A: A magnified pathological image of the resected specimen reveals some diverticula in the sigmoid colon (HE stain, 10 ×); B: A magnified pathological image of the marked square in Figure 4A (diverticulosis) shows active inflammation characterized by a marked thickening of the muscularis propria, fibrous thickening of interstitial tissue, and a marked infiltration of inflammatory cells with no evidence of malignancy (100 ×).

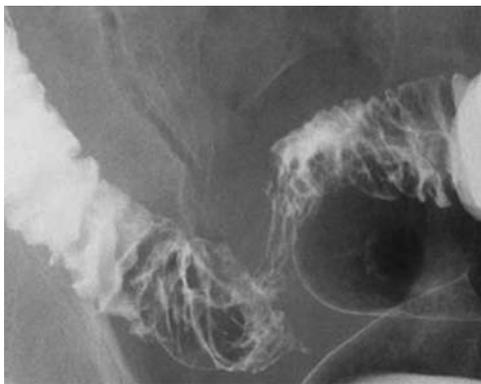


Figure 4 Barium enema revealed a segmental stenosis approximately 10 cm long, mucosal wall thickening, and cobblestone-like mucosa in the sigmoid colon.



Figure 5 Colonofiberscopy detected severe stenosis in the sigmoid colon approximately 25 cm from the dentate line, and the scope could not be advanced further. Mucosal surfaces in the visible range were edematous and red with a partial polyposis-like appearance.

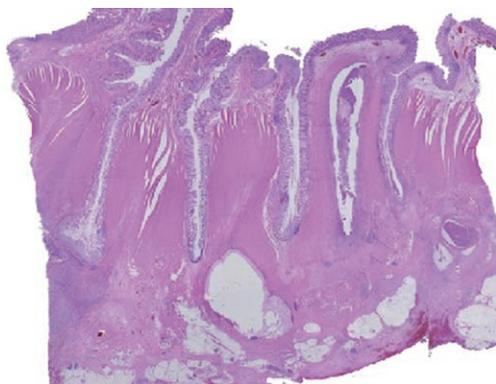


Figure 6 Pathological examination of the resected specimen. Pathological examination of the resected specimen revealed no change in the mucosal epithelial surface, but multiple diverticula penetrated the muscularis propria with a marked thickening of the muscularis propria, fibrous thickening of interstitial tissue, severe infiltration of inflammatory cells, and a cystic change that contained inflammatory exudate and abscess formation.

FDG uptake in the affected portion of the sigmoid colon. A marked increase in FDG uptake was also observed in the surrounding lymph nodes, which suggested a metastasis of the sigmoid cancer to para-aortic lymph nodes (T3N1M1). Partial sigmoidectomy was performed. Patho-

logical examination of the resected specimen revealed no changes in the mucosal epithelial surface, but a marked infiltration of inflammatory cells was observed (Figure 6). The patient was diagnosed with sigmoid diverticulitis, which led to circumferential stenosis due to severe and chronic inflammation.

DISCUSSION

Colonic diverticulosis is generally asymptomatic, but it can lead to diverticulitis, diverticular hemorrhage, and other complications in rare cases. A colonic diverticulum is a false diverticulum without muscularis propria, and the occurrence of diverticulitis leads to the spread of inflammation beyond the intestine. Only 0.09% of patients with diverticulosis develop diverticulitis and subsequent stenosis, which occurs more frequently in the left colon^[5].

The two cases in this study presented difficult preoperative differential diagnoses, which included benign lesions, such as intestinal tuberculosis and metastatic colon cancer, and malignancies with minimal tumor exposure on the mucosal surface, such as diffusely infiltrating colon cancer. However, no characteristic macroscopic findings were obtained, and biopsies did not provide a definitive diagnosis.

Benign lesions such as intestinal tuberculosis do not require immediate diagnosis because these lesions are post-healing changes. However, malignancies such as colon cancer require early diagnosis. The criteria for the differentiation of diverticulitis and cancer using barium enema X-ray results have been reported by Berman and Kirsner^[4]. The cases in this report could have been diagnosed as malignant using these criteria due to the stenosis length, the presence of an hourglass-shaped circumferential stenosis, clear borders and steep margins, despite their almost normal mucosal appearances. PET-CT was performed to differentiate between benign and malignant lesions, and an increased uptake was observed in both cases.

A delayed PET-CT reveals an increased uptake during active inflammation, which is a malignant pattern. However, the PET-CT of lung lesions reveals malignant lesions with higher maximum standardized uptake value levels than inflammatory lesions^[6]. A greater increase in F-FDG uptake at 60 s has been demonstrated in malignant tumors compared to inflammatory lesions in animals^[7].

Cut-off value for a malignant diagnosis using FDG has not been established. **Colon cancer arising from colonic diverticulum** has been reported previously, but Haubrich *et al.*^[2] demonstrated no correlation between colonic diverticulum and colon cancer. Conversely, a significantly higher prevalence of colon cancer in patients with and without multiple sigmoid diverticula was observed in a study conducted with a population of Japanese patients by Mihara *et al.*^[8]. Patients with colonic diverticulosis who develop colon cancer tend to develop diverticulitis at the same site^[9]. Therefore, a diagnosis of colon cancer should be considered in a patient with low levels of FDG uptake on PET-CT.

A lower false-positive rate for the diagnosis of inflammatory lesions has been demonstrated using MRI-DWI compared to PET in animals, but the sensitivity and specificity of MRI-DWI were similar to PET-CT^[10]. These results suggest that MRI-DWI is a useful diagnostic tool when the differentiation between colon cancer and inflammation is difficult.

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 San Francisco, CA 3000,
 United States

January 19-21, 2012
 2012 Gastrointestinal Cancers
 Symposium
 San Francisco, CA 94103,
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January 20-21, 2012
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 Association Clinical Congress of
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 Miami Beach, FL 33141,
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 The Future of Obesity Treatment
 London, United Kingdom

February 16-17, 2012
 4th United Kingdom Swallowing
 Research Group Conference
 London, United Kingdom

February 23, 2012
 Management of Barretts
 Oesophagus: Everything you need
 to know
 Cambridge, United Kingdom

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 2012
 Montreal, Canada

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March 30-April 2, 2012
 Mayo Clinic Gastroenterology and
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 San Antonio, TX 78249,
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 27th Annual New Treatments in
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 9th International Symposium on
 Functional GI Disorders
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 Singapore, Singapore

April 15-17, 2012
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 Colorectal Cancer Congress 2012
 Prague, Czech

April 18-20, 2012
 The International Liver Congress
 2012
 Barcelona, Spain

April 19-21, 2012
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April 20-22, 2012
 Diffuse Small Bowel and Liver
 Diseases
 Melbourne, Australia

April 22-24, 2012
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 La Jolla, CA 92093, United States

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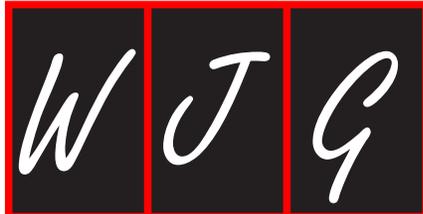
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 Las Vegas, NV 89085, United States

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 Modern Technologies in
 Diagnosis and Treatment of
 Gastroenterological Patients
 Dnepropetrovsk, Ukraine

November 4-8, 2012
 The Liver Meeting
 San Francisco, CA 94101,
 United States

November 9-13, 2012
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 of Liver Diseases
 Boston, MA 02298, United States

December 1-4, 2012
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In press

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

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

No author given

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

Volume with supplement

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

No volume or issue

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

Books

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

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- 15 Morse SS. Factors in the emergence of infectious dis-

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- 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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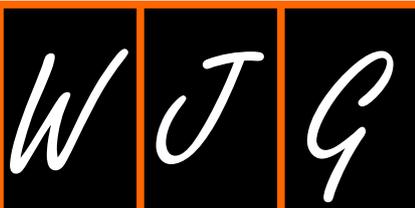
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Metabolic syndrome after liver transplantation: Preventable illness or common consequence?

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Abstract

The metabolic syndrome is common after liver transplant being present in approximately half of recipients. It has been associated with adverse outcomes such as progression of hepatitis C and major vascular events. As the United States population ages and the rate of obesity increases, prevention of the metabolic syndrome in the post-transplant population deserves special consideration. Currently, the metabolic syndrome after transplant appears at least two times more common than observed rates in the general population. Specific guidelines for patients after transplant does not exist, therefore prevention rests upon knowledge of risk factors and the presence of modifiable elements. The current article will focus on risk factors for the development of the metabolic syndrome after transplant, will highlight potentially modifiable factors and propose potential areas for intervention. As in the non-transplant population, behavioral choices might have a major role. Opportunities exist in this regard for health prevention studies incorporating lifestyle changes. Other factors such as the need for immunosuppression, and the changing characteristics of wait listed patients are not modifiable, but are important to know in order to identify persons at higher risk. Although immunosuppression after transplant is unavoidable, the contribution of different agents to the development of components of

the metabolic syndrome is also discussed. Ultimately, an increased risk of the metabolic syndrome after transplant is likely unavoidable, however, there are many opportunities to reduce the prevalence.

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Key words: Liver transplantation; Diabetes mellitus; Dyslipidemias; Hypertension; Metabolic syndrome X; Obesity; Hypertension; Immunosuppression

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INTRODUCTION

Liver transplantation is a life saving and life changing procedure for patients with advanced chronic liver disease, hepatocellular carcinoma and acute liver failure. The exceptional liver transplant recipient has normal fitness^[1] and can literally climb mountains^[2]. However, the more typical patient has impaired fitness^[3], gains weight^[4-6] and develops the metabolic syndrome^[7-10]. In fact, the prevalence of the posttransplant metabolic syndrome (PTMS) ranges from 44%-58%^[7-10], much higher than the 23% observed in the United States population^[11]. Is the high prevalence of the metabolic syndrome after liver transplant an unavoidable consequence driven by factors such as immunosuppression? Or is it a reflection of behavioral

tendencies in liver transplant recipients that can be modified to prevent illness? Although a definite conclusion is not likely, data can be found to support both possibilities. After a brief overview of the scope of the problem, the current manuscript will examine the factors which contribute to the PTMS and potential means of prevention.

PROBLEM DEFINED

The National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III)^[12] defined the components of the metabolic syndrome as follows; (1) impaired fasting glucose (≥ 100 mg/dL); (2) Abdominal obesity (> 102 cm in men, > 88 cm in women); (3) hypertriglyceridemia (≥ 150 mg/dL or drug therapy for triglycerides); (4) low levels of high density lipoprotein (HDL) (< 40 mg/dL in men, < 50 mg/dL in women or drug treatment for low HDL); and (5) elevated blood pressure ($\geq 130/85$ mmHg or drug treatment for hypertension). The presence of 3 or more of these components defines the metabolic syndrome. Complications of advanced liver disease can confound the diagnosis of the metabolic syndrome in the pretransplant setting. The presence of ascites alters waist circumference. Vasodilation and decreased effective circulating volume found with portal hypertension results in lowered systemic blood pressure. Synthetic dysfunction observed with end stages of liver disease immediately prior to transplant can result in lowered serum glucose and lipid values. A study evaluating the metabolic syndrome prior to liver transplantation found a rate of 5.4%^[10]. Pretransplant rates for hypertension were 9%-19%^[8-10,13], diabetes, 10%-22%^[7-10,13], dyslipidemia, 3%-43%^[9,10] and obesity, 11%-38%^[7,10].

Studies of the metabolic syndrome after transplant are summarized in Table 1. The rate of the metabolic syndrome may be underestimated in some reports as diabetes is substituted for impaired fasting glucose, hypertension is substituted for elevated blood pressure and body mass index (BMI) is substituted for elevated waist circumference. The presence of the metabolic syndrome after transplant was associated with more rapid progression of hepatitis C^[8] and an increased rates of major vascular events^[9,10]. Although no prospective data are available, it would be reasonable to expect that the metabolic syndrome might contribute to graft loss and patient death.

PRETRANSPLANT RISK FACTORS

The transplant population has been changing with regard to indication and recipient characteristics. Hepatitis C is declining as an indication for transplant while non-alcoholic fatty liver disease (NAFLD) is increasing and the average age of transplant candidates is rising. With these changes, risk factors for the metabolic syndrome are becoming more common in liver transplant candidates and are important predictors of PTMS development. Although studies evaluating risk factors to predict PTMS

are relatively small, certain factors identified were consistent across multiple series. A summary of risk factors is presented in Table 2. Obesity before transplant is a key factor in predicting the metabolic syndrome after transplant. Both pretransplant weight^[9] and BMI^[7,10,14] were correlated with post transplant metabolic syndrome. Not surprisingly, persons who were obese prior to transplant were often obese after transplant^[7]. The rate of obesity in wait-listed patients varies by transplant indication. Persons awaiting transplant with cryptogenic cirrhosis, which often results from NAFLD, were found to be more commonly obese than age and gender matched controls^[15]. NAFLD has increased annually as an indication for liver transplantation and is projected to become the most common indication for transplantation over the next 10-20 years^[16,17]. Review of Scientific Registry of Transplant Recipients data showed recipients characterized as other/unknown causes, which includes NAFLD, has steadily increased in the past 10 years currently representing 23.6% of recipients, almost equaling the percentage of recipients with hepatitis C^[18]. The proportion of obese persons awaiting transplant will presumably increase with the indication for transplant changing to a higher proportion of recipients with NAFLD. Compounding the effect of obesity, transplant for cryptogenic cirrhosis was found to be a risk factor for PTMS when controlling for other factors including pretransplant BMI^[9,10].

Other risk factors for the PTMS have been identified. Pretransplant diabetes was found to predict PTMS in multiple series^[7,10,14]. In fact, in one study, persons with pretransplant diabetes had nearly a 6 fold higher odds of having the metabolic syndrome after transplant^[10]. Age was additionally predictive of the metabolic syndrome after transplant^[9,10]. This is particularly important as the recipient population in the United States is aging. In 2009, nearly 75% of transplant recipients were above the age of 50, compared to 1993 where only 42% of recipients were 50 years of age or greater^[18]. Other pretransplant factors that were associated with the development of the metabolic syndrome after transplant in at least one series include hypertriglyceridemia^[10], low HDL^[10] and transplantation for hepatitis C or alcohol cirrhosis^[9].

A ROLE FOR PREVENTION PRIOR TO TRANSPLANT?

A preventive intervention could target persons with chronic liver disease, before they require transplant. The long term benefit of weight loss in an obese patient with cirrhosis is not known. In limited series from weight loss surgery, fibrosis improvement in cirrhotic patients after weight loss was inconsistent^[19-21]. However, in selected persons with compensated cirrhosis, diet and weight loss are reasonable recommendations. The potential for a weight loss intervention in reducing future need for transplant in obese persons with cirrhosis is an area in need of study. An exercise program in persons with advanced cirrhosis awaiting transplant may have additional

Table 1 Studies examining the prevalence of the metabolic syndrome after liver transplant

Study	Number subjects (n)	Metabolic syndrome (%)	Elevated glucose (%)	Elevated blood pressure (%)	Increased waist circumference (%)	Dyslipidemia (%)	Adverse outcome reported with metabolic syndrome
Bianchi <i>et al</i> ^[7]	296	45	60	53	32	37 increased TG 50 low HDL	Not studied
Laryea <i>et al</i> ^[9]	118	58	61 (diabetes)	62 (HTN)	36 (BMI > 30 kg/m ²)	45 increased TG 48 low HDL	More cardiovascular events
Hanouneh <i>et al</i> ^[8]	82	50	52 (diabetes)	64 (HTN)	45 (BMI > 28 kg/m ²)	Not reported	Increased fibrosis with HCV
Kallwitz <i>et al</i> ^[88]	172	65	68	Not reported	53	42 increased TG	More cardiovascular events
Laish <i>et al</i> ^[10]	252	52	40 (diabetes)	58 (HTN)	31	47 increased TG 49 increased HDL	More cardiovascular events

HTN: Hypertension; TG: Triglycerides; HDL: High density lipoprotein; BMI: Body mass index; HCV: Hepatitis C virus.

Table 2 Factors associated with the metabolic syndrome after transplant

Pretransplant risk factors	Posttransplant risk factors
Weight ^[9]	Change in BMI ^[7]
Body mass index ^[7,10,14]	
Cryptogenic cirrhosis ^[9,10]	
Alcohol cirrhosis ^[9]	
Hepatitis C cirrhosis ^[9]	
Pretransplant diabetes ^[7,10,14]	
Age ^[9,10]	
Triglycerides ^[10]	
High density lipoprotein ^[10]	

BMI: Body mass index.

benefits. Sarcopenia^[22] and decreased functional capacity measured by a six-minute walk test^[23] was associated with mortality in persons with cirrhosis. Clearly, increasing physical activity in cirrhotic patients at various degrees of synthetic dysfunction has potential utility and deserves further study.

Existing data indicate that as liver disease progresses to the point of needing transplant, participation in life-style modification becomes increasingly limited by malnutrition, muscle loss and reduced exercise capacity. Protein calorie malnutrition is common, and occurs more frequently with advanced liver disease^[24]. In cirrhosis, a shift toward a catabolic state with utilization of stored fat as a primary energy source during rest and exercise has been observed^[25,26]. The catabolic state induced by an overnight fast in a cirrhotic patient is roughly equivalent to a 36 h fast in a healthy subject^[27]. In a state of protein malnutrition, there is no rationale for a calorie restricted diet in a patient that is potentially requiring transplant. Alterations in physical activity are additionally vital for clinically significant weight loss, with a recent recommendation of greater than 250 min/wk^[28]. There are limitations for cirrhotic patients to achieve this goal. In health-related quality of life measures, persons awaiting liver transplant reported decreased scores relating to physical functioning^[29]. Oxygen consumption during peak exercise is often severely impaired in liver transplant candidates, and is correlated with disease severity^[30]. Additionally, cirrhotic patients develop anaerobic metabolism at low work loads, an effect that also becomes more pronounced as liver dis-

ease progresses^[31]. Studies have shown decreased muscle mass^[22] and strength^[31] in cirrhotic patients which may further limit the ability to exercise. The increase in portal pressure which can occur during exercise could potentially increase the risk of complications such as variceal bleeding is another barrier to exercise^[32].

POST TRANSPLANT RISK FACTORS FOR METABOLIC SYNDROME

As obesity prior to transplant is a risk factor for PTMS, it is intuitive that weight gain after transplant might predict the metabolic syndrome. However, data are mixed. The overall change in BMI after transplant was associated with the metabolic syndrome in one series^[7] but not in another^[9]. It is possible that ascites pre transplant may have resulted in the underestimation of weight gain post transplant in the later series. Weight gain after transplant is well described, however. In one series, the proportion of overweight and obese persons after transplant was 57% compared to 38% prior to transplant^[7]. In a large series of almost 600 liver transplant recipients, the median weight gain at 1 years and 3 years was 5.1 kg and 9.5 kg, respectively^[6]. In studies with longitudinal data, it appears that most weight gain occurs within the first year after liver transplant^[5,33,34]. Data from our center echoes these findings and is shown in Figure 1^[35]. Weight gain after transplant should be viewed in the context of persons returning to health after illness. Aggregate data from three studies which measured the rate of an elevated waist circumference after transplant totaled 36.9%^[7,10,35]. Although the populations are not matched, the absolute number is strikingly similar to the 38.6% rate reported for the United States population^[11]. In a study comparing rates of obesity, the standard prevalence ratio was not significantly higher in persons after liver transplant compared to the general United States population^[36].

Despite the weight gain over time after transplant, time since transplant was not associated with the prevalence of PTMS^[7,9,10]. This finding suggests factors resulting in PTMS develop soon after transplant and factors in addition to obesity require further scrutiny. Immunosuppression is one such factor. The overall contribution of immunosuppression to the development of the metabol-

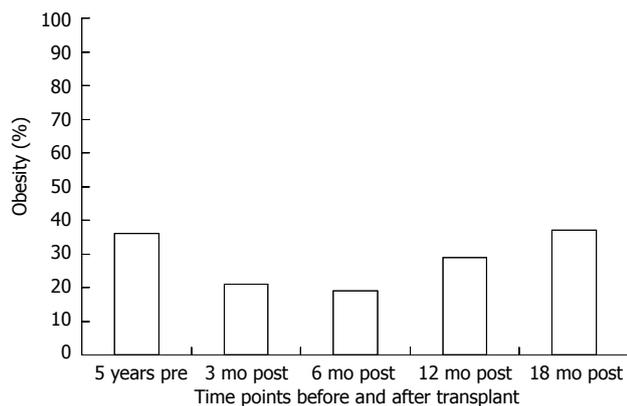


Figure 1 The prevalence of obesity at time points before and after liver transplant.

ic syndrome after transplant is difficult to measure, since immunosuppression is unavoidable. Although, the choice of calcineurin inhibitor (CNI) was not associated with the development of the metabolic syndrome^[7,8], different immunosuppressive agents have been shown to increase risk for various components of the metabolic syndrome. With regard to weight gain after transplant, one series found more weight gain with cyclosporine in the first year^[6]. However, the effect was not seen at 2 years. Another series found a higher overall BMI in cyclosporine treated patients, but no difference in rates of elevated waist circumference^[7]. Weight loss occurred in a majority of liver transplant recipients after switching to tacrolimus from cyclosporine^[37]. Although corticosteroids are often associated with weight gain, this effect after transplant was found in some^[5,38], but not all series^[6,34].

Post transplant diabetes

When compared to the general population, a higher prevalence of posttransplant diabetes mellitus (PTDM) has been observed in liver transplant recipients^[36]. Much of this risk can be related to immunosuppression with both calcineurin inhibitors and steroids playing a major role. Both CNIs have been associated with decreased insulin sensitivity and reduced insulin release^[39]. The reduced insulin release might result from CNI induced damage to pancreatic beta cells^[40]. Comparing the CNIs, most studies show higher rates of PTDM with tacrolimus use compared to cyclosporine^[41-46]. New onset PTDM was found in 27% of recipients on tacrolimus compared to 22% on cyclosporine [hazard ratio (HR) 1.24, 95% confidence interval: 1.07-1.43] in an analysis of over 15 000 recipients from United Network for Organ Sharing data^[45]. Switching tacrolimus to cyclosporine was found to improve PTDM^[47]. However, the absolute rates may not tell the entire story. Lowered serum levels of tacrolimus were associated with better glycemic control^[48]. Corticosteroid use with CNIs has an influence on PTDM rate. Corticosteroid use increases the risk of diabetes through decreasing insulin production and peripheral sensitivity and increasing hepatic gluconeogenesis^[49]. Avoidance of

corticosteroids and the use of induction therapy played an important role in decreasing rates of PTDM^[45,50-57]. In steroid free regimens using tacrolimus and an induction agent, PTDM was seen in less than 10% of recipients^[50,53]. Other series showed that patients receiving cyclosporine are more likely to require corticosteroids^[38,58]. Tacrolimus was associated with less graft loss and rejection^[43] potentially lessening the need for concomitant immunosuppressive agents such as steroids. Further studies are needed to compare rates of PTDM between the two CNIs used with induction therapy.

There are multiple studies examining risk factors in addition to immunosuppression for PTDM. Impaired glucose tolerance prior to transplant consisting of abnormal glucose levels^[59,60] or pretransplant diabetes^[7,44,61], was shown to be associated with post transplant diabetes. Hepatitis C as the transplant indication was linked to higher rates of PTDM in multiple series^[7,41,45,60-63]. Furthermore, eradication of hepatitis C led to improved glycemic control^[62]. In addition to hepatitis C, alcohol cirrhosis was additionally associated with PTDM^[7,44]. The number of episodes of acute cellular rejection^[64,65] and the number of steroid boluses^[62] were tied to higher rates of PTDM. Other factors correlated with PTDM include male gender^[44,61,66], older age^[41,45,67], African American race^[45] and a BMI > 25 kg/m²^[45,60]. Donor factors including age and diabetes were additional risk factors for PTDM^[45]. Insulin resistance after transplant was associated with a pretransplant BMI > 30 kg/m², older age, increased triglycerides, HCV infection and steroid boluses^[10].

Post transplant hypertension

Hypertension is another metabolic component with a higher standardized prevalence ratio in liver transplant recipients compared to the general population^[36]. Immunosuppression appears to have a profound effect in this setting. CNIs are known to increase sympathetic tone, result in vasoconstriction and cause sodium dependent volume expansion^[68]. Hypertension was observed less often with tacrolimus than with cyclosporine^[37,38,56,58,69]. Additionally, changing CNIs from cyclosporine to tacrolimus resulted in decreased rates of hypertension^[70,71]. Hypertension is observed with corticosteroid use^[49]. However, decreasing rates of hypertension with steroid sparing immunosuppression regimens were seen in a series using cyclosporine^[54,57] but not observed in series using tacrolimus^[50,51].

Post transplant dyslipidemia

Unlike diabetes and hypertension, the rate of hyperlipidemia was not found to be higher in persons after liver transplant compared to the general population^[36]. The definition of hyperlipidemia varied widely in studies and few used NCEP-ATP III definitions for dyslipidemia. This is important as one study found the rate of hypertriglyceridemia to be higher than that of elevated cholesterol after transplant^[72]. Although the absolute rate of hyperlipidemia after transplant may not be elevated compared to the general population, various risk factors

and choice of immunosuppressive agent may influence the prevalence of hyperlipidemia. Pretransplant hepatocellular disease and posttransplant renal dysfunction were found to be associated with hypertriglyceridemia after transplant^[72]. With regard to immunosuppressive agent, cyclosporine was associated with more hyperlipidemia^[38,56,69,73,74] and hypertriglyceridemia^[38] compared to tacrolimus. Additionally, changing from cyclosporine to tacrolimus improved hyperlipidemia in multiple series^[37,70,71]. The reason for this effect with cyclosporine could be related to inhibition of bile salt synthesis resulting in hyperlipidemia^[75]. Long term corticosteroid use may additionally contribute to hyperlipidemia^[57,76]. Steroid free or sparing regimens were associated with improved lipid levels^[52,53], including less hypertriglyceridemia in one series^[51]. Sirolimus, an agent used either in conjunction or in place of a CNI, is associated with high rates of dyslipidemia. With sirolimus, dyslipidemia was observed in 55% of patients, the majority of which required therapy^[77]. This finding observed with sirolimus use might result from changes in insulin signaling pathways resulting in excess triglyceride production and secretion^[78]. Rates of dyslipidemia were less when sirolimus was combined with tacrolimus compared to cyclosporine^[79].

In summary, it appears that higher rates of the metabolic syndrome after transplant compared to the general population might be attributed to increased rates of PTDM and hypertension. Although obesity does not appear more prevalent in post transplant populations, weight gain after transplant is pervasive. These findings could lead to a two-fold plan to reduce the metabolic syndrome after transplant. The first would be prevention of excess weight gain through behavioral changes that could start before transplant and continue afterward. The second would be focusing on immunosuppressive strategies to lower rates of diabetes, hypertension and hyperlipidemia after transplant, especially in persons with multiple risk factors present prior to transplant.

PREVENTION AFTER TRANSPLANT

After transplant, patients have an improved functional capacity and can perform tasks independently^[80]. The use of a structured exercise program increased exercise capacity and fitness for the first six months after transplant followed by a plateau^[81]. The improvement in fitness was three times greater than expected in a sedentary person undergoing a similar training regimen. Multiple studies have shown that although exercise performance improved after transplant, it remained lower than predicted values for age matched controls^[5,81,82]. Despite the improvement in fitness and quality of life, many persons after transplant remain sedentary^[81,83]. Only a quarter of persons were found to be physically active after transplant, and those that were physically active had less hypertension and decreased BMI^[83]. Clearly there is a role for programs dedicated to increase physical activity after transplant. There are little data regarding nutritional composition

and caloric intake after transplantation. Up to two thirds of subjects were found to have more than recommended energy intake^[84]. Overall, more data is required to assess the contribution of caloric intake to weight gain after transplant.

There is very limited data regarding behavioral changes and exercise therapy after liver transplantation. A single randomized trial evaluated the effects of exercise and dietary counseling after transplantation. An improvement in cardiorespiratory fitness and quality of life was reported in the intervention group, but no changes were noted in body composition or muscle strength^[82]. There was a trend toward improved body composition in the 37% of subjects that adhered to the intervention^[82]. There are no data regarding the impact of an exercise program on the prevalence of the metabolic syndrome or singular components after transplant. Behavioral therapy after transplant represents an area with vast potential for important research. It is additionally important to note that the primary goal of intervention in this setting should be the prevention of weight gain immediately after transplant as opposed to the treatment of the metabolic syndrome once present.

Additional therapies for weight loss have been tried after transplant. A study using orlistat found a reduction in waist circumference, but not body mass index in a small group of patients^[85]. Concern over interference with the absorption of immunosuppression limited the use of this agent. Bariatric surgery at the time of transplant and in the post transplant period was described in case reports^[86,87]. However, bariatric surgery should be done in a way that preserves access to the biliary system and to minimize interference with the absorption of immunosuppression. As a result of these obvious pitfalls, bariatric surgery during and after liver transplant has not been widely performed.

CONCLUSION

The transplant population is aging and, consistent with the overall population, becoming more often overweight or obese. Immunosuppression likely contributes to an increased prevalence of the metabolic syndrome in liver transplant recipients, especially through increased rates of diabetes and hypertension. These features make it likely that the metabolic syndrome after transplant will become more prevalent. A number of preventative measures could be considered to reduce the burden of the metabolic syndrome in liver transplant recipients.

Patients should be screened for risk factors for post transplant metabolic syndrome at the time of transplant evaluation. Consistent risk factors include diabetes, obesity and cirrhosis from NAFLD. When assessing obesity, screening for the highest lifetime BMI may be beneficial. Identification of at risk patients will allow special focus on preventive effort after transplant.

Although there are no data supporting a definitive increase in risk resulting from the choice in calcineurin

inhibitor, immunosuppression is modifiable. Minimization of corticosteroid use, possibly in conjunction with induction agents, appears to be beneficial in reducing components of the metabolic syndrome. The choice of CNI could be individualized based on the presence of components of the metabolic syndrome prior to transplant. For example, tacrolimus may be a better agent for someone with long standing pretransplant hypertension.

Consideration should be given for early eradication of hepatitis C, especially in those persons at risk for post-transplant diabetes.

Preventative efforts through behavioral changes should be made before and after transplant. At minimum, improved fitness prior to transplant is associated with better survival. After transplant, a multidisciplinary approach with increased activity, diet modifications and behavioral therapy should be explored. Since the metabolic syndrome develops early after transplant, intervention should begin as soon as medically feasible.

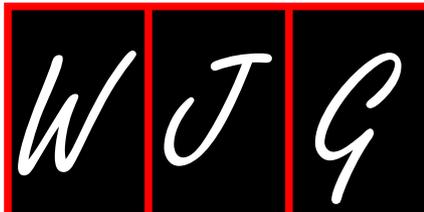
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Cellular and molecular mechanisms of intestinal fibrosis

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Abstract

Fibrosis is a chronic and progressive process characterized by an excessive accumulation of extracellular matrix (ECM) leading to stiffening and/or scarring of the involved tissue. Intestinal fibrosis may develop in several different enteropathies, including inflammatory bowel disease. It develops through complex cell, extracellular matrix, cytokine and growth factor interactions. Distinct cell types are involved in intestinal fibrosis, such as resident mesenchymal cells (fibroblasts, myofibroblasts and smooth muscle cells) but also ECM-producing cells derived from epithelial and endothelial cells (through a process termed epithelial- and endothelial-mesenchymal transition), stellate cells, pericytes, local or bone marrow-derived stem cells. The most important soluble factors that regulate the activation of these cells include cytokines, chemokines, growth factors, components of the renin-angiotensin system, angiogenic factors, peroxisome proliferator-activated receptors, mammalian target of rapamycin, and products of oxidative stress. It soon becomes clear that although inflammation is responsible for triggering the onset of the fibrotic proc-

ess, it only plays a minor role in the progression of this condition, as fibrosis may advance in a self-perpetuating fashion. Definition of the cellular and molecular mechanisms involved in intestinal fibrosis may provide the key to developing new therapeutic approaches.

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Key words: Inflammatory bowel disease; Intestinal fibrosis; Extracellular matrix; Molecular mediators; Myofibroblasts; Inflammatory cells; Epithelial cells; Mesenchymal cells; Endothelial cells

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INTRODUCTION

Fibrosis is a chronic and progressive process characterized by an excessive deposition of extracellular matrix (ECM) components, such as collagens. It is believed to follow chronic tissue inflammation and ultimately leads to organ scarring and subsequent loss of function. Fibroproliferative disease may affect almost all tissues and organs, including the skin, kidneys, lungs, cardiac and vascular systems, eyes, liver, pancreas and intestine. Tissue fibrosis is a leading cause of morbidity and mortality. It has been estimated that 45% of deaths in the United States can be attributed to fibrotic disorders^[1].

Intestinal fibrosis is usually considered to be a com-

mon complication of several enteropathies with distinct initiating pathophysiology, such as inflammatory bowel disease (IBD), radiation enteropathy, graft-versus-host disease, collagenous colitis, eosinophilic enteropathy, drug-induced enteropathy, sigmoid diverticulitis, solitary rectal ulcer, cystic fibrosis, intra-peritoneal fibrotic adhesions, desmoplastic reaction in gastrointestinal tumors (familial adenomatous polyposis-FAP), desmoid tumors, gastrointestinal (GI) stromal tumors (GISTs) and post-surgical intestinal adhesions and strictures leading to intestinal stenosis and obstruction^[1-3].

Of these enteropathies, IBD is the main cause of intestinal fibrosis since this disease is characterized by a persistent immuno-mediated intestinal inflammation. In IBDs, both ulcerative colitis (UC) and Crohn's disease (CD), fibrosis follows the distribution and location of inflammation. In UC, the deposition of ECM is restricted to the mucosal and submucosal layers of the large bowel. In CD, fibrosis can involve the entire bowel wall of the GI tract including the mucosa, submucosa, muscularis mucosa, muscularis propria and serosa layers^[2]. The increase of ECM in the tissue, with collagens and fibronectins being the major components, can ultimately lead to development of intestinal strictures and obstruction. Fibronectin has been shown to co-localize with aggregations of fibroblasts^[2]. Intestinal strictures may be composed of a combination of fibrosis and inflamed tissue. In CD, these may occur anywhere in the GI tract, but most frequently affect the terminal ileum. The increased rate of clinical complications related to small bowel fibrosis in CD is likely related to the smaller diameter of the bowel lumen, rather than to more severe fibrosis.

Intestinal injury is almost invariably followed by an acute inflammatory response. This is usually followed, in turn, by physiologic healing of the damaged tissue and restoration of the normal structure and function of the intestine. If this does not occur, chronic inflammation can develop, characterized by continuous events of injury and repair that may lead to the development of fibrosis. Injury to the intestine is not an uncommon phenomenon, even in otherwise healthy individuals. In most instances, wound healing leads to normal restitution and resolution of the tissue damage. In IBD, it is still unclear which factor triggers the road to chronicity. In addition, once intestinal inflammation is chronic, it is not yet understood what sets the stage for the later development of intestinal strictures.

In contrast to the intensive investigations focusing on the immunological mechanisms related to the early phases of intestinal inflammation and repair, the pathophysiology of chronic mucosal wound healing and the late events of repair leading to fibrosis remain largely unexplored.

It appears to be widely accepted that chronic intestinal inflammation invariably leads to fibrosis. However, this process does not occur in all patients. Chronic intestinal inflammatory diseases exist, such as celiac disease or lymphocytic colitis, that are not complicated by fibrosis and stricture formation. These findings indicate that

distinct mechanisms of inflammation and restitution/fibrosis exist. It is crucial to explore this area since various pathways could be targeted separately, which would thus allow tailored treatment for wound healing abnormalities, especially in IBD.

Several lines of evidence suggest that inflammation is necessary to trigger the onset of the fibrotic process, but subsequently plays a minor role in progression of the disease^[1]. Anti-inflammatory treatment in IBD and in other chronic inflammation-associated fibrotic conditions in various organs (lung, liver, kidney) does not prevent evolution of fibrosis once the process of excessive ECM deposition has started. Mechanisms that regulate fibrosis, therefore, appear to be distinct from those regulating inflammation. The lack of efficient and well-tolerated anti-fibrotic drugs is partly due to the fact that the main and specific cellular and molecular pathways leading to fibrosis remain to be identified.

Various findings suggest that innate and adaptive immune mechanisms acting in chronic inflammation may divert the healing process towards fibrogenesis. A potential mechanism has been attributed to plasticity of the adaptive CD4 T cell immune response. Although CD4 T helper (Th) 1 and Th2 cells are reasonably stable after differentiation occurs, mature Th17 and Foxp3 regulatory T cells (Tregs) can transform into other subsets, i.e., exhibit plasticity^[4]. This appears to be driven by the transcription factors related orphan receptor (ROR) γ t for Th17 cells and Foxp3 for Treg cells. A potential escape mechanism could be the micro RNA system: small sequences of RNA that interact with genes and alter their transcription^[5]. Changes in microRNA expression, induced by biological treatment, may result in non-response to the therapeutic agents.

Fibrogenesis is a "physiological process" triggered by the onset of inflammation that may lead either to tissue repair or fibrosis depending on the balance between production of ECM proteins and enzymatic degradation. Intestinal fibrosis is related to the abnormal function of activated intestinal mesenchymal cells (proliferation, migration, contraction, ECM production) namely, ECM-producing cells or myofibroblasts. Myofibroblasts are activated by a variety of mechanisms including paracrine signals derived from immune and non-immune cells, autocrine factors secreted by myofibroblasts, and pathogen-associated molecular patterns (PAMPs) derived from micro-organisms that interact with pattern recognition receptors such as the toll-like receptors (TLRs)^[1]. The most investigated soluble factors that regulate the activation of the ECM-producing cells include cytokines [interleukin (IL)-6, IL-13, IL-17, IL-21, tumor necrosis factor (TNF)- α], chemokines [monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1], growth factors [transforming growth factor (TGF)- β 1, connective tissue growth factor (CTGF), platelet derived growth factor (PDGF), insulin-like growth factor (IGF)-1 and 2, epidermal growth factor (EGF)], components of the renin-angiotensin (ANG) system (RAS), angiogenic

factors [e.g., vascular endothelial growth factor (VEGF)], peroxisome proliferator-activated receptors (PPARs), mammalian target of rapamycin (mTOR), and products of oxidative stress^[1]. All these molecules are being investigated as potential targets of anti-fibrotic drugs. Pharmacological modulation of tissue ECM deposition by reducing activated ECM-producing cells and their profibrogenic effects (proliferation, motility, contraction, ECM production) could be useful in the prevention and treatment of intestinal fibrosis^[1].

ECM degradation is mediated by the matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). The fine balance between MMPs and TIMPs appears to be disturbed in chronically impaired wound healing in IBD. It is unclear which specific MMPs and TIMPs are involved and how they are regulated in this process. Nevertheless, effective pharmacological modulation of the MMP/TIMP-system could be helpful in the reversal of already established tissue fibrosis^[6].

In IBD, current therapeutic agents, mainly corticosteroids, aminosalicylates, immunosuppressants (e.g., azathioprine and methotrexate), and even the more recent biologic drugs, such as anti-TNF antibodies, can improve intestinal inflammation but seem unable to significantly improve fibrosis^[7-9]. Surgical correction, by means of intestinal resection or stricturoplasty, is necessary in up to 75% of CD patients during the course of their disease^[10-12]. However, surgical resection is associated with a high rate of recurrent stricturing disease and the need for repeated surgery is high; therefore, exploration of new therapeutic approaches has now become mandatory^[11,13,14].

RELATIONSHIP BETWEEN INFLAMMATION AND FIBROSIS

Wound healing is an important biological process that allows the replacement of dead or damaged cells after tissue injury and involves a *regenerative phase*, where injured cells are replaced by cells of the same type, thus leaving no evidence of damage, and a phase known as *fibroplasia*, where normal parenchymal tissue is replaced by the deposit of new ECM components^[1].

In physiological conditions, the normal turnover of ECM, in each tissue, is regulated by several factors that influence synthesis and degradation. Indeed, under normal physiological conditions, continuous ECM remodeling occurs consisting of the production and balanced degradation of ECM components^[15]. Myofibroblasts are the predominant mucosal cells that synthesize components of the ECM and ECM breakdown is mediated by proteolytic enzymes derived from various cell types of which the MMP family represent a large and important component^[15,16].

Fibrosis is progressive but considered reversible. Several studies have shown that fibrosis may progress independently of inflammation^[1,15].

The inflammatory process may be acute or chronic based on its duration and the cells involved. Acute in-

flammation is a rapid response to injurious agents while chronic inflammation is a process of prolonged duration in which the inflammatory cascade lasts for weeks or months and under certain circumstances a lifetime^[15].

During a chronic inflammatory process, the physiological sequence of events related to inflammation (coagulation cascade, inflammatory response, and fibroproliferative response) is activated for a prolonged time period. If the network of negative feedback mechanisms that terminate the proliferative and fibrotic response fails to operate, the healing process can become pathologic causing continuous activation of the fibroproliferative response and excess of ECM deposit that progressively leads to substantial changes in normal tissue architecture with loss of organ function^[1,15,17].

Inflammation is induced and sustained by an infiltrate of immune cells, such as T cells, macrophages and neutrophils in the intestinal mucosa, which determine tissue damage characterized through a loss of epithelial cells and degradation of ECM in the lamina propria leading to the formation of ulcerations. Subsequently, release of inflammatory mediators from damaged epithelial and/or endothelial cells leads to an anti-fibrinolytic-coagulation cascade and formation both of blood clots and a provisional ECM^[15,17,18].

Clotting releases thrombin, a potent inducer of platelet degranulation through which a vast store of bioactive mediators (including thromboxanes, prostaglandins, chemokines and cytokines) are secreted promoting further clot formation and stimulation of the inflammation process. Another consequence of platelet degranulation is the release of potent chemotactic and growth-promoting cytokines at the site of inflammation which enhances inflammatory responses by recruitment of immune cells and activation of immune and non-immune cells and the release of oxygen radicals, cathepsins and ECM-degrading enzymes. Neutrophils are the first cell type recruited and represent the most abundant inflammatory cell in the early stages of wound healing. Activated neutrophils degranulate, releasing inflammatory and pro-fibrogenic cytokines, and subsequently die followed by the recruitment of macrophages. During this initial leukocyte migration phase, the activated macrophages and neutrophils eliminate tissue debris, dead cells, and any invading organisms. They also produce cytokines and chemokines, which initiate and amplify the wound-healing response. These factors are also mitogenic and chemotactic for endothelial cells, which surround the injury and form new blood vessels as they migrate toward the center of the ulceration^[18].

When inflammation becomes chronic it induces myofibroblast activation that can determine an excessive accumulation of ECM, which promotes the formation of a permanent fibrotic scar. ECM consists of several molecular components including collagens (I-VI), elastin, glycoproteins, glycosaminoglycans and proteoglycans (Table 1). ECM is not an inactive structure, but directly regulates the inflammatory response and the process of healing and fibrosis by focal adhesion with immune and

Table 1 Extracellular matrix molecules involved in wound repair and fibrosis

Collagens	Fibrillar type collagens I-III-V Non-fibrillar collagen type IV
Glycoproteins	Laminin Entactin/nidogen Fibronectin Tenascin Sparc/BM40 Thrombospondin
Proteoglycans	Glycosaminoglycans (hyaluronic acid) Heparan sulfate Chondroitin sulfate Perlecan
ECM-modifying proteins	Matrix metalloproteinases Tissue inhibitor of metalloproteinases

ECM: Extracellular matrix.

non-immune cells as well as myofibroblasts^[19].

In summary, the events triggered by chronic injury that are involved in fibrogenesis are: (1) immediate damage to the epithelial/endothelial barrier; (2) release of chemokines and cytokines; (3) recruitment of inflammatory cells (immune and non-immune); (4) release of reactive oxygen species (ROS) and pro-fibrogenic cytokines and growth factors; (5) activation of ECM-producing cells; and (6) accumulation of ECM proteins.

Innate immune mechanisms

The causes leading to chronic intestinal inflammation in IBD are unknown. It has been suggested that, in genetically predisposed subjects, it could be the result of a dysregulated immune response to intra-luminal antigens, such as resident luminal bacteria, bacterial products, or dietary antigens: in a chronic defective mucosal barrier, the continuous exposure of lamina propria immune cells to the luminal antigens may trigger, amplify and maintain the local inflammation.

Genetic factors and luminal microbes, in addition to their role in triggering the IBD, are also directly, or indirectly, involved in the development of intestinal fibrosis in IBD.

The luminal bacteria express PAMPs, including lipopolysaccharide (LPS) components of bacterial cell walls, bacterial DNA, and double-stranded RNA, able to bind to pattern recognition receptors expressed by a wide variety of immune and non-immune cells, such as the extracellular TLRs and the intracellular nucleotide oligomerization domain/caspase recruitment domain (NOD/CARD)-like receptors^[20].

TLRs are expressed on immune cells as well as non-immune cells, and are involved in innate immunity by recognizing specific patterns of microbial components.

The TLR family, comprised of 10 members, are able to act as primary sensors of microbial products triggering a host defence response against invading pathogens and activating signaling pathways that induce the expression of immune and pro-inflammatory genes^[20].

Bacterial components induce the activation of an intracellular signaling cascade *via* TLRs that involves the preserved intra-cytoplasmic Toll/interleukin-1R (TIR) domain and TIR domain-containing adaptors, such as MyD88. A MyD88/interleukin-1 receptor-associated kinase signaling is thus generated, which regulates innate immunity and can strongly influence the course of the inflammatory response. An increased mucosal expression of TLRs in the intestine of patients with IBD has been reported^[21,22]. The TLR activation can be considered a pivotal event in the immunological response in IBD patients^[21], suggesting that over-expression of certain TLRs could be one of the underlying mechanisms leading to the abnormal host reaction to commensal bacteria, as seen in IBD^[22]. Another important aspect of this defective immune tolerance to commensal bacteria seems to be TLR polymorphisms. Various studies have demonstrated the correlation between *TLR4* polymorphisms, at Asp299Gly and Thr399Ile, and the development of IBD^[23,24]. Indirect evidence also suggests the involvement of an aberrant innate immune response in intestinal fibrogenesis. Antibodies directed against microbial peptides represent good serological markers that could help in the identification of fibrotic CD. Patients with a stronger immune response to microbial peptides are more likely to develop earlier complicated CD, including the earlier occurrence of fibrotic CD^[25,26].

Nevertheless, the exposure of subepithelial cells to bacterial ligands seems not only to lead to immune-mediated inflammation, but also to direct mesenchymal cell activation.

Studies performed on animal models of IBD have demonstrated not only the presence of TLRs in intestinal fibroblasts and the role of PAMPs in their activation, but have also revealed a close correlation between pathogen-mediated fibroblast activation and the progression of fibrosis. The TLR ligands could directly lead to differentiation of fibroblasts into activated myofibroblasts and TLR expression in non-immune cells can be considered another key event leading to tissue scarring and to the development of fibrosis^[27-29].

The role of TLRs in the fibrosis-promoting signals has been frequently observed in the liver in which hepatic stellate cells (HSCs), the main precursors of ECM-producing cells, are sensitized by TLR4 activation; this receptor is functionally expressed by HSCs and directly stimulates myofibroblasts to enhance TGF- β signaling^[30]. An increase of LPS during hepatic fibrogenesis induces a TLR4-mediated downregulation of bone morphogenetic protein and activin membrane-bound inhibitor (BAMBI), a pseudoreceptor that decoys TGF- β by lacking an intracellular kinase domain^[30,31]. Thereby, the TLR4-MyD88-dependent downregulation of BAMBI induces an unrestricted activation of the TGF- β pathway in HSCs. The LPS-TLR4-MyD88 signaling cascade results are thus closely linked with TGF- β -mediated collagen deposition and promotion of liver fibrosis^[29,30].

The role of TLRs has been investigated in the dam-

aged tissue of several organs, including the intestine, even if their involvement in fibrogenic progression has not been completely elucidated.

An increased expression of TLR 2, 3, 4, 6 and 7 has been observed in the intestinal fibroblasts isolated from patients with CD^[21,22,32]. These receptors are activated by their respective microbial ligands and promote the differentiation of fibroblasts into collagen-producing myofibroblasts to induce a fibrogenic response^[32]. In addition, an increased production of fibronectin and upregulated levels of α -smooth muscle actin (α -SMA) in human intestinal myofibroblasts exposed to TLR ligand have been reported, thus confirming the pro-fibrogenic activity of TLRs and the direct link between bacterial innate immunity and intestinal fibrosis in IBD in man^[33]. Furthermore, TLR activation of gut myofibroblasts induces an increased secretion of CXC chemokine ligand 8, a ligand that was initially characterized as a neutrophil chemotactic factor, but seems to exhibit both an angiogenic and angiostatic activity that can lead to the progression of intestinal fibrosis^[34].

Another key factor able to activate an innate immune response by intracellular recognition of PAMPs of selected microorganisms is NOD2, an intracellular peptidoglycan receptor for muramyl dipeptide encoded by the *NOD2/CARD15* gene present on chromosome 16. *NOD2/CARD15* polymorphisms seem to be, in part, involved in the pathogenesis of CD. Considerable evidence confirms, indeed, a relationship between the mutation of *NOD2/CARD15* genes and CD, either alone or in combination with mutation of *TLR* (especially *TLR4*) or *ATG16L1* (an autophagy gene)^[35].

The major *NOD2/CARD15* polymorphisms associated with CD are Arg702Trp, Gly908Arg and Leu1007fsinsC and these appear to produce defects in the host defence response against invading bacteria that can lead to a persistent intracellular infection with chronic stimulation of inflammatory cells^[36]. NOD2 is present in several cell types such as monocytes and macrophages where it seems to decrease the TLR-induced production of pro-inflammatory Th1 cytokines^[37]. NOD2 is also expressed in Paneth cells. A correlation between *NOD2/CARD15* mutations and the downregulation of mucosal α -defensin has been repeatedly proven^[38,39]. Furthermore, NOD2 can induce autophagy by recruiting the critical autophagy protein ATG16L1 on the plasma membrane during bacterial internalization.

In addition, there is a growing body of evidence proving that the main *NOD2/CARD15* variants are closely related to ileal disease, a stenosing phenotype and to a greater need for abdominal surgery in CD patients^[26]. Cells carrying *NOD2* variants show an enhanced pro-inflammatory response to various intestinal microbes and lead to an increased TGF- β production and collagen deposition by T cells^[40]. All these findings provide evidence that may encourage the clinical use of *NOD2/CARD15* genotyping, both as a marker of CD and as a prognostic factor of the need for early surgery due to stricturing and

fibrostenosing disease^[26].

IBD, however, is a polygenetic disease. The development of genome-wide association scanning technologies has led to the discovery of more than 100 confirmed IBD loci^[41-43]. Some, such as the Th 17 pathway genes (*IL23R*, *IL12B*, Janus kinases 2 (*JAK2*), signal transducers and activators of the transcription 3 (*STAT3*), are shared between CD and UC, others are phenotype-specific (autophagy genes such as *ATG16L1*, *IRGM* and *NOD2* for CD; epithelial barrier genes *HNF4a*, *E-Cadherin*, *LAMB1* and *IL-10* for UC). Variants of some of these genes would also be excellent candidates involved in the development of fibrosis in IBD^[44-46].

Most studies have focused on the involvement of non-immune cell types in the progression of fibrosis in IBD, including mesenchymal cells (smooth muscle cells, fibroblasts, myofibroblasts), epithelial cells, nerve cells and platelets^[47-49]. In addition, mucosal microvascular cells play a central role in the microcirculation regarding the onset and maintenance of IBD^[47]. Much evidence indicates that neoangiogenesis, the growth of blood vessels from those already present, plays a crucial role in IBD^[48]. The microcirculation and endothelial cells perform a crucial task in intestinal immune homeostasis: endothelial cells regulate the type and the number of leukocytes that migrate from the blood flow to the interstitial space and specialized vascular cells, such as those found in the endothelium of venules, control selectively the influx of a specific subset of T-cells^[49].

Despite the evidence regarding the role of endothelial cells and neoangiogenesis in the inflammatory process, very limited data are available on their role in intestinal fibrosis.

CELLULAR MECHANISMS

The key event that leads to the development of intestinal fibrosis is not only recruitment of immune and inflammatory cells, but also the exposure of mesenchymal cells to a variety of inflammatory mediators that are able to maintain these cells in a persistent state of trans- and de-differentiation between fibroblasts, myofibroblasts and smooth muscle cell phenotypes. All mesenchymal cell types can directly or indirectly contribute to intestinal fibrosis by producing large amounts of ECM proteins with different architectural and barrier functions, and are able to regulate several growth factors acting in a paracrine and autocrine fashion^[50-52]. Each single mesenchymal cell type can be identified by the expression of specific cellular markers, as reported in Table 2.

Several conditions (such as ischemia, radiation, chemicals, microbes, products of oxidative stress, drugs, autoimmunity and allergies) promote the release of inflammatory mediators during tissue injury, leading to activation of mesenchymal and non-mesenchymal cells that produce ECM, also referred to as ECM-producing cells (Figure 1).

Cellular processes that lead to abnormal wound healing are mainly represented by activation (proliferation,

Table 2 Cell types involved in intestinal fibrosis

Cell type	Positive markers
Fibroblasts	Vimentin, FSP-1, N-cadherin (high), collagen type I, prolyl 4-hydroxylase
Intestinal subepithelial myofibroblasts	Vimentin, α -SMA, cadherin-11, FSP-1, collagen type I
Interstitial cells of Cajal	Vimentin, c-Kit receptor
Smooth muscle cells	Vimentin (low), α -SMA, Desmin, collagen type I
Stellate cells	Vitamin A, GFAP, α -SMA
Pericytes	α -SMA, desmin (low), MCSP, RGS5, PDGFR- β , CD80, CD86, CD 13, CD90
Epithelial cells	ANG I and II, ET-1, collagen type I
Endothelial cells	E-cadherin, cytokeratins, CD31, vWF, VE-cadherin, N-cadherin (low), vimentin (low)
Bone marrow stem cells	
Hematopoietic stem cells	CD45, CD34, CD14
Mesenchymal stem cells	CD105, CD73, CD44, CD71, CD90
Fibrocytes	CD45, CD34, CD11, CD13, CD14, CD80, CD86, collagen type I, α -SMA

FSP-1: Fibroblast specific protein 1; MCSP: Melanoma chondroitin sulfate proteoglycan; RGS5: Regulator of G protein signaling-5; GFAP: Glial fibrillary acidic protein; α -SMA: α -smooth-muscle actin; VE-cadherin: Vascular endothelial cadherin; vWF: von Willebrand factor; cKit: Tyrosine-protein kinase kit or CD117; ANG I and II: Angiotensin I and II; ET-1: Endothelin-1; CD: Cluster of differentiation. High and low indicate level of expression.

migration, contraction, ECM production) of fibroblasts and myofibroblasts. Nevertheless, it has become clear that the cellular source of the ECM proteins not only involves mesenchymal cells, such as fibroblasts, myofibroblasts and smooth muscle cells, but also other cell populations, such stellate cells, pericytes, as well as bone-marrow and intestinal stem cells^[15,50]. Recently, it has also been observed that myofibroblasts may derive from non-mesenchymal cell transformation such as epithelial-to-mesenchymal transition and endothelial-to-mesenchymal transition^[51].

Fibroblasts

Fibroblasts are a heterogeneous population of cells present in the interstitium of all normal tissues and organs where they play a pivotal role in maintaining structural integrity, regulating matrix homeostasis and taking part in healing and regenerative processes and/or in pathogenesis of scarring.

An acute injury or acute inflammation can launch activation of fibroblasts driving them towards a fibrogenic phenotype. This activated fibroblast can be engaged either in normal wound healing or fibrosis. Fibroblast activation is characterized by a post-transcriptional or post-translational up-regulation of ECM secretion.

Fibroblasts co-express vimentin, fibroblast-specific protein 1 (FSP-1, S100A4), N-cadherin, and prolyl 4-hydroxylase (Table 2).

The increase in the resident fibroblast population is

a pivotal mechanism for the development of intestinal fibrosis. Several growth factors found in the inflamed gut, such as IGF- I , basic fibroblast growth factor (bFGF), EGF, CTGF, PDGF, and pro-inflammatory cytokines, such as interleukins (IL-1 β , IL-6) and TNF- α , increase their proliferation rate^[27].

TGF- β 1, one of the most potent profibrogenic factors, shows a bifunctional role in fibroblast proliferation with either growth stimulation or growth inhibition and blocking of cell differentiation depending upon the concentration used or the organ from which the cells are derived^[51,52]. TGF- β 1 can also act in an indirect mode by up-regulation of the PDGF receptor, increasing synthesis of CTGF and promoting expression of IGF- I , all factors that directly affect proliferation^[15,53,54].

Another mechanism that further increases the number of activated fibroblasts is the migration from non-affected tissue areas into and through the surrounding ECM, under the effect of a chemotactic gradient (chemotaxis) or with active random movement (chemokinesis) to the wound area. In the intestine, the cell migration is generally an essential factor for physiological wound repair, and is a fundamental process during the progression of pathological conditions such as fibrosis in IBD. Migration of fibroblasts can be induced by autocrine stimuli, like fibronectin, and paracrine factors, such as PDGF-AB, IGF- I , EGF and TGF- β 1, all of which appear to be fibronectin-dependent^[51,55,56]. Mediators of active inflammation, such as TNF- α , IFN- γ or PGE2, can inhibit fibroblast migration further indicating distinct mechanisms of inflammation and fibrogenesis^[57,58].

Myofibroblasts

Myofibroblasts represent a highly contractile cell type that is thought to be critical for tissue repair and the pathogenesis of fibrogenetic diseases^[59,60]. These cells exhibit a “hybrid” phenotype between fibroblasts and smooth muscle cells and, when activated, synthesize high levels of ECM, particularly collagen, glycosaminoglycans, tenascin and fibronectin^[61]. Besides their normal activities in growth and differentiation of tissues, the myofibroblasts play a central role in wound healing. During normal wound repair of a tissue injury, a closely regulated sequence is induced, including activation and proliferation of myofibroblasts that are present in the wound bed where they play essential roles in wound contraction and connective tissue restoration, e.g, through the production of ECM and basement membrane molecules^[11,15,17].

Myofibroblasts can also play a role in the up- or down-regulation of the inflammatory response by the secretion both of chemokines and cytokines^[11,17]. When these processes are not controlled, deranged, or repeated, as occurs in several fibroproliferative diseases, the normal resolution stages are abrogated and the proliferation of myofibroblasts continues, inducing excessive accumulation of the ECM and leading to alterations in the tissue architecture and ultimately to organ failure. Therefore, fibrotic disease is a major pathological end point of activated and proliferating

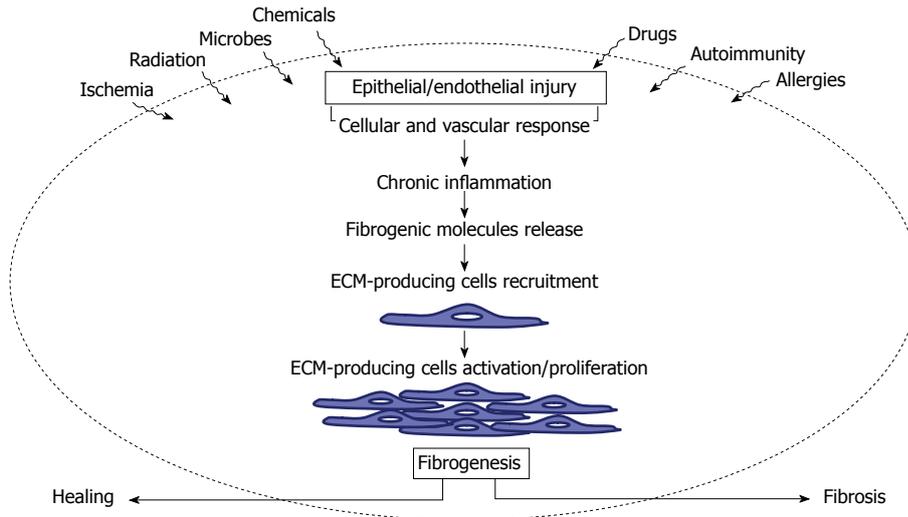


Figure 1 Mechanisms of fibrogenesis. Inflammatory response to tissue damage promotes the release of fibrogenic molecules. These molecules increase activation and proliferation of ECM-producing cells. ECM: Extracellular matrix.

erating myofibroblasts in most, if not all, tissues^[1,17].

Myofibroblastic cells contain smooth muscle cytoskeletal markers (in particular α -SMA) together with three filaments (vimentin, desmin or myosin), with variable expression depending on tissue, species and environmental factors^[61,62]. Based on immunohistochemical staining of these filaments in a given tissue, a classification system has been proposed^[61,62]. Myofibroblasts that express only vimentin (V-type myofibroblasts), those that express vimentin and desmin (VD-type), those that express vimentin, α -SMA and desmin (VAD-type), those that express vimentin and α -SMA (VA-type), and those that express vimentin and myosin (VM-type)^[61,62].

Two types of myofibroblasts are present in the intestinal mucosa in physiological conditions, the interstitial cells of Cajal (ICC) and the intestinal sub-epithelial myofibroblasts (SEMFs)^[61,62].

ICC are located in the submucosa and muscularis propria in association with the smooth muscle layer of the gut, while SEMFs are mainly located at the base of the intestinal crypts in the lamina propria. ICC are pacemaker cells which regulate gastrointestinal smooth muscle motility, facilitate the propagation of electrical events and modulate neurotransmission^[63]. ICC express vimentin and the c-Kit receptor (Table 2).

SEMFs, also called pericryptal fibroblasts, are a syncytium of α -SMA-positive mesenchymal cells, which reside subjacent to the basement membrane of the small and large intestines^[61,62]. SEMFs form a three-dimensional network and are in connection with each other by gap and adherent junctions, but also maintain connections with epithelial cells through fenestrations in the basement membrane; they also interact with intestinal macrophages. SEMFs express α -SMA and vimentin suggesting that they are members of the VA class of myofibroblasts. They may also express smooth muscle myosin (and thus may be referred to as VAM-type myofibroblasts), although the expression of myosin is less than that ob-

served in the corresponding smooth muscle cells in the same tissue^[61,62].

It is unknown whether SEMFs and ICC differentiate from a common precursor. Instead, it has been clearly demonstrated that mediators which act on myofibroblasts, promoting their proliferation and ECM production, are numerous and include: PDGF, EGF, IGF-1 and 2, CTGF, IL-1, IL-13, stem cell factor (SCF), endothelins (ET-1, -2, -3), ANG II, TGF- α , TGF- β , bFGF and PPAR γ ; these factors are all relevant for the transdifferentiation of fibroblasts to myofibroblasts^[15].

Smooth muscle cells

In physiological conditions when there is a need for replacement of connective tissue following injury, the production of collagen is devolved to cells of mesodermal origin. Other cells do not synthesize collagen or may produce only minute quantities; however, under abnormal stimuli collagen expression may become considerably enhanced in these cells^[61,62]. Airway smooth muscle cells (SMCs) appear to play an important role in controlling and perpetuating airway inflammation and fibrosis in chronic airway diseases^[64].

SMCs are one of the three interrelated cell phenotypes into which intestinal mesenchymal cells can differentiate (the other two being fibroblasts and myofibroblasts) and are mainly identified by α -SMA, desmin and collagen type I^[27] (Table 2).

Several studies have shown that SMCs are an important cellular component of IBD. In UC, their behavior leads to a considerable thickening of the muscularis mucosa and, in CD, to a remarkable thickening of the bowel wall, with subsequent stricture formation and obstruction. These cells actively contribute to the development of fibrosis in IBD by inducing production of collagen and MMPs in response to several inflammatory mediators, such as TGF- β and IL-1 β . SMCs are also able to release significant amounts of IL-6, contributing to the

inflammatory process^[65].

Stellate cells

Stellate cells were initially described in the liver as mesenchymal cell precursors important in retinoic acid metabolism, where they represent the site of fat or vitamin A storage. Thereafter, the important contribution of these cells to hepatic and pancreatic fibrosis has been shown^[66,67].

These cells are identified through the expression of vitamin A, glial fibrillary acidic protein (GFAP) and α -SMA (Table 2).

In the liver, it has been clearly shown that HSCs are the main contributors to fibrogenesis. Acute or chronic inflammation can promote activation of HSCs through transdifferentiation from quiescent vitamin A-rich cells into myofibroblast-like cells with strong proliferative, fibrogenic and contractile ability. Moreover, the presence of stellate cells has been detected also in other organs, such as the pancreas, gut, lung, uterus, kidney and deferent duct, even if their functions remain to be fully elucidated^[51,66,67].

Interestingly, there is very limited information regarding intestinal stellate cells, although recently these have been isolated and cultured from the human intestinal mucosa^[51]. In IBD, these cells show a higher proliferation rate, faster differentiation into myofibroblasts, and an earlier and higher collagen production than those from the normal non-IBD mucosa^[51].

Pericytes

Pericytes are cells derived from non-differentiated mesenchymal cells. They surround the endothelial cells of capillaries and small blood vessels.

Pericytes stain positive for α -SMA, desmin, melano-ma chondroitin sulphate proteoglycan, platelet-derived growth factor receptor (PDGFR)- β , and regulator of G protein signaling-5. Pericytes also express costimulatory molecules such as CD80, CD86, CD13 and CD90, suggesting that they may be replenished by circulating fibrocytes. Furthermore, pericytes can express ANG I and II, endothelin-1 and collagen type I (Table 2). The pattern of these pericyte markers differs according to the type of vessel, organ and also pathological condition.

Pericytes control endothelial cell differentiation, endothelial signaling, angiogenesis and ECM degradation. These multiple functions can be exerted due to their location between the interstitium and endothelium^[68]. Moreover, on account of their intermediate phenotype between vascular-SMC and fibroblasts, pericytes represent a useful reserve of fibroblasts during wound healing and are, therefore, considered an excellent contributor to inflammation-associated fibrosis on account of their ability to differentiate into collagen-producing fibroblast-like cells^[51]. Pericytes increase the deposition of ECM proteins in proximity to the blood vessels during the initial phase of fibrotic processes. In addition, the involvement of pericytes in intestinal fibrogenesis has been recently

highlighted, even if their contribution remains to be defined^[51].

Epithelial and endothelial cell transformation

The main fibrogenic cells (fibroblasts, myofibroblasts) may also derive from non-mesenchymal cells, including epithelial and endothelial cells, *via* transformation.

Epithelial-to-mesenchymal transition (EMT) or endothelial-to-mesenchymal transition (EndoMT) is a key process in tissue development, carcinogenesis and organ fibrosis, and is characterized by dramatic changes in cell phenotype and function. Both are a consequence of disruption of the local basement membrane, loss of epithelial cell adhesion, reprogramming of the signaling machinery, *de novo* synthesis of α -SMA, rearrangement of cytoskeletal proteins and transmigration of the epithelial cells through the basement membrane into the interstitial space^[69]. Epithelial or endothelial cells assume a spindle-shape morphology, lose classical cell markers and gain typical fibroblast or myofibroblast markers, such as FSP-1, α -SMA or vimentin, and show the capacity to produce interstitial collagens and fibronectin (Table 2). All these changes are due to the high plasticity of epithelial and endothelial cells. Therefore, these cell types can be considered a multipotent progenitor tissue, which can display alternative developmental pathways following injury.

Recent data on the contribution of EMT and EndoMT in intestinal fibrosis are now available. It has been shown in animal models and in human primary cells that EMT and EndoMT can contribute to intestinal fibrogenesis^[70,71].

Bone marrow and intestinal stem cells

Stem cells are a non-differentiated cell type that do not increase in number but generate a range of differentiated progeny that may continue to be divided *via* a process of clonal growth. They are also defined as non-specialized cells that reside in a particular tissue and renew themselves thus becoming capable of producing all the specialized cell types of the tissue^[72].

Reserves of adult stem cells include the bone marrow, blood stream, cornea and retina, dental pulp, liver, skin, gastrointestinal tract and pancreas. Bone marrow contains hematopoietic and mesenchymal stem cells, which are able to migrate to the majority of organs and differentiate into various cell types. These cells may represent a source of fibroblasts during inflammatory and fibrotic disease; indeed, stem cells can migrate from the bone marrow to sites of injury, during pathological conditions, and differentiate to mesenchymal cells able to express tissue connective proteins^[73].

While HSCs give rise to 3 classes of blood cells (leukocytes, erythrocytes and thrombocytes), and are characterized by several specific markers, including CD45, CD34 and CD14, the mesenchymal stem cells (MSCs) are multipotent and can differentiate into several cell types such as osteoblasts, chondrocytes, myocytes and myofibroblasts. MSCs express CD105, CD73, CD44, CD71 and CD90. Both HSCs and MSCs express vimentin, che-

mokines and chemokine receptors, adhesion molecules and integrins^[74].

A class of bone marrow-derived cells that become progenitors for mesenchymal cells is represented by fibrocytes that circulate in the peripheral blood. These cells appear to be involved in intestinal repair and fibrosis in IBD^[50,51,73,74]. Fibrocytes express hematopoietic markers (CD45, CD34, CD11, CD13, CD14, CD80, CD86), as well as collagen I and α -SMA^[27,51,74-76] (Table 2).

The ability of fibrocytes to differentiate into fibroblasts and contractile myofibroblasts has been highlighted. This leads to the production of ECM components and ECM-modifying enzymes by differentiated fibrocytes that migrate to the affected tissues during both normal tissue repair and inflammatory fibrotic processes. Under physiological conditions, fibrocytes, after their maturation in the bloodstream, may contribute to the local population of macrophages and dendritic cells, whereas during an inflammatory process, numerous fibrocytes differentiate into mesenchymal cells synthesizing ECM. This behavior demonstrates the close relationship between fibrocyte proliferation, migration and differentiation, as well as tissue fibrogenesis^[27].

Fibrocyte functions that lead to tissue fibrosis are modulated by IL-1, TGF- β and serum amyloid P (SAP), a serum protein that inhibits the maturation process in the circulation but promotes differentiation of fibrocytes within the tissue^[75]. Furthermore, fibrocytes themselves produce growth factors, such as CTGF and TGF- β , inflammatory cytokines and chemokines, that in turn promote the proliferation of resident fibroblasts and their differentiation into myofibroblasts^[75].

The contribution of fibrocytes to tissue damage and progression of fibrosis has been shown in several pathological conditions, including asthma, nephrogenic fibrosis, systemic sclerosis, atherosclerosis, chronic pancreatitis, chronic cystitis and tumor-associated stromal reactions^[77].

Another aspect of intestinal fibrosis under investigation is the presence and the involvement of local stem cells in the fibrotic process. Undifferentiated intestinal stem cells (ISCs) give rise to daughter or progenitor cells, which can subsequently differentiate into mature cell types such as columnar cells, goblet cells, neuroendocrine cells and Paneth cells^[72,74,76].

Differentiation of ISCs is induced by various transduction pathways and appears to be involved in the pathogenesis of IBD^[78]. It has been highlighted that a potential mechanism linked to the development and progression of Crohn's disease is the differentiation of ISCs into Paneth cells^[78]. These cells produce different broad spectrum antimicrobial peptides, principally the α -defensins HD-5 and HD-6. In small intestinal Crohn's disease, both these Paneth cell products are specifically reduced. Mechanisms for defective antimicrobial Paneth cell function are complex due to the impaired functions of various pathways including the NOD2, Wnt pathway transcription factor TCF7L2 (also known as TCF4), autophagy factor ATG16L1, endosomal stress protein XBP1, toll-like receptor TLR9,

calcium-mediated potassium channel KCNN4, as well as to the inactivation of HD-5.

A better understanding of all these mechanisms may lead to the development of new therapeutic approaches for the prevention and/or treatment of intestinal fibrosis progression.

MOLECULAR MECHANISMS

Intestinal fibrosis results from the activation of a large variety of cell types that act synergistically and are exposed to an extremely complex microenvironment, under the control of various biological mediators, such as growth factors, cytokines, chemokines, proteolytic enzymes, complement components, vasoactive amines and peptides. The most important of these molecules include, specifically, TGF- β , activins, CTGF, PDGF, IGF-1 and 2, EGF, ET-1, -2, -3, RAS, various cytokines such as IL-1, -4, -6, -13, -17, -21, -22, -23, TNF- α , ROS, PPAR γ , mTOR, MMPs and TIMPs. All these molecules play an important role in the activation of the acute and chronic inflammatory response. In addition, they also regulate the fibrogenic processes stimulating ECM accumulation, independently of inflammation^[1,15,27], acting in autocrine, paracrine, or endocrine pathways. All these profibrotic molecules may be considered as a target for new antifibrotic treatment approaches.

TGF/Smad proteins

Studies on transgenic mice over-expressing TGF- β have revealed the development of fibrosis in several organs, including skin, kidney, lung, heart, blood vessels, liver, pancreas and intestine^[15].

TGF- β is a multifunctional polypeptide hormone acting in essentially all cells, influencing distinct functions including proliferation, differentiation, apoptosis, immunoregulation, regulation of the inflammatory response, restitution and healing, as well as fibrosis^[79]. At a cellular level, TGF- β affects virtually all stages of the chronic inflammatory and fibrotic disease processes.

The profibrotic effects of TGF- β are numerous, including influx and activation of ECM-producing cells, as well as promoting EMT and EndoMT. The biological action of TGF- β that contributes to several fibrotic diseases is the regulation not only of the synthesis but also the breakdown of ECM proteins, including collagens, fibronectins and proteoglycans^[27].

Multicellular organisms present more than 60 TGF- β family members. These include 3 TGF- β s, 5 activins and at least 8 bone morphogenetic proteins (BMPs), all encoded by distinct genes. The three mammalian TGF- β isoforms, TGF- β 1, 2 and 3, are secreted as latent precursor molecules (LTGF- β) containing an amino-terminal hydrophobic signal peptide region, the latency associated peptide (LAP) region and the C-terminal potentially bioactive region. The LTGF- β is usually linked to latent TGF- β -binding proteins (LTBP), requiring activation into a mature form for receptor binding and subsequent

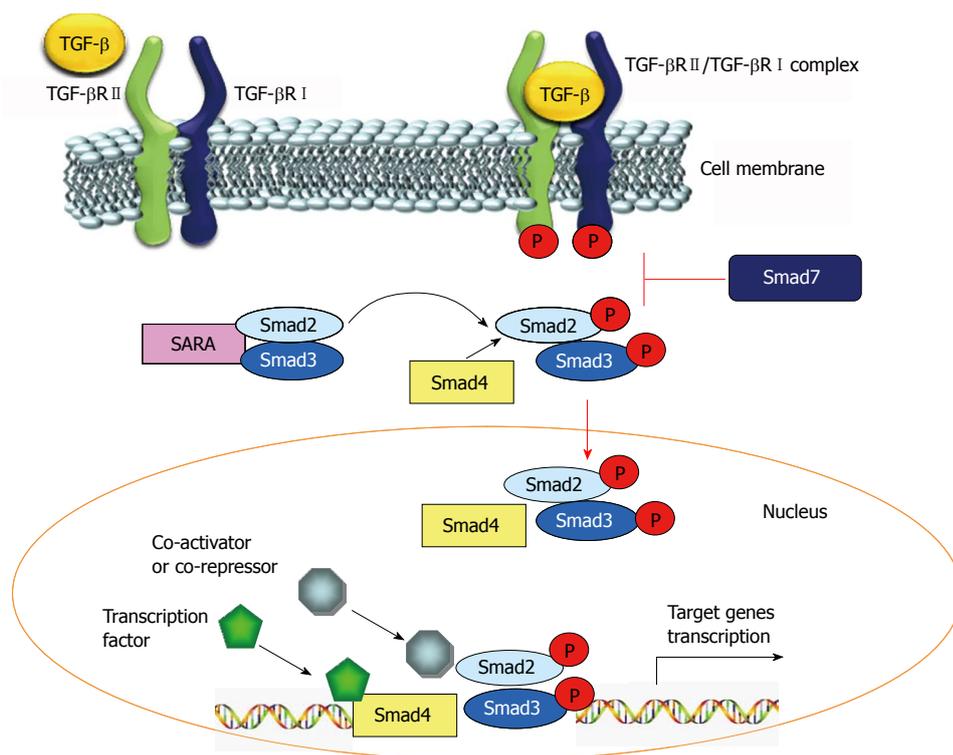


Figure 2 Transforming growth factor- α signaling and induction of gene transcription. Smad: Small mother against decapentaplegic; SARA: Smad anchor for receptor activation; TGF- β : Transforming growth factor β .

activation of signal transduction pathways. The LTBP is removed extracellularly either by proteolytic cleavage by various proteases such as plasmin, thrombin, plasma transglutaminase, or endoglycosidases, or by physical interactions of the LAP with other proteins, such as thrombospondin-1^[80].

The actions of the TGF- β family are mediated through at least three types of TGF- β receptors, TGF- β R I, R II and R III. TGF- β R I and R II are two different transmembrane serine/threonine kinase receptors with single transmembrane domains, which form homo- and heterodimer complexes that bind TGF- β , inducing phosphorylation of a family of proteins designated as Smads that transduce the ligand signal from the cell surface to the nucleus^[80] (Figure 2).

Smads are a family of 8 related proteins, which function as signaling intermediates for the TGF- β superfamily of ligands. Type I receptors specifically recognize and phosphorylate the ligand-specific receptor-activated Smad (R-Smad) and include Smad1, Smad5 and Smad8 (downstream of the BMP), and Smad2 and Smad3 (downstream of TGF- β and activin), that are recruited to activated T β RI by a membrane-bound cytoplasmic protein called Smad anchor for receptor activation (SARA). Upon ligation of TGF- β to its receptors, the phosphorylated Smad2 and 3 form a complex with the common mediator Smad (Co-Smad), such as Smad4. Co-Smad acts as a convergent node in the Smad pathways downstream of the TGF- β superfamily receptors, complexing R-Smad. R-Smad/Smad4 complexes are then translocated into the nucleus by a mechanism involving the cytoplasmic

protein importin; they may then function as transcription factors, binding DNA either directly or in association with other DNA-binding proteins^[80] (Figure 2).

A third group of Smad proteins, the inhibitory Smads such as Smad6 or Smad7, antagonize TGF- β signaling by interfering with the ligation of Smad2/3 to the activated receptor complex^[80].

Using a combined cDNA microarray/promoter transactivation approach, several new Smad gene targets have been identified, among which are *COL1A1*, *COL3A1*, *COL5A2*, *COL6A1*, *COL6A3*, and *TIMP-1*, indicating that the Smad signaling pathway is crucial for the simultaneous activation of several fibrillar collagen genes by TGF- β ^[80]. About 60 other ECM-related genes were also identified as immediate-early gene targets downstream of TGF- β ^[81].

Several studies suggest that disruption of the TGF- β /Smad signaling pathway, either by the loss of Smad3 or the increase of Smad7 expression, prevents the development of tissue fibrosis in various organs, including the skin, kidney, lungs, liver and intestine^[82-84].

Differences in small bowel and colonic morphology, as well as in the expression of collagens I-VI, α -SMA, TGF- β 1, Smad7, and other factors, have been reported in Smad3 knockout mice compared to the littermate wild-type controls^[85]. Using this tool made it possible to define the key role of the TGF- β /Smad signaling pathway in chronic intestinal inflammation and fibrosis^[85]. In fact, a significant reduction of trinitrobenzene sulfonic acid (TNBS)-induced intestinal fibrosis has been reported in knockout mice as compared to wild-type mice^[84].

Smad3 may take part in recruitment of fibroblasts to the site of injury, as well as in differentiation of fibroblasts to myofibroblasts and regulation of collagen synthesis. Furthermore, the loss of Smad3 interferes with the effects of TGF- β on chemotaxis and auto-induction of inflammatory cells^[84,86]. These findings indicate that the TGF- β /Smad signaling pathway is the key element in determining the progressive nature of intestinal fibrosis.

The effects of TGF- β on *ECM* gene expression and subsequent development of tissue fibrosis may be related to additional mechanisms, such as CTGF, the expression of which is controlled by TGF- β in a Smad-dependent manner^[87].

TGF- β preferentially transduces intracellular signaling through Smad proteins, but signal mechanisms that stimulate *ECM* accumulation can also be activated by other molecules such as members of the mitogen-activated protein kinase (MAPK) family including extracellular signal-regulated kinase (ERK), c-Jun N terminal kinase and p38 kinases, as well as members of the JAK and STAT protein family^[88].

Studies on liver fibrosis demonstrate that both MAPK and Smad signaling, independently and in synergy, stimulate HSC activation, thus increasing collagen and α -*SM1* gene expression^[88,89].

The JAK-STAT signaling pathway, activated by TGF- β , is involved in fibrogenesis and can be negatively regulated on multiple levels^[90]. Suppressors of cytokine signaling may inhibit STAT phosphorylation by binding and inhibiting JAKs or competing with STATs for phosphotyrosine binding sites^[91]. Protein inhibitors of activated STATs (PIAS) may act directly in the nucleus on STAT proteins. PIAS1 and PIAS3 inhibit transcriptional activation by STAT1 and STAT3, respectively, by binding and blocking their access to the DNA sequences^[92].

TGF- β 1 may also influence the imbalance between enhanced production and deposition and impaired degradation of *ECM* components. In IBD, the tissue expression of MMP-1, 2, 3 and 9 is increased in relation to that of TIMP-1 and 2, compared with controls^[92]. How the balance of MMPs and TIMPs is regulated is still not clear, but blocking TGF- β 1 in cultures of intestinal biopsies upregulated the expression of MMP-3 but not of TIMP-1, thus suggesting a new role for TGF- β 1 in IBD tissue remodeling^[93].

Activins

The activins are members of the TGF- β superfamily with broad and complex effects on cell growth and differentiation. The activins interact with heterodimeric serine/threonine kinase receptor complexes to activate Smad transcription factors and the MAP kinase signaling pathways. Important functions of activins, in particular of activin A, in tissue inflammation, repair and fibrosis of several organs, including the intestine, have been reported^[94-96].

Activin is a dimeric protein consisting of β_A and β_B subunits connected by disulfide linkages resulting in three different configurations with similar functions: the ho-

modimeric activin A ($\beta_A\beta_A$) and activin B ($\beta_B\beta_B$), and the heterodimeric activin AB ($\beta_A\beta_B$).

Their action is mediated by two heteromeric receptor complexes consisting of two transmembrane receptors (type I or II) with intrinsic serine/threonine kinase activities, similar to the TGF- β mechanism of signal transduction, while its biological effects seem to be inhibited by another activin-binding protein, namely follistatin^[94].

Several studies have shown an increase in activin levels in IBD and in many other inflammatory diseases, thus giving rise to the hypothesis that it plays a significant role in the inflammatory response as well as in fibrosis^[95].

Munz *et al.*^[96] reported an increase in the expression of activin, particularly the activin β_A -subunit, in surgical specimens from the gut of patients with IBD, showing a strong correlation with the degree of inflammation. *In situ* hybridization studies revealed the highest levels of activin mRNA in the mucosa and submucosa of highly inflamed areas. These findings suggest that activins play a novel and important role during the inflammatory processes of the gut^[96].

CTGF

TGF- β has been regarded as a pivotal growth factor in the formation and maintenance of connective tissues but it has been clearly proven that the effects on *ECM* gene expression and subsequent development of tissue fibrosis are related to other factors, such as CTGF, the expression of which is controlled by TGF- β in a Smad-dependent manner. Northern blot and *in situ* hybridization studies have demonstrated that CTGF is co-expressed with TGF- β in principally every fibrotic disorder and CTGF has been considered a possible key determinant of progressive fibrosis^[97].

CTGF is a cysteine-rich mitogenic peptide that binds heparin and is secreted by fibroblasts after activation with TGF- β . In the adult mammal, CTGF acts as a downstream mediator of TGF- β action on connective tissue cells, where it stimulates cell proliferation and *ECM* synthesis. It does not appear to act on epithelial cells or immune cells. Since the biological actions of TGF- β are complex and affect several different cell types, CTGF may serve as a more specific target for selective intervention in processes involving connective tissue formation during wound repair or fibrotic disorders^[97].

CTGF is an interesting molecule for future anti-fibrotic therapies as it is possible that inhibition of CTGF might block the pro-fibrotic effects of TGF- β , without affecting TGF- β 's immunosuppressive and anti-inflammatory effects. In addition to TGF- β , a number of other regulators of CTGF expression have been identified, including VEGF, TNF- α , shear stress, cell stretch and static pressure, hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radical ($HO\cdot$) and nitric oxide (NO)^[98].

Outlining the mechanisms that underlie *CTGF* gene regulation in normal and fibrotic cells might help in the design of future intervention strategies aimed at targeting specific interference with CTGF expression at sites

of progressive fibrosis. In addition, forms of treatment targeting CTGF effects have been proposed which might lead to a favorable outcome of wound repair.

PDGF

PDGF consists of two related peptide chains, PDGF-A or PDGF-1 (16 kDa, 124 amino acids), and PDGF-B or PDGF-2 (14 kDa, 140 amino acids) linked by disulfide bonds. All possible isoforms, i.e., PDGF-AA, PDGF-BB and PDGF-AB, are biologically active. More recently, two additional PDGF genes and proteins have been identified, namely PDGF-C and PDGF-D^[99].

PDGF is synthesized mainly by megakaryocytes. It is stored in the α granules of platelets from which it is released after platelet activation. Other cell types also synthesize PDGF, including macrophages, endothelial cells, fibroblasts, glial cells, astrocytes, myoblasts, smooth muscle cells, and a number of tumor cell lines. Several factors induce PDGF synthesis, including hypoxia, thrombin and several cytokines and growth factors such as IL-1, IL-6, TNF- α , TGF- β , FGF- β and EGF^[100].

Two tyrosine kinase receptors, called PDGFR α and PDGFR β , have been described. Binding of the ligand leads to receptor dimerization, which initiates signaling. Ligand configuration and pattern of receptor expression influence the formation of different receptor dimers. Generally, mesenchymal cell expression of PDGFRs is low in normal conditions, but increases dramatically during inflammation.

PDGF plays crucial roles during development. Increased PDGF activity has been linked with several diseases and pathological conditions. Its expression is significantly increased in the inflamed intestine of patients with IBD. Intestinal fibroblasts, intestinal subepithelial myofibroblasts and the interstitial cells of Cajal are activated and proliferate in response to the PDGF family (mainly PDGF-BB)^[51]. PDGF also enhances migration of fibroblasts, and its effects seem to be fibronectin-dependent. Increased activity of PDGF is also responsible for an excessive deposition of ECM in fibrotic processes within several organs, including the intestine.

IGFs

IGFs (IGF- I and -II) are polypeptides composed of a single chain of 70 and 67 amino acids, respectively. They are potent mitogens since they have stimulatory effects on proliferation and inhibitory effects on apoptosis in epithelial cells. IGF- I is the predominant post-natal IGF, whereas IGF- II is a predominantly fetal IGF. IGF- I interacts with IGF- I receptor type I (IGF- I R) on the cell surface. Subsequent phosphorylation by a receptor tyrosine kinase initiates the intra-cellular signaling process. IGF- II also interacts with a receptor type II (mannose-6-phosphate IGF- II R)^[101].

In the intestine, IGF- I interacts principally with fibroblasts and epithelial and endothelial cells. The entire GI tract expresses IGF- I and -II and their respective receptors, the latter being localized in both the mucosal

and muscularis layers. Expression of insulin-like growth factor binding protein (IGFBP)-6 in the intestine has also been described; IGFBPs are proteins that, by binding to IGF, are able to modulate its bioavailability and activity.

Expression of IGF- I is locally increased in several bowel diseases. During bowel inflammation, pro-inflammatory cytokines are able to induce IGF- I expression by mesenchymal cells. The IGF- I, in turn, could regulate the proliferation of these or other cell types acting in a paracrine manner on epithelial cells or in an autocrine manner on intestinal mesenchymal cells^[102]. IGF- I plays a relevant role in the deposition of collagen and fibrosis. It has been shown to be up-regulated in the bowel of animals with experimental intestinal fibrosis and of patients with CD^[102]. In an experimental model of rat colitis, an up-regulated IGFBP and collagen expression and down-regulated collagenase expression were shown, confirming the important role of IGF- I in collagen synthesis in colitis, mediated by IGFBPs^[103]. It has been hypothesized, moreover, that IGF- I, through IGFBP-5, is able to modulate proliferation of fibroblasts/myofibroblasts and collagen synthesis^[104].

In addition, in contrast with the local expression, the circulating levels of IGF- I and IGFBP-3 seem to be reduced in patients with IBD^[105].

EGF

EGF is the prototype member of a family comprising different peptides with a similar primary structure that bind to a family of EGF receptors and have similar biological effects: they are potent mitogens and are also able to modify several properties of non-proliferating cells^[106].

Several tyrosine kinase receptors are now known for EGF family members, such as human EGF receptors 1-4 (HER1, HER2, HER3 and HER4). Since these receptors can heterodimerize, they can form at least 10 different dimers that likely bind to distinct EGF-like molecules and deliver different signaling when activated^[106].

Binding of EGF induces the phosphorylation of several intracellular proteins regulating transcription, translation, cell architecture, cell proliferation, and the production of inflammatory mediators.

EGF is a 53-residue peptide and is also known as urogastrone, since it was initially isolated from urine and was found to inhibit gastric acid secretion^[107]; it is expressed in exocrine pancreas, duodenum, breast milk, colostrum and gastric juice^[108].

EGF can be isolated from the lumen of the intestine; its receptors have been detected in the bowel, especially on monocytes and myofibroblasts.

EGF has numerous functions within the GI tract: it stimulates cell proliferation, inhibits gastric acid secretion, up-regulates intestinal electrolyte and nutrient transport, induces expression of several enzymes, enhances epithelial restitution and stimulates angiogenesis.

EGF stimulates fibroblast proliferation and ECM production in idiopathic pulmonary fibrosis; moreover, it stimulates a more pronounced ECM production in inter-

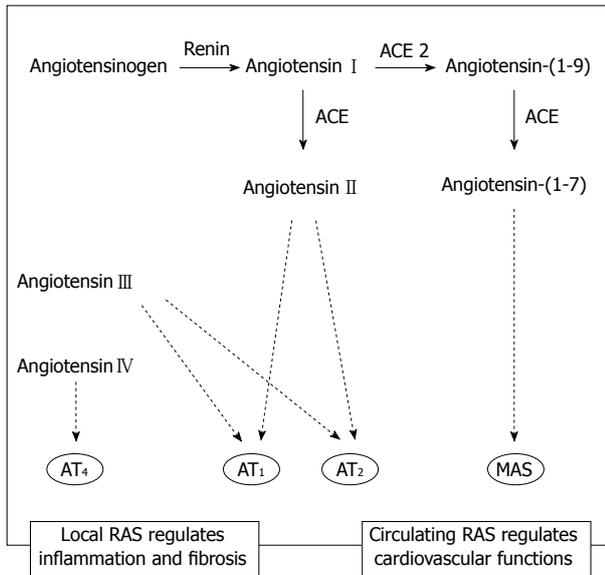


Figure 3 Components and effects of the renin-angiotensin system. ACE: Angiotensin-converting enzyme; AT1: Angiotensin II type 1 receptor; AT2: Angiotensin II type 2 receptor; AT4: Angiotensin II type 4 receptor; MAS: Mas receptor; RAS: Renin-angiotensin system.

stitial pneumonia fibroblasts than in normal fibroblasts, thus suggesting an important role of this growth factor in fibrotic processes^[109].

The role of EGF in IBD is not clear. EGFR expression is generally upregulated in TNBS-induced colitis^[110]. EGF administered intraperitoneally and subcutaneously reduces the severity of TNBS-induced colitis in rats^[110]. EGF appears to be involved in the regulation of migration of human colonic fibroblasts and myofibroblasts^[111,112]. Further studies are needed to fully elucidate the role of EGF and EGFR in IBD and intestinal fibrosis.

Endothelins

Endothelins are a group of polyfunctional cytokines. So far, four endothelins [(ET)-1, ET-2, ET-3 and ET-4]) and two endothelin receptors (ET_A and ET_B) have been recognized.

Endothelins strongly induce vasoconstriction and may provoke local ischemia. They can also induce the release of pro-inflammatory cytokines, alter the intestinal permeability and stimulate leukocyte adhesion in submucosal venules of the intestine, probably through up-regulation of cell adhesion molecules on the endothelium and leukocytes^[113-115].

Adhesion of circulating leukocytes to the endothelium could be one of the initial steps in the pathogenesis of IBD, since adhesion is a crucial event in the recruitment of leukocytes to the inflamed tissue. In addition, several of the pro-inflammatory cytokines playing a key role in IBD, such as IL-1 β and TNF- α , can remarkably increase endothelin production^[116,117].

It has been shown that bosentan, a non-selective endothelin receptor antagonist, reduces intestinal inflammation in a murine model of IBD^[118]. Probably the reduction of inflammation induced by this drug was due to a reduction in adherent leukocytes leading to a reduction of the

mucosa infiltrate of inflammatory cells.

In vivo studies performed on cardiac, renal and pulmonary tissue have shown the role of endothelins in fibrosis^[119]. ET1 stimulates colonic myofibroblast activation (differentiation, migration and contraction), effects prevented using an ET-1 antagonist^[120]. These findings suggest a possible role for ET1 also in the development of intestinal fibrosis.

RAS

The relevance of the renin-ANG system, usually viewed as an endocrine system regulating physiological and vital cardiovascular processes, has expanded in the last decade to include independently regulated local systems in several tissues, new functions of the RAS and new active products of ANG II^[121,122] (Figure 3).

Besides circulating RAS, local RASs also exist in various organs and tissues, such as the heart, blood vessels, kidney, liver, pancreas, intestine, nervous system, reproductive system, as well as lymphatic and adipose tissue^[123-125]. It has been reported that the ANG converting enzyme (ACE) produces the decapeptide ANG I and octapeptide ANG II, respectively. In addition, alternative enzymes to renin and ACE generate a number of bioactive peptides from ANG I and/or ANG II, such as ANG III, ANG IV and ANG-(1-7). ANG II, together with these bioactive peptides, mediates their specific functions *via* the respective cellular receptors of target tissues and organs^[123]. In the last few years, the understanding of the RAS function has changed considerably, following the discovery of other ANG forms [such as ANG III, IV or (1-7)], additional enzymes involved in their generation (such as ACE2, aminopeptidase A or prolyl endopeptidase) and receptors (such as ANG II type 4 receptor and Mas). RAS, therefore, is not represented by a simple linear proteolytic cascade, but by a series of extremely complex reactions (Figure 3). All components of the RAS exist in the large bowel in adults^[126]. In the colon, many cells, such as epithelial cells, vascular endothelial cells, mesenchymal cells and inflammatory cells, express ANG II receptors.

Local RAS has novel functions including the regulation of cell growth, differentiation, proliferation and apoptosis, generation of ROS, expression of cytokines (such as IL-6, TNF- α), activation of endothelial cells, as well as tissue inflammation, ECM production and fibrosis^[123].

These different roles make some of the RAS components attractive therapeutic targets in various chronic diseases, including fibrosis.

ANG II, moreover, plays a role in the pathogenesis of chronic fibrogenetic diseases of various organs, including kidney, heart, lung, pancreas, liver and intestine, through the regulation of both inflammatory and fibrotic processes^[127,128]. In such conditions, fibrosis is mediated, at least in part, through ANG II induction of TGF- β ^[13].

Several components of RAS are over-expressed in the fibrotic liver, both in human and animal models^[129]. In particular, it would appear that ANG II is able to induce proliferation of myofibroblasts and stellate cells,

to stimulate inflammatory cells and to induce the release of several profibrotic molecules, such as TGF- β , CTGF and IL-1 β ^[130]. Blocking RAS, by using ACE inhibitors or ANG II type I (AT1) receptor antagonists, reduces hepatic fibrosis^[131].

In human lung fibroblasts, ANG II is able to modify PDGF-D, IL-4 and IL-7 expression and to induce higher levels of collagen and elastin. Using candesartan, an inhibitor of AT1 receptor, these effects have been shown to be suppressed^[132].

In experimental models of kidney damage, RAS inhibitors (ACE inhibitors and AT1 antagonists) have shown beneficial effects on proteinuria, cell growth, inflammation and fibrosis, thus suggesting that ANG II could be involved in the fibrotic process activating mononuclear cells, increasing proinflammatory mediators and regulating matrix degradation^[132].

New insights into the role of RAS in the development and modulation of chronic intestinal inflammation and related fibrosis have been reported^[133]. The direct effect of ANG II on the pathogenesis of immune-mediated colitis was assessed using mice genetically deficient in angiotensinogen (*Ang*^{-/-}), which is the precursor of ANG II^[133]. TNBS-induced acute colitis was less severe in the *Ang*^{-/-} mice compared to wild-type (*Ang*^{+/+}) mice.

Both ANG II and TGF- β 1 are overexpressed in intestinal fibrosis and stenosis, particularly in Crohn's disease^[134,135]. The production of TGF- β 1, indeed, is strongly stimulated by the local activation of ANG II^[13]. An up-regulation of ANG II in the colon of rats and mice with experimentally induced colitis also supports the role of RAS in intestinal inflammation and fibrosis^[13,136].

Daily administration of the ACE inhibitor captopril in rats with chronic TNBS-induced colitis significantly reduced the macroscopic and microscopic pattern of both colonic inflammation and fibrosis, decreased the colon collagen content, and reduced TGF- β 1 mRNA levels by about 60%. The antifibrotic mechanism of captopril could be related to the inhibition of ANG II-mediated TGF- β 1 overexpression, and/or to a direct downregulation of TGF- β 1 transcripts^[13]. Likewise, the use of losartan, a specific AT1 receptor antagonist, significantly improved the macro- and microscopic scores of experimentally-induced colorectal fibrosis and reduced TGF- β 1 concentration, thus suggesting that this drug has a preventive effect on colorectal fibrosis complicating TNBS-induced chronic colitis by a downregulation of TGF- β 1 expression^[137].

In view of these data, RAS could be considered as a future target for new antifibrotics in IBDs.

Cytokines

Cytokines regulate the inflammatory process, mediating the interactions between activated immune cells and non-immune cells. They can enhance an inflammatory response or reduce inflammation and promote healing.

IBDs are mainly characterized by an enhanced CD4⁺ T cell proliferation and trafficking into the intestinal

mucosa and by alterations in the cytokine profile of the main activated Th cells, resulting in a disturbed balance between pro- and anti-inflammatory cytokines. The abnormal lamina propria T-cell activation and the consequent resistance to apoptosis are, indeed, considered as key events in the pathogenesis of IBD.

Upon activation, naïve CD4⁺ T cells can differentiate into different subsets depending on the surrounding cytokine milieu. CD4⁺ Th cells are currently divided into four major subsets, based on their expression profile of transcription factors and secreted cytokines: Th1, Th2, Th17 and Treg.

Th1 cells are characterized by the secretion of interferon γ (INF- γ). The differentiation of Th1 cells is mainly induced by IL-12 and can be further enhanced by INF- γ . Th1 cells express the transcription factors T-bet and STAT4. INF- γ is a pro-inflammatory cytokine which together with IL-12 induces the differentiation of macrophages and the production of other pro-inflammatory cytokines, such as IL-1 β , IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF- α ^[138].

Th2 cells produce primarily IL-4, IL-5 and IL-13. Th2 cells develop in the presence of IL-4 and require the transcription factors GATA3 and STAT6.

Th1 and Th2 negatively regulate each other through their specific cytokine actions: IL-12 represses the induction of Th2 cells, whereas IL-4 inhibits Th1 cell development.

Microarray experiments have established that the various gene-expression profiles associated with different pathological conditions are strongly influenced by Th1- and Th2-polarization of the chronic inflammatory responses^[139].

The Th1 response seems to be involved mainly in CD, whereas the Th2 response occurs mainly in UC^[140]. However, during the late stages of IBD, both Th1 and Th2 cytokines play a pivotal role in the progression of intestinal fibrosis. Several findings have demonstrated that IL-1 β and TNF- α , two Th1 pro-inflammatory cytokines, stimulate fibroblast proliferation and collagen deposition, whereas the development of a Th2 cell response, and particularly IL-4 and IL-13, upregulates the expression of several genes including those that encode procollagen-I, procollagen-III, MMP-2 and MMP-9 as well as TIMP-1^[139,141].

In addition, the other two subsets of CD4⁺ T cells, represented by the Th17 and Treg cells, are involved in the development of intestinal fibrosis in alternate ways^[142].

Th17 cells, which are strongly associated with autoimmune pathology, are characterized by the production of IL-17A, IL-17F, IL-21, IL-22 and TNF- α ^[142]. Differentiation of Th17 cells needs the combined action of TGF- β , IL-6 and IL-21 in mice, whereas IL-6 and IL-21 can be replaced by IL-23 or IL-1 β in humans. These cytokines induce the transcription factors ROR γ t (mice) or RORc (human) and STAT3. Development of Th17 cells is suppressed by INF- γ and IL-4, which promote Th1 and Th2

Table 3 Predominant pro-fibrotic cytokines

Cytokines	Role in fibrosis	Ref.
IL-1/IL-33	Activate myofibroblasts	[144,146]
	Stimulate TGF expression	[145]
	Regulate chemokines, cytokines and MMPs secretion	[144]
IL-4/IL-13	Activate myofibroblasts	[147]
	Increase deposition of collagen I, III and IV	[148]
	Induce production of latent TGF- β and activate TGF- β	[152,153]
IL-6	Promote fibrocyte differentiation	[154]
	Stimulates proliferation of fibroblasts	[157]
	Modulates TGF- β and TGF- β R II	[156]
IL-21/IL-22	Its neutralization improves fibrosis	[155]
	Stimulate the expression of IL-4, IL-13 and IL-17	[159]
	Increase secretion of ECM degrading enzymes	[160]
IL-17/IL-23	Maintain the integrity of the epithelial barrier	[161]
	Inhibit deposition of collagen	[163]
	Maintain chronic inflammation in the gut	[143]
TNF α	Induce activation and proliferation of myofibroblasts	[165]
	Stimulate deposition of collagen	[164]
	Stimulates intestinal myofibroblast proliferation	[170]
IL-17/IL-23	Stimulates deposition of collagen	[171]
	Inhibits collagen degradation through TIMP-1 induction	[172,173]

TNF: Tumor necrosis factor; ECM: Extracellular matrix; IL: Interleukin; TGF- β : Transforming growth factor- β ; TIMPs: Tissue inhibitors of metalloproteinases; MMPs: Matrix metalloproteinases.

cells, respectively. Once Th17 cells have developed, IL-23 is needed for stabilization and further expansion of these cells. Also, IL-1 β and IL-6 can act to enhance the development and expansion of these cells. The intestine of patients with IBD presents higher IL-17 levels than in healthy subjects. There is emerging evidence that IL-17 strongly interacts with IL-23, the expression of which is considerably increased in the inflamed intestine; its selective depletion with monoclonal antibodies has been demonstrated to greatly attenuate T-cell mediated colitis in mice^[143].

Treg cells, which control effector T-cell responses, produce the anti-inflammatory cytokines IL-10 and TGF- β . The differentiation of Treg is induced by TGF- β but inhibited in the presence of pro-inflammatory cytokines. Treg are characterized by the expression of the transcription factor Foxp3 and STAT5 and the expression of CD25 on their surface.

There is a growing body of evidence proving that the inflammatory cascade which influences the progression of intestinal fibrosis can involve many Th1, Th2, Th17 and Treg cytokines, such as IL-1 β , IL-4, IL-6, IL-13, IL-17, IL-21, IL-22, IL-23, IL-33 and TNF- α (Table 3). Consequently, the use of biological therapies antagonizing these cytokines could be of value in the treatment of intestinal fibrosis and, therefore, may represent the targets of new antifibrotic drugs.

IL-1 and IL-33: IL-1 belongs to a family of proinflammatory cytokines that are rapidly expressed following tissue damage. Besides its role in acute and chronic inflammation, IL-1 is involved in tissue remodeling following chronic inflammation in various organs.

IL-1 contributes to the development of fibrosis during chronic intestinal inflammation through various mechanisms, such as regulating intestinal myofibroblast activation, the induction of chemokines (e.g., IL-8, MCP-1) and MMP secretion, and the turnover of ECM^[144]. Furthermore, IL-1, in combination with TNF and INF- γ , is able to increase the TGF- β -induced epithelial-mesenchymal transition (EMT), an important cellular process of fibrogenesis^[145].

Recently, it has been reported that IL-33, a novel member of the IL-1 family, induces mucosal pathology *in vivo* and may lead to the development of fibrosis and angiogenesis^[146]. TLR-3 is one of the strongest promoters inducing IL-33, which activates myofibroblasts and pericytes.

IL-4 and IL-13: Some Th-2 cytokines (IL-4 and IL-13) are present in elevated levels in fibrotic processes and induce activation and differentiation of fibroblasts to myofibroblasts and also induce production of collagens^[1,17,159].

IL-4 appears to be involved in the development of pulmonary fibrosis; in particular, activation of the IL-4R α pathway in macrophages seems to be fundamental in silica-induced pulmonary fibrosis^[147]. Furthermore, it was demonstrated that IL-4 increases the mRNA expression of collagen I, III and IV in cultured human hepatic fibroblasts; these effects require STAT-6 activation^[148]. IL-4 level, finally, correlated with cardiac fibrosis in patients with heart failure^[149].

Inhibition of IL-13 reduces hepatic and skin fibrosis^[150,151]. In experimental studies a marked reduction of hepatic fibrosis by IL-13 blockade has been reported^[150,151]. IL-13 signaling through the corresponding receptor IL-13R α induces production of latent TGF- β (TGF- β bound to LAP) in macrophages and, indirectly, contributes to its activation by stimulating the synthesis of enzymes, such as MMPs and cathepsins, able to remove LAP^[152]. In TNBS-induced colitis, fibrosis development is dependent upon IL-13 binding to the IL-13 receptor to induce TGF- β ^[153]. In the same way, if IL-13 signaling is inhibited TGF- β is produced in reduced amounts and fibrosis does not occur^[153]. Soluble IL-13R α 2-Fc is a highly effective decoy receptor of IL-13 which can reduce the progression of established fibrotic disease. IL-10 has also been shown to inhibit fibrosis in numerous experimental models^[1]. The IL-13 decoy receptor and IL-10, by suppressing collagen deposition, act as endogenous factors that slow the progression of fibrosis.

Both IL-4 and IL-13 appear to be responsible for promoting fibrocyte differentiation from CD14+ peripheral blood monocytes, without induction of their proliferation. In dermal fibroblasts, IL-4 and IL-13 stimulate ERK1/2 pathways, which are involved in the modulation

of collagen gene expression^[154].

IL-6: In addition to a potent proinflammatory action, IL-6 may also have a relevant role in the development of fibrosis. In the cardiac allograft model, IL-6 neutralization improved graft fibrosis^[155]. The role of IL-6 in fibrosis has been further confirmed by the finding of its ability to modulate TGF- β and TGF- β R II mRNA and protein levels in mouse skin^[156] and to strongly stimulate proliferation in normal and keloid fibroblasts, as well as increase levels of STAT-3, which contributes to several processes including collagen production^[157]. IL-6 is markedly increased in CD where it appears to stimulate fibrogenetic mesenchymal cells^[158].

IL-21 and IL-22: IL-21 appears to be related to IBD-associated intestinal fibrosis. IL-21 promotes fibrosis by enhancing the development, survival and migration of Th2 cells. Moreover, it stimulates the expression of IL-4 and IL-13 receptors in macrophages, inducing their activation^[159], stimulates the secretion of ECM-degrading enzymes by fibroblasts and the secretion of the T cell chemoattractants by epithelial cells^[160]. IL-21 is produced in excess in CD compared to controls. IL-21 together with IL-6 is critical in the development of Th17 cells.

IL-22, primarily produced by Th17 cells, presents bi-functional characteristics, pro- and anti-inflammatory, depending on the local milieu. In the intestine, it is involved both in mucosal defense and in wound healing processes. IL-22 plays a protective role in IBD by enhancing barrier integrity and epithelial immunity of the intestinal tract^[161]. A mouse model of UC has, in addition, demonstrated that IL-22 is able to activate the innate immune pathway by enhancing STAT3 activation, particularly within colonic epithelial cells, and by inducing both the restitution of mucus-producing goblet cells and the STAT3-dependent expression of mucus-associated molecules. These studies might, therefore, suggest a protective role for this cytokine in intestinal inflammation^[162]. The involvement of IL-22 in the regulation of fibrotic processes has been shown in a mouse model of hypersensitivity pneumonitis (that generally progresses to lung fibrosis), in which direct blockade of IL-22 enhanced the deposition of collagen in the lung. These findings reveal a protective pathway of this cytokine in the development of lung fibrosis and could suggest an analogous role also in the intestine^[163].

IL-17 and IL-23: The IL-23/IL-17 axis plays a role in normal intestinal homeostasis, although the precise actions of these cytokines remain to be fully elucidated. In the normal intestine, constitutive production of small amounts of both IL-23 and IL-17 may act to protect the epithelial layer fortifying tight junction formation between epithelial cells and inhibiting bacterial colonization. In inflammation, activated dendritic cells produce large amounts of IL-23, which could activate innate immune cells to produce pro-inflammatory cytokines such as IL-1 β , TNF- α and IL-6. IL-23 also induces an in-

creased production of IL-17 and INF α , both by T cells and non-T cells. Recently, it has been shown that targeting IL-23 by employing a p40 peptide-based vaccine improves TNBS-induced acute and chronic murine colitis with a significant decrease in collagen deposition^[164]. The main action of IL-17 may be to promote the production of chemokines that recruit and activate granulocytes, further increasing inflammation^[143]. IL-17 was also found to be a potent activator of mesenchymal cells, especially of myofibroblasts^[165].

In vivo, fibrotic models show enhanced IL-17A expression and activation of IL-17A-associated signaling pathways. Studies performed on pulmonary fibrosis models showed evidence that IL-17A increased synthesis and secretion of collagen and induced epithelial-mesenchymal transition in alveolar epithelial cells in a TGF- β 1-dependent manner^[166].

Moreover, it has been demonstrated that IL-1 β -mediated pulmonary fibrosis is IL-17A-dependent^[167] and that IL-17A blocking is able to attenuate myocarditis-induced cardiac fibrosis and ameliorate ventricular function; further confirming its role in the fibrogenetic process^[168].

A study on Th17 cells in human peripheral blood demonstrated that the expansion of IL-23R+ CD4+ cells was promoted by the combination of IL-7 and IL-12, which are also able to increase the secretion of IFN- γ , while IL-12 alone stimulated these cells to secrete predominately IL-17^[169]. On the basis of these data, IL-7 and IL-12 have similarly been identified as anti-fibrotic cytokines, even if their effective role in the fibrotic process remains to be established.

TNF- α

Another central mediator of the fibrotic process in IBD is TNF- α , a proinflammatory cytokine with important immunomodulatory properties, abundantly expressed in the intestine of patients with CD and UC. This cytokine is produced from various cells such as monocytes/macrophages, adipocytes and T cells, and has different effects including further macrophage and T cell activation, stimulation of granuloma formation and expression of adhesion molecules in endothelial cells with consequent recruitment of neutrophils and other immune cells at the site of inflammation.

TNF- α is able to induce intestinal fibrosis by up-regulating collagen accumulation; moreover, it has mitogenic effects in intestinal myofibroblasts and is able to extend the inflammatory state thus increasing the expression of other inflammation mediators, such as IL-6 and IL-1 β , and activating NF κ B-dependent pathways, a transcription factor that increases the expression of cytokines, enzymes and adhesion molecules^[170].

TNF- α acts by binding to two immunologically distinct TNF- α receptors of approximately 55 kDa (TNF-R1) and 75 kDa (TNF-R2). Studies performed on intestinal myofibroblasts have shown that TNFR2 is essential for TNF- α -induced cell proliferation and collagen synthesis^[171].

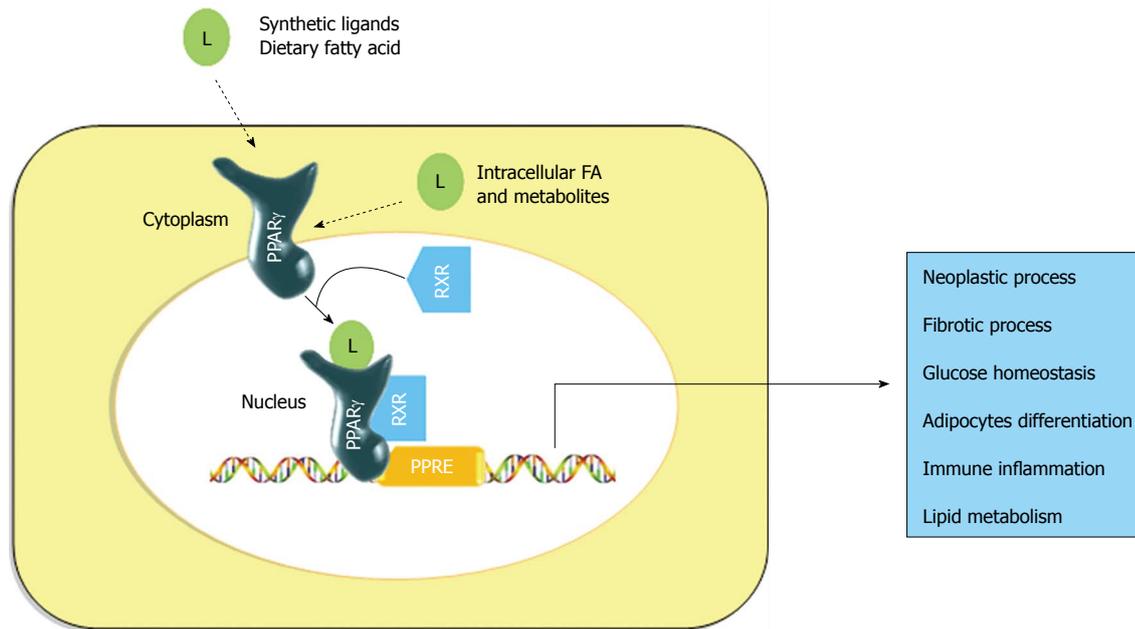


Figure 4 Activation of the peroxisome proliferator activator receptor- γ pathway. Regulation of several genes involved in fibrogenesis. PPAR- γ : Peroxisome proliferator activator receptor- γ ; PPRE: PPAR response elements; RXR: Retinoid X receptors; L: Ligand; FA: Fatty acids.

Furthermore, TNF- α induces TIMP-1 expression and reduces MMP-2 activity and collagen degradation. Inhibition of collagen degradation, through TIMP-1, and induction of collagen gene transcription are responsible for the effects of TNF- α on total collagen accumulation; moreover, TNF- α also appears to have additional effects on collagen synthesis when combined with IGF- I , a growth factor implicated as a key pro-fibrogenic mediator during intestinal inflammation *in vivo*. IGF- I and TNF- α synergistically stimulate intestinal myofibroblast proliferation and collagen production^[172,173].

The role of TNF- α in IBD was confirmed by the therapeutic benefits of anti-TNF- α monoclonal antibodies. In several clinical trials, anti-TNF- α drugs, such as infliximab, adalimumab and certolizumab pegol, induced clinical remission and healing of mucosal lesions in about one third of patients with CD^[174-176].

There is not strong evidence regarding the effectiveness of anti-TNF- α drugs to reduce intestinal fibrosis in IBD.

ROS

Infiltrating leucocytes in the inflamed mucosa produce a large amount of ROS. ROS include various species ($O_2^{\cdot -}$, H_2O_2 , $HO\cdot$) and are involved in several processes, such as regulation of signal transduction, activity of phagocytes, oxidative damage of proteins, lipids and nucleic acids^[177].

Superoxide is mainly generated from the uncoupling of the mitochondrial electron-transport system and from several enzymes including NADPH oxidases, cyclooxygenases, lipoxygenases and nitric oxide synthases. Hydrogen peroxide is directly obtained from oxygen, spontaneously or enzymatically (through the action of the enzyme superoxide dismutase); it can also be the result of lipid

metabolism in peroxisomes. In the presence of metals, hydrogen peroxide can be involved in $HO\cdot$ formation^[178].

ROS, moreover, can negatively affect cell signaling by altering the transcription process and phosphorylation of proteins, including transcription factors.

ROS are involved in acute and chronic inflammatory processes that include a neutrophil or macrophage infiltrate, such as acute and chronic infections, autoimmune conditions such as IBD, arthritis and other inflammatory conditions^[179]. In several pathological states the increase of ROS depends on an over-expression or increased activity of enzymes involved in their production. Increased activity of the NADPH oxidase enzyme is associated with pulmonary, cardiac, hepatic and intestinal fibrosis^[179].

Patients with idiopathic pulmonary fibrosis have a lower antioxidant capacity compared to healthy subjects^[180-182]. It has been reported that antioxidants protect rats against experimental pulmonary fibrosis^[183,184]. It is thus likely that attenuating oxidative stress in tissues could prevent fibrosis caused by ROS.

A recent study showed that mice with NADPH oxidase deficiency had phagocytes which did not produce ROS and they did not develop lung fibrosis induced by bleomycin^[185]. Inhibition of ROS production in this experimental model was associated with alterations in IL-6 levels and in the MMP-9/TIMP-1 ratio, molecules involved in pulmonary fibrosis and remodeling^[185].

The role of ROS has been widely studied in liver fibrosis and substantial data suggest its importance in the transformation of HSCs into activated collagen-producing cells^[186]; ROS appear to be a key mediator in collagen gene regulation^[187].

ROS could also be involved in intestinal fibrosis. It has been shown that inhibition of oxygen radical secre-

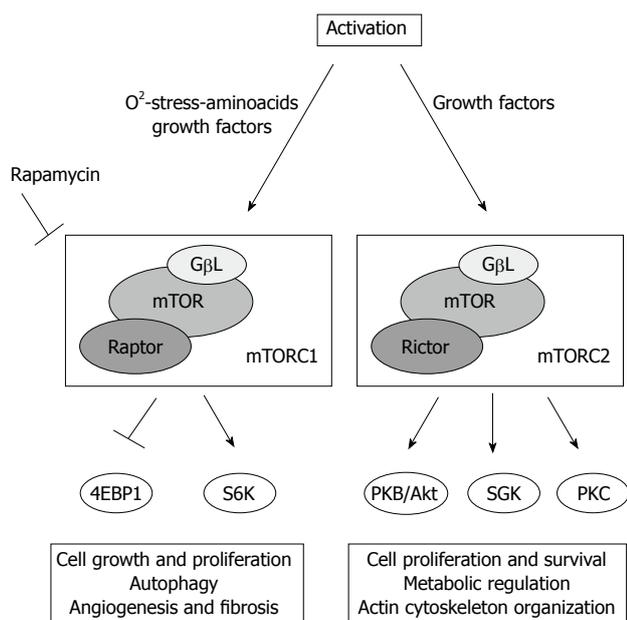


Figure 5 Features of mammalian target of rapamycin. mTORC1: Mammalian target of rapamycin complex1; mTORC2: Mammalian target of rapamycin complex 2; 4EBP1: 4E binding protein; S6K: S6 kinase; PKB/Akt: Protein kinase B; SGK: Serum- and glucocorticoid-inducible kinase; PKC: Protein kinase C.

tion improves experimental colitis in mice^[188].

PPAR-γ

Novel molecules involved in IBD and in tissue fibrogenesis appears to be PPARs^[189]. Three different isoforms of PPARs have been identified, termed PPAR-α, PPAR-γ and PPAR-δ, each one encoded by specific genes and with distinct patterns of tissue distribution but with similar structure and function. PPARs are nuclear receptors, which regulate gene transcription by binding to retinoid X receptors (RXR) as functional heterodimers in response to a variety of endogenous and exogenous ligands. The PPAR/RXR complex modulates gene target expression by binding to the PPAR response elements (PPREs) in the gene promoter^[190]. These nuclear receptors present three main domains: the N-terminal domain containing a ligand-independent transactivation function; the DNA-binding domain containing two zinc-finger motifs responsible for binding on PPREs to regulate transcription of PPAR-responsive genes; the ligand-binding domain (LBD) containing a ligand-dependent activation function. The LBD shows the presence of a wide hydrophobic region which could explain the increased ability of PPARs to bind a wide variety of natural and synthetic ligands^[191,192] (Figure 4).

In particular, the PPAR-γ isoform, identified mainly in the colorectal mucosa but also in adipocytes, the liver, vascular tissue and several inflammatory cells (monocytes and macrophages, dendritic cells, B and T cells), seems to be involved in several physiological processes, such as differentiation of adipocytes, glucose homeostasis, lipid metabolism, inflammatory and immune processes, as well as fibrosis^[189,190]. Several ligands, either natural, such

as arachidonic acid metabolites (15-d-PGJ₂) and some eicosanoids, or synthetic, such as thiazolidinediones and some non-steroidal anti-inflammatory drugs, appear to be responsible for the activation of PPAR-γ (Figure 4).

PPAR-γ activation seems to be strongly related to the TGF-β/Smads pathway. The stimulation of PPAR-γ with specific ligands, indeed, interferes with the Smad3 pathway by directly antagonizing Smad3 or downregulating the CTGF expression that promotes TGF-induced synthesis of collagen^[189,193]. PPAR-γ agonists inhibit fibroblast migration and proliferation^[194] as well as the transdifferentiation of epithelial and mesenchymal cells in activated myofibroblasts^[195], one of the key points in fibrosis development. PPAR-γ ligands repress TGF-β-induced myofibroblast differentiation and activation by targeting the PI3K/Akt and Smad3 pathways, respectively^[196,197]. Overexpression of PPAR-γ prevents the development of tissue fibrosis, whereas its loss increases susceptibility to fibrosis^[198,199]. All these findings could explain the ability of PPAR-γ to interfere in multiple phases of the tissue fibrotic processes. Therefore, PPAR-γ should be regarded as providing innate protection from excessive fibrogenesis and as a potential new target for the development of novel compounds with anti-fibrotic properties^[200]. Several PPAR-γ ligands with selective activity are under development. Experimental studies have shown that PPAR-γ agonists attenuate fibrosis in various organs including lung, kidney, pancreas, liver and intestine, antifibrotic effects that are abolished by the use of PPAR-γ selective antagonists^[201-206]. The PPAR-γ coding gene is considered a potential susceptibility gene for IBD^[189].

Mammalian target of rapamycin

In the 1970s, during a discovery programme for novel anti-microbial agents from natural sources, a soil sample from Easter Island (known as Rapa Nui) was found to contain *Streptomyces hygroscopicus*, a bacterium that produces a potent antifungal metabolite; the antibiotic was isolated and called rapamycin (Rapa + mycin). TOR was originally identified in *Saccharomyces cerevisiae* and, in subsequent biochemical studies, also in mammals and therefore was named the mTOR.

mTOR is a 289 kDa phosphatidylinositol 3-kinase-related kinase^[207]. mTOR signaling is activated by hormones, growth factors, amino acid levels, stress and alterations in cellular energy status^[208] (Figure 5). Among the signal inputs, growth factor- and hormone-induced mTOR activation is the best characterized and is believed to be mediated through the PI3K pathway and PKB/Akt.

mTOR forms at least two distinct complexes. mTOR complex 1 (mTORC1) is composed of mTOR, GβL and Raptor and is responsible for sensing the nutrient; mTORC2 consists of mTOR, GβL and Rictor and is involved in the organization of actin. mTORC1 is sensitive to rapamycin whereas mTORC2 is insensitive to rapamycin.

The best characterized downstream targets of mTORC1 are S6K1 and 4E-BPI which control protein synthesis and cell growth and proliferation, respectively, as

well as autophagy, angiogenesis and fibrosis^[208] (Figure 5). Rapamycin induces a translational arrest by preventing phosphorylation of S6K-1 and 4E-BPI by mTOR^[209].

It has been demonstrated that mTOR strongly enhances expression of hypoxia-inducible factor (HIF)1- α , a subunit of HIF which is a transcription factor that mediates expression of several genes, the products of which play a considerable role in inducing angiogenesis^[210]. Two of the key gene products induced by HIF are VEGF and angiopoietin-2, which represent the main driving factors of neo-angiogenesis.

It has been shown that mTOR signaling is required for angiogenesis and plays a key role in endothelial cell proliferation in response to hypoxia. Hypoxia, indeed, rapidly promotes and sustains mTOR phosphorylation, whereas mTOR inhibition by rapamycin specifically abrogates hypoxia-mediated amplification of endothelial proliferation and angiogenesis^[211,212]. Moreover, it has been recognized that mTOR-dependent HIF1- α expression is sensitive to rapamycin. Inhibition of mTOR with rapamycin can reduce the process of angiogenesis by blocking VEGF, TNF- α and PDGF α production through inhibition of HIF1- α expression and its transcriptional activation^[213-215]. mTOR might, therefore, play an important role in the abnormal angiogenesis associated with IBD. It has been reported that mTOR is an effector of EGFR- and ANG type 1 receptor-induced fibrosis^[216,217].

mTOR signaling is considered an attractive target for antifibrotic intervention. mTOR inhibitors constitute a relatively new category of immunosuppressive and anti-neoplastic drugs^[218]. These share a unique mechanism of action that is focused on the inhibition of the mTOR. Their clinical applications have recently expanded significantly to cover a wide spectrum of immune and non-immune-mediated disorders, including IBD as well as solid organ transplantation, various solid organ and hematological malignancies, metabolic problems such as diabetes mellitus and obesity, and even fibrotic conditions, including skin, pulmonary, renal, hepatic and intestinal fibrosis^[215-217,219-223].

It has been reported that rapamycin exerts direct antifibrotic activities both by reducing the number of fibroblasts and myofibroblasts and by down-regulating the production of fibrogenic cytokines, such as IL-4, IL-6, IL-17 and TGF- β 1, and the synthesis of type I and III collagen^[224-226]. Application of rapamycin to cultures of fibroblasts dose- and time-dependently downregulates the expression of cytoplasmic PCNA, cyclin D1, α -SMA, fibronectin and collagen^[227]. Rapamycin may improve the defective autophagy associated with fibrostenotic CD^[26,35,42,207].

mTOR might, therefore, also play important roles in IBD-associated fibrosis. Combined immunosuppressive and anti-fibrotic actions of rapamycin and its analogues may result in a promising treatment approach to fibrotic chronic enteropathies such as CD. This has been confirmed by two case reports in which two patients with severe refractory CD were successfully treated

with two different analogues of rapamycin: sirolimus and everolimus^[228,229]. In addition, a recent clinical trial demonstrated the effectiveness of everolimus to maintain steroid-induced remission in patients with moderate-to-severe active CD^[230].

MMPs

An increased turnover of ECM components leads to an intensive remodeling of connective tissue. A delicate balance exists between synthesis and degradation of ECM components. Disturbance of this balance may result either in progressive organ destruction, as seen in formation of ulcers and fistulae, or excessive deposition of collagen, resulting in fibrosis^[231-233]. Degradation of all ECM components is regulated by the enzymatic activity of the predominant and large family of MMPs. In addition to playing a central role in ECM turnover, MMPs proteolytically activate or degrade a variety of non-matrix substrates including chemokines, cytokines, growth factors and junctional proteins. Thus, they are increasingly recognized as critical players in inflammatory response and fibrogenesis^[231-233].

MMPs are a group of calcium-activated and zinc-dependent endopeptidases that are secreted as proforms (inactive zymogens)^[231-233]. They are produced by various cell types, including mesenchymal cells, T-cells, monocytes, macrophages and neutrophils, in response to inflammatory stimuli, such as IL-1, TNF- α , or TGF- β 1^[233]. The MMP family consists of at least 25 distinct members^[234], which can be subclassified according to their substrate specificities: collagenases (e.g., MMP-1, -8, -13, -18), gelatinases (MMP-2, -9), stromelysins (MMP-3, -7, -10, -11), elastase (MMP-12), membrane types (MMP-14, -15, -16, -17, -24, -25) and others (MMP-19, -20, -23, -26, -27, -28). All MMPs become activated *via* proteolytic cleavage and are regarded as the major enzyme group capable of degrading ECM components such as collagens, laminins and fibronectins, including basement membranes. Once one type of MMP is activated it can catalyze activation of other MMP proforms.

MMP activity is controlled by specific and non-specific inhibitors such as TIMPs and α 2-macroglobulin, respectively^[16,232,233]. TIMPs are produced by the same cell types as MMPs and form a 1:1 complex with activated MMPs. The fine balance between MMPs and TIMPs regulates the turnover of ECM both under physiological conditions and in tissue remodeling during inflammation and wound healing. Thereby, the imbalance, due to reduced MMP activity and/or increased expression of TIMPs, may lead to excessive deposition of ECM proteins driving fibrogenesis^[115,30,51].

In IBD, overexpression of several MMPs has been reported, which might lead to inflammation and tissue injury, facilitating the migration and invasion of fibroblasts into the bowel wall, ultimately leading to fibrosis^[235-238]. Addition of recombinant MMP-3 to an *ex vivo* gut model caused extensive tissue injury, and inhibition of MMPs almost completely abolished tissue destruction^[239]. The expression of MMP-1, -3, -7 and -13, as well as TIMP-3,

was increased in the wound edges of ulcerated tissue in UC and CD^[240]. Levels of MMP-1, -2, -3 and -9 relative to TIMP-1 and -2 are increased in inflamed, compared to non-inflamed, IBD tissue homogenates, regardless of the presence of fibrosis^[92,231].

TGF- β 1 may lead to fibrosis by creating an imbalance of TIMP1/MMP expression in favor of TIMP-1^[241]. Stricture-derived myofibroblasts in CD overexpress TIMP-1^[242] and the expression of TGF- β 1 and TIMP-1 is increased in the mucosa overlying strictured compared to non-strictured intestine, while MMP-3 and -12 are down-regulated^[243]. In support of this notion, blocking TGF- β 1 in cultures of intestinal biopsies upregulates the expression of MMP-3, but not of TIMP-1^[93]. In a murine model of chronic inflammation, fibrosis is associated with an increase in TIMP-1^[244].

A possible genetic basis has been explored: SNPs in genes encoding MMPs and TIMPs have been reported. An SNP at the TIMP-1 site is associated with increased susceptibility for CD, and an SNP at the MMP-3 site may increase the chance of stenotic complications^[245].

An imbalance in MMP:TIMP expression and enhanced levels of the messages for fibrogenic cytokines and ECM proteins were also reported in late radiation enteritis and *Schistosoma mansoni*-induced chronic colitis^[246,247]. Despite the fact that the expression of collagens, MMPs and TIMPs simultaneously increased, quantification of net collagen deposition showed an overall accumulation of collagen. These findings indicate that the intestine affected by chronic inflammation is subjected to an active process of fibrogenesis as well as fibrolysis, with a balance toward fibrogenesis. This demonstrates that established fibrotic tissue is not scarred fixed tissue but is subjected to a dynamic remodeling process.

Taken together, all these data strongly support the hypothesis that an imbalance of tissue-degrading enzymes and their inhibitors may cause intestinal fibrosis^[232,233,248]. MMPs and TIMPs may, therefore, be considered as targets for new anti-fibrotic treatment approaches.

CONCLUSION

Intestinal fibrosis is a highly complex process involving the dynamic actions of numerous molecules which are able to regulate activation of ECM-producing cells during tissue damage and repair. The specific molecules determining the balance between physiologic repair following acute inflammation *versus* the excessive accumulation of ECM leading to fibrosis remain to be identified.

Strong evidence indicates that inflammation triggers fibrosis, which, once established, may progress independently. Available anti-inflammatory drugs have been shown to be ineffective in the prevention and treatment of the fibrosis. It is critical to elucidate the cellular signals promoting fibrogenesis that act independently of inflammatory pathways and immuno-inflammatory response.

Definition of the cellular and molecular mechanisms involved in intestinal fibrosis will represent the key to the

development of new therapeutic approaches for the treatment of fibro-stenosing enteropathies, particularly CD.

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Current knowledge on esophageal atresia

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Abstract

Esophageal atresia (EA) with or without tracheoesophageal fistula (TEF) is the most common congenital anomaly of the esophagus. The improvement of survival observed over the previous two decades is multifactorial and largely attributable to advances in neonatal intensive care, neonatal anesthesia, ventilatory and nutritional support, antibiotics, early surgical intervention, surgical materials and techniques. Indeed, mortality is currently limited to those cases with coexisting severe life-threatening anomalies. The diagnosis of EA is most commonly made during the first 24 h of life but may occur either antenatally or may be delayed. The primary surgical correction for EA and TEF is the best option in the absence of severe malformations. There is no ideal replacement for the esophagus and the optimal surgical treatment for patients with long-gap EA is still contro-

versial. The primary complications during the postoperative period are leak and stenosis of the anastomosis, gastro-esophageal reflux, esophageal dysmotility, fistula recurrence, respiratory disorders and deformities of the thoracic wall. Data regarding long-term outcomes and follow-ups are limited for patients following EA/TEF repair. The determination of the risk factors for the complicated evolution following EA/TEF repair may positively impact long-term prognoses. Much remains to be studied regarding this condition. This manuscript provides a literature review of the current knowledge regarding EA.

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Key words: Esophageal atresia; Tracheoesophageal fistula; Esophageal stenosis; Long-gap; Gastro-esophageal reflux

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INTRODUCTION

Thomas Gibson first described esophageal atresia (EA) associated with tracheoesophageal fistula (TEF) in 1697. However, it was not until 1941 that Cameron Haight performed the first successful surgical repair of this anomaly following innumerable attempts by other surgeons^[1-3].

EA with or without TEF remains the most common congenital anomaly of the esophagus. Although EA with or without TEF is a relative rare condition, this complex anomaly is still a challenging problem in pediatric surgery^[1,2]. In developing countries, many infants that present with EA with TEF exhibit pneumonitis due to late referral, and these patients usually have a low birth weight.

The overall incidence of EA/TEF ranges from one in every 2500 to 4500 live births^[1-9]. The vast majority of cases are sporadic, although the incidence is higher in twins. It has been reported that the relative risk for EA/TEF in twins was 2.56 higher than in singletons^[6-8].

An improvement in survival has been observed over the most recent decades. This finding is likely multifactorial and largely attributable to advances in neonatal intensive care, neonatal anesthesia, ventilatory and nutritional support, antibiotics, surgical materials and techniques^[3,5,9,10]. Indeed, mortality is currently limited to those cases with coexistent severe life-threatening anomalies. It should be noted that the care and treatment of EA is a measure of the surgical expertise and facilities available in a particular center or country. Despite an increased number of patients with severe associated anomalies, survival rates as high as 95% have been reported in centers offering the best neonatal care^[11]. Today, practically all patients without concomitant severe malformation survive. Moreover, the high mortality of very low birth weight patients, patients with severe cardiac malformation and of infants with long-gap defects has significantly decreased. Because of this increase in survival, morbidity associated with EA/TEF repair has become an important issue during the follow-up of these children.

There are a limited number of reports concerning the long-term outcome of patients with EA. Relationships between esophageal dysmotility, gastroesophageal reflux, esophagitis and epithelial metaplastic changes, including esophageal cancer, should be studied further^[3,5,8,9]. The present manuscript provides gastroenterologists with a literature review focused on EA with the aim of providing recent data regarding the embryology, diagnosis, therapeutic approaches, complications and outcomes of this very common congenital anomaly.

CLASSIFICATION

The classification of EA anomalies is determined by the location of the atresia and the presence of any associated fistula to the trachea. In this respect, five different variants have been clinically described. The first classification was published by Vogt in 1929 and was modified by Gross in 1953. Thus, two classifications are used today. The primary types of congenital EA are EA with distal TEF (85%, Vogt IIIb, Gross C), isolated EA without TEF (8%, Vogt II, Gross A), TEF without atresia or H-type TEF (4%, Gross E), EA with proximal TEF (3%, Vogt III, Gross B) and EA with proximal and distal TEF

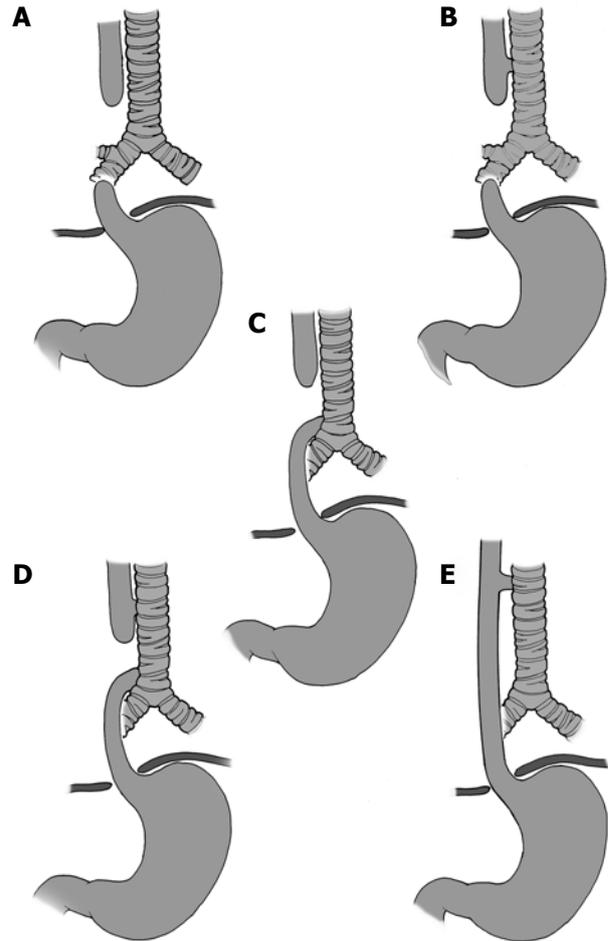


Figure 1 Classification of esophageal atresia/tracheoesophageal fistula. A: Esophageal atresia (EA) without tracheoesophageal fistula (TEF); B: Proximal TEF with distal EA; C: Distal TEF with proximal EA; D: Proximal and distal TEF; E: TEF without EA or "H"-type TEF.

(< 1%, Vogt IIIa, Gross). Figure 1 illustrates the EA classification scheme. An understanding of these anatomical variants is important to aid in medical and surgical management^[1,4,5,12,13].

EMBRYOLOGY

Successful treatment of esophageal anomalies requires knowledge of their embryological origin. Although significant improvements in clinical treatment have been made in recent years, our understanding of the etiology of these defects is still incomplete^[14-17].

The primitive digestive tube (PDT) emerges from the primitive endoderm and subsequently gives rise to the esophagus and trachea. There are three primary theories that attempt to explain this phenomenon^[6,14-19]. The first theory postulates that the evagination of a tracheal diverticulum begins with the PDT, which grows rapidly in the caudal direction, resulting in the separation of the trachea and esophagus (what remains of the PDT). In the context of this developmental mechanism, tracheoesophageal malformations result from tracheal growth failure^[14].

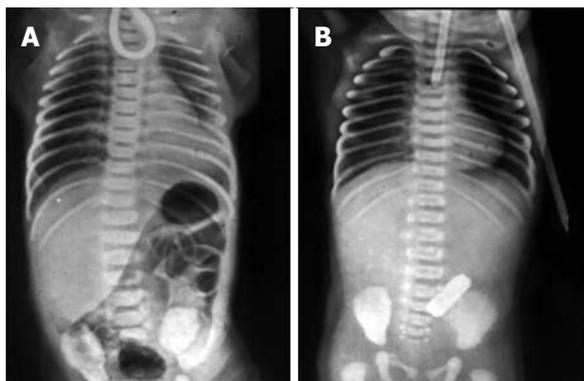


Figure 2 Plain X-rays of the chest and abdomen of two neonates with esophageal atresia. A: The non-progression of an orogastric catheter in the blind esophageal pouch and the presence of air in the stomach diagnose esophageal atresia with distal tracheoesophageal fistula; B: The radiopaque tube in the blind esophageal pouch and the absence of air in the stomach identify esophageal atresia without tracheoesophageal fistula.

Another theory suggests that the formation of a mesenchymal septum in the coronal plane of the PDT, separating the trachea ventrally and the esophagus dorsally from the distal to the proximal ends of PDT. A failure in this process would then result in a tracheoesophageal malformation^[14]. In these two theories, the origin of EA is a cellular rearrangement of the remaining, distal PDT^[14].

The third theory combines elements of the first two and suggests that rapid growth of the tracheal diverticulum occurs in concert with a mesenchymal septation of the PDT, separating the trachea from the esophagus. Unlike the previous theories, however, in this proposed mechanism, EA is believed to result from the loss of a portion of the previously formed tube due to regression toward the main part of the embryo^[14].

Although syndromic cases of EA/TEF are rare, examining the specific genetic anomalies involved may provide valuable information regarding the abnormal developmental processes leading to EA/TEF. Many genes and genetic pathways have been implicated in the development of EA/TEF, but few have been shown to be involved in humans, animals, or both^[15].

There are also molecular and morphogenetic factors related to EA, such as apoptosis, the Sox2, Shh, Gli-2, Gli-3, Pcsk5 and FOX genes and the transcription factors Nkx2.1 and Tbx4^[4,5,15-19]. A failure in the expression of these genes or in the apoptotic programs that they regulate is responsible for EA. However, a whole understanding of these processes remains incomplete^[4,15]. In addition, environmental factors have been suggested to increase the risk for the development of tracheoesophageal anomalies^[15]. Further studies are required for a universally accepted explanation for the pathophysiology of EA/TEF.

DIAGNOSIS

The diagnosis of EA is most commonly made during the first 24 h of life but may be made either antenatally or

may be delayed^[1,5,7-9,20].

Ultrasound (US) scanning is currently a routine method used in prenatal care between the 16th and 20th week of gestation. The suspicion of EA is based on the presence of polyhydramnios and the absence of the gastric bubble, but these are non-specific criteria^[7,21]. US features highly suggestive of EA/TEF are only observed in a small minority of fetuses with EA/TEF (< 10%) on prenatal scans. The combination of polyhydramnios and the absence of a stomach bubble in two previous reports of small patient series gave modest positive predictive values of 44% and 56%, respectively^[7,21].

Dilatation of the blind fundus of the upper segment of the atresic esophagus, the “upper pouch sign”, may also be observed during fetal deglutition at approximately the 32nd gestational week^[7,21]. Moreover, an upper pouch sign may not be detected even with specific examination^[7].

The diagnostic criterion of EA through the use of magnetic resonance imaging is the non-visualization of the intra-thoracic portion of the esophagus. This imaging modality is complementary to ultrasound due to a high percentage of false positives when images are analyzed in an isolated fashion^[21,22]. Even with advances in technological imaging, no ideal prenatal diagnostic method for EA exists.

In the delivery room, the primary sign of EA is the impossibility of the passage of an orogastric catheter beyond 11 or 12 centimeters^[23]. In the nursery, the most important clinical signs are abundant salivation, episodes of cyanosis and suffocation during breastfeeding^[1,4,7]. The confirmation of the diagnosis of EA should be made with a simple chest X-ray using air as contrast in the proximal pouch to avoid aspiration of contrast fluid. If a distal TEF is present, air in the stomach will be present on X-ray films and abdominal distension may be evident^[24]. Figure 2 shows the plain X-rays of two neonates with different forms of EA.

Tracheobronchoscopy has been proposed as an imaging method to detect EA during the preoperative period^[3,22,25]. This technique is used to determine the anatomy of the TEF with respect to the carina, to identify other airway anomalies and to occlude the TEF with a balloon, facilitating mechanical ventilation and avoiding both gastric distension and gastroesophageal reflux. In cases of presumed isolated EA, bronchoscopy also helps to rule out the presence of the less common proximal TEF^[5,22].

PREOPERATIVE PERIOD

Once the diagnosis of EA has been established, the infant needs to be transferred to a regional pediatric surgical center with intensive care support facilities. The aim of the preoperative treatment of EA is to improve the general state of the newborn so that definitive surgery can be carried out under the best possible conditions^[1,4,5].

Classification and risk factors

There are three primary classifications of preoperative

risks regarding EA: the Waterston, Montreal and Spitz classifications^[1,3,10,11,26,27].

According to Waterston, the risk factors to be considered are birth weight (BW), the presence or absence of pneumonia and complications from associated congenital anomalies. In this classification scheme, patients are categorized into group A (BW > 2500 g, with no other complications), group B (BW between 1800 g and 2500 g with no other complications or BW > 2500 g with moderate pneumonia/congenital anomaly) or group C (BW < 1800 g, with no other complications or BW > 2500 g with severe pneumonia/severe congenital anomaly)^[1,3,11,27].

In the Montreal classification scheme for EA, factors such as dependence on mechanical ventilation (MV) and associated congenital anomalies are considered to be of high prognostic significance^[1,11]. Patients are classified into group I (isolated major anomaly, isolated dependence on MV or the presence of non-significant anomalies) and group II (presence of severe congenital anomalies or dependence on MV associated with one major anomaly).

Spitz drafted the most recent classification method by associating BW and cardiac anomalies (CA) as risk factors for EA. In this classification scheme, patients are divided into group I (BW > 1500 g, without CA), group II (BW < 1500 g or the presence of CA) and group III (BW < 1500 g with CA)^[11,21,26,27].

These classification systems serve as guides for the determination of the type of treatment for each case of EA. Some authors no longer consider BW to be a risk factor^[26].

Associated congenital anomalies

What is perhaps of major clinical importance is the high frequency of anomalies associated with EA, with a frequency of over 50%, which may greatly impact both treatment and outcome. In addition to the high frequency of anomalies, their unequal distribution between patients is also important from a clinical perspective. Patients with isolated EA without TEF exhibit anomalies in as many as 65% of cases, while a much lower frequency is observed in patients with TEF without atresia (10%)^[1,7,28].

The most common associated malformation occurs in the cardiovascular system (23% of cases), followed by musculoskeletal malformations (18%), anorectal and intestinal malformations (16%), genital-urinary malformations (15%), anomalies of the head and neck (10%), mediastinal anomalies (8%) and chromosomal anomalies (5.5%)^[5]. Of the observed cardiac anomalies, the most common are ventricular septal defects and tetralogy of Fallot.

A concurrence of congenital anomalies unassociated with a genetic disturbance is referred to as VACTERL [vertebral, anal, cardiac, tracheal, esophageal, renal, and limb (pattern of congenital)] association, which is diagnosed if the patient with EA has 2 or more anomalies of the vertebral, anorectal, cardiac (excluding patent ductus arteriosus and patent foramen ovale), renal/genitourinary, or limb systems. Cardiac anomalies are the most common ones. This broad spectrum of anomalies suggests an al-

teration during the early stages of embryogenesis related to a deficiency in the regulation of the *Sbb* gene^[29]. The CHARGE (coloboma, heart, atresia choanal, retarded growth, genital hypoplasia, ear deformities) association may also include EA^[7].

Life-threatening anomalies, including Potter's Syndrome (bilateral renal agenesis, pulmonary hypoplasia, typical dysmorphic facies), cerebral hypoplasia and chromosomal anomalies, such as trisomy of chromosomes 13, 14 and 18, may be present. These severe conditions directly predict adverse outcomes^[5]. Similarly, infants with totally uncorrectable major cardiac defects or with grade IV intraventricular hemorrhage should be considered for non-operative management^[11].

Nasr *et al.*^[30] demonstrated in 2010 that normal clinical and radiologic examination predicts the absence of significant cardiac abnormalities on echocardiography in 100% of cases. Therefore, these authors conclude that routine pre-surgical echocardiography may not always be necessary, but should be reserved for infants with abnormal clinical and/or radiologic findings.

Vascular components are often overlooked in the investigation of anomalies associated with EA^[31]. The presence of the right aortic arch in association with EA is most often discovered during the surgical intervention for EA correction. According to Babu *et al.*^[32], this condition occurs in 2.5% to 5% of cases of EA, with a greater frequency in males. The method of choice for detecting this anomaly is echocardiography, although this technique is not routinely employed during the investigation of EA. Following the discovery of this anomaly, a left thoracotomy should be performed to correct the EA^[32].

The persistence of the left superior vena cava (LSVC), which results from the persistence of the left inferior cardinal vein, is observed in nearly 10% of infants with EA. Depending on the trajectory of the LSVC, complications may occur, such as thrombosis of the coronary sinus and arrhythmia^[31,33].

Antibiotic prophylaxis

Colonization by bacterial flora of the digestive tract in newborns with EA is related to the establishment of enteral nutrition. However, strains of *Pseudomonas* and *Serratia* have been isolated in the portion of the esophagus that is present in these infants. Antibiotic prophylaxis using amoxicillin and clavulanate is therefore indicated in such patients^[1,3,34].

Neonatal care

Neonatal care includes stabilization of the infant's respiratory status with avoidance of endotracheal intubation; suction tube drainage of the blind, proximal esophageal pouch; semi-prone positioning of the child to minimize the risk of gastroesophageal reflux; and aspiration *via* the occult fistula of the distal trachea^[5]. Monitoring of vital signs and vascular access should also be performed as precautionary measures.

SURGICAL TREATMENT

The surgical treatment of EA is considered urgent but not an emergency, except in premature infants with respiratory distress^[1,3].

Anesthetic care has focused on minimizing ventilation through the fistula, usually by placing the end of the endotracheal tube distal to the fistula, preventing gastric distension/perforation and/or ventilator compromise^[1,7,25]. However, if the fistula is located at the level of the carina, distal placement of the endotracheal tube is impossible. Gastric distention can complicate the ventilation of patients with large TEFs. Gastric distension can also result in the aspiration of gastric contents or in elevation of the diaphragm, leading to decreased tidal volumes, decreased venous return, cardiovascular collapse and ultimately perforation. This results in tension pneumoperitoneum^[1,7,25].

The semi-prone position is the position of choice, with the right side elevated at 45° and the right arm placed over the head. Anesthesia is maintained during the surgical procedure with a volatile anesthetic agent. The patient is ventilated with positive-pressure MV and hydrated with a crystalloid solution^[34].

The primary correction of EA and TEF is the best treatment option in the absence of severe malformations^[35-38]. Standard right posterolateral extrapleural thoracotomy below the tip of the scapula is extremely useful and allows the repair of other complex anatomic variants^[2]. If a right-sided aortic arch is observed on preoperative echocardiography, a left thoracotomy must be performed and the chest is entered through the fourth intercostal space. Care should be taken to avoid entry into the pleura. Extrapleural dissection proceeds posteriorly and superiorly to identify the azygos vein. Division of the azygos vein arch allows for full exposure of the posterior mediastinum. The TEF and vagus nerve are often encountered beneath the azygos arch. This procedure begins with the closure of the fistula. The TEF is divided near the trachea and sewn with fine non-absorbable sutures, which is followed by the correction of the EA. The upper atresic esophageal pouch is identified with downward tension on an oro-esophageal tube, and its dissection is facilitated by the placement of a traction suture at the end of the pouch. Blunt and sharp dissection can mobilize the proximal pouch to the level of the thoracic inlet. Esophageal continuity is accomplished using a single-layer, end-to-end anastomosis with monofilament absorbable sutures^[5]. With respect to unstable patients, however, the procedure should be performed in steps^[35].

There is limited evidence to support the use of a trans-anastomotic tube. The majority of surgeons do not routinely use an intercostal catheter if the repair is extrapleural. Alabbad *et al*^[39] observed that a trans-anastomotic feeding tube may lead to a shorter total parenteral nutrition duration and decreased cholestasis. It was also demonstrated that central venous catheters tended to be removed earlier when trans-anastomotic tubes were used, decreasing the risk of future infection. Furthermore,

hospital stays tended to be shorter^[39]. However, this study provides only preliminary evidence of the benefits of trans-anastomotic tubes. Larger prospective studies will be required to conclusively demonstrate these benefits and to ensure that this technique does not increase anastomotic leaks.

No ideal replacement for the esophagus is available. Nonetheless, the substitute should function as similarly as possible to the original tissue^[35-37]. The reconstruction of the esophagus using only its atresic portions is preferable to the use of any other material, even in cases of long-gap EA^[40-46]. However, a number of authors do not agree with this procedure^[47,48].

Long-gap EA still presents a challenge to pediatric surgeons. A long gap between the two ends of the esophagus in cases of EA has been defined as a gap longer than 3 cm or greater than the height of 2 vertebral bodies^[49].

The historical treatment of EA has included a gastrostomy, the estimation of the extent of the gap between the proximal and distal esophagus and proximal pouch decompression. These measures allow time for potential lengthening of the esophagus with linear patient growth. Delayed surgical intervention is accomplished at approximately three months of age with attempts at achieving a primary anastomosis. Failed attempts at primary anastomosis may then be addressed with colonic interposition, reverse or antegrade gastric tube interposition, gastric transposition, or free jejunal graft interposition. Alternatively, these approaches have been attempted during the neonatal period as a primary repair^[5,50-52].

The ideal surgical treatment for patients with long-gap EA has not been determined, and the topic is still very controversial. In fact, it remains somewhat difficult to determine which surgical procedure achieves the best results. The infant's own functional esophagus is superior to any esophageal replacement. Familiarity with different techniques used to preserve this tissue is therefore important. A number of techniques are proposed for the treatment of long-gap EA. One is the dissection and mobilization of the distal esophageal stump using circular myotomy^[41]. Some authors believe that this technique can be used without vascular impairment of the esophagus due to the abundant irrigation of the esophagus, and that it is preferable to surgery under tension^[41]. Others believe that this technique may result in necrosis of the distal stump and consequent leaking of the anastomosis^[47].

Suboptimal results with these strategies have led to the development of techniques that attempt to elongate the esophagus sufficiently to bridge the gap. This principle was the basis of the technique described by Foker *et al*^[43]. The so-called Foker technique consists of the use of external traction sutures to elongate the esophageal portions and to approximate one stump to the other prior, thus completing the anastomosis. As the esophagus exhibits spontaneous growth during the first three months of life, the technique is performed following this period to ensure that the esophagus has the necessary thickness

to support the traction. This procedure is extremely beneficial despite the requirement for a second thoracotomy, and its success is ensured by esophageal mobilization and the secondary anastomosis^[43,44,53]. A complication that may occur with this technique is the undesired cutting of the esophagus by the sutures^[43]. To avoid this outcome, a modification of the Foker technique has emerged, in which small tubes of silastic are attached to the terminal portions of the two esophageal stumps and the thoracic wall, where the tension is applied^[43].

Nagaya *et al.*^[54] proposed a method for assessing the tension of the anastomosis by placing a metal clip in the trachea at the site of the TEF and assessing the distance between this clip and both the site of anastomosis and the esophago-gastric junction. This method is based on the notion that the stretching of the esophagus is directly related to the tension of the anastomosis and that this information can predict possible postoperative complications. Stretching of up to 5 mm is considered well tolerated^[54].

Tamburri *et al.*^[55] showed that Kimura's technique^[56] is a useful surgical option for a select group of patients with (1) a complex long-gap EA that requires a primary esophagostomy; or (2) any type of EA with the development of severe complications following a primary repair and that required a secondary esophagostomy. The operative procedure consists of an initial cutaneous esophagostomy, multistaged extrathoracic esophageal elongation (ETEE), and definitive esophageal anastomosis. The ETEE includes the mobilization and dissection of the esophagus up to the cricoid cartilage level, with the opening placed on the previously made superior clavicle surgical scar. Variable elongation of the esophagus is achieved following completion of the dissection. The esophagus is again exteriorized as an esophagostomy at a level of a few centimeters below the previous esophagostomy site^[55,56].

More recently, Stringel *et al.*^[57] reported another technique used to repair long gap EAs without anastomosis. This technique consisted of suture approximation and subsequent endoscopic and fluoroscopic placement of string for guided dilations^[57].

In addition to techniques that employ the patient's own esophagus, there are those that use pedicle grafts from the jejunum or colon, which are indicated when the motility of the esophagus does not allow adequate oral feeding and gastric transposition^[5,36,47,48]. The creation of a jejunal graft involves a cross-sectional cut proximal to the ligament of Treitz, separating the first two mesenteric arteries from the peripheral arch. A second cut is made at the level of the third mesenteric artery. The distal portion of the proximal jejunum is removed and transferred to the thorax, leaving on the proximal portion of the jejunum. An anastomosis is then made between the esophageal stumps and the graft. The justification for this technique is that the peristalsis remains in the jejunal graft, but it does not have a tendency to elongate^[48]. With respect to the colon pedicle technique for esophago colic anastomosis, the graft is chosen from the portion of the colon irrigated by the left colic artery and transferred to

the thorax posterior to the mediastinum. The colon also needs to be attached to the muscles of the neck. This technique should only be employed after four months of life to ensure adequate vascularization of the colon^[36,58]. The advantage of using the colon is the presence of long marginal arches, which allows for ample mobilization of extensions to the cervical region. Another advantage is the graft's extreme resistance to gastric fluid due to the continuous production of mucus. The most feared complication is necrosis of the transposed loop^[36,58].

A number of authors prefer gastric transposition^[1,3,37]. This technique has the advantages of being simple, of using the vascularization of the stomach and of easily raising the stomach to the neck for the anastomosis. The primary disadvantages are respiratory distress and late-onset gastric leakage^[37].

For thoracoscopic repair of EA, the patient is placed in a 3/4 prone position and the trocars are inserted at the angle of the scapula in the right axillary region and between the vertebra and the angle of the scapula at the 7th to 8th right intercostal space. The azygos vein and TEF are linked, maintaining the separation between the portions of the esophagus until the mobilization of the proximal stump to avoid retraction of the distal stump toward the diaphragm^[34,59]. A preferentially termino-terminal anastomosis is performed between the esophageal portions, beginning with the posterior wall of the esophagus^[34]. Thoracoscopy is not indicated for the correction of long-gap EA, as this defect requires more invasive dissection and there may be difficulty with the anastomosis of the proximal and distal portions of the esophagus, leading to an increased surgical time, which does not justify the minimally invasive repair^[34].

Cases of "H-type" TEF without EA (Type E) are unique in their presentation and repair^[5]. Infants with this anomaly often present with a long-standing history of recurring aspiration and multiple episodes of pneumonia. The diagnosis is often made following a contrast esophagram or an upper gastrointestinal series by observing the fistula at the lower cervical esophagus or by bronchoscopy. The operative approach for these cases begins with a rigid bronchoscopy and attempts to cannulate the fistula with a small feeding tube or Fogarty catheter. This approach will often aid fistula identification at the time of the operation. The fistula is approached through a right lateral, oblique cervical incision with the neck extended. Dissection begins anterior to the sternocleidomastoid muscle with lateral reflection of the carotid sheath. Exposure of the esophagus continues into the upper mediastinum with identification of the fistula. Primary repair of the tracheal and esophageal portions of the fistula are performed with interrupted sutures. Separation of the adjacent suture lines is aided by interposition of mobilized strap muscles^[5].

Some infants require respiratory support, especially those who are premature; those with associated cardiac anomalies; those in whom the diagnosis was initially missed; and in those with a splinted diaphragm secondary

to gastric distension^[1,7]. In infants requiring respiratory support, great care should be taken during intubation and in supervising ventilation. This challenging and potentially rapidly fatal situation appears to be best managed by an emergency ligation of the distal fistula with a transpleural approach. In the majority of cases, ligation of the fistula improves respiratory status. The thoracotomy is closed pending resolution of respiratory distress over the subsequent few days. Endoscopic placement of a Fogarty balloon catheter, while conceptually an elegant temporizing maneuver, requires a high degree of expertise and has not been proven to be a successful approach in most centers^[1,7].

POST-OPERATIVE PERIOD

In most centers, the infant returns to the neonatal intensive care ventilated and with the neck flexed to reduce anastomotic tension. When the esophageal anastomosis has been performed under tension, the infant is electively paralyzed and mechanically ventilated for 5-7 d postoperatively. If the surgeon has inserted an transanastomotic feeding tube, feeding through the tube should progress slowly, usually beginning 48 h following the surgery^[39]. When the infant can swallow saliva, oral feeding may be started. A routine contrast study appears unnecessary in many cases, but if there is any doubt regarding the integrity of the anastomosis, a water-soluble contrast study should be performed^[1,7].

The primary complications during the postoperative period are leak and stenosis of the anastomosis, gastroesophageal reflux (GER), esophageal dysmotility, fistula recurrence, scoliosis, deformities of the thoracic wall and respiratory disorders^[1-5,7,37,47,60-65].

The outcome following EA/TEF repair is variable. Some patients have an uneventful postoperative period, while others experience several esophageal or respiratory complications that can significantly affect their health through adulthood and can predict the patient's ability to develop adaptive behaviors^[7,49,62-65].

Anastomotic leaks

Anastomotic leaks are considered minor or major. These leaks occur in 15%-20% of patients but are a significant disruption to recovery in less than a third of these cases^[1-5,7,37,38]. Leaks result from the small, friable lower segment, ischemia of the esophageal ends, excess anastomotic tension, sepsis, poor suturing techniques, the type of suture, excessive mobilization of the distal pouch and increased gap length^[66].

Minor leaks are spontaneously reabsorbed by the body, with the vast majority healing within a few days. These leaks are nonetheless associated with a greater incidence of subsequent stricture development. Alternatively, major leaks may cause tension pneumothorax and require the placement of a drain or an early thoracotomy. The thoracotomy is carried out with the intention of repairing the anastomosis. However, if there has been a complete

disruption that precludes any attempt at re-anastomosis, the repair includes a cervical esophagostomy, the closure of the distal esophagus and a subsequent esophageal replacement. Antibiotics and continuous suctioning of the upper pouch may be instituted to reduce saliva egress from the esophagus. The institution of either parenteral nutrition or trans-anastomotic tube feeding is also recommended. A contrast study prior to oral feeding should be performed at the discretion of the surgeon^[1-5,7,37].

A close association exists between anastomotic leakage and the tension of the anastomosis on the suture line^[66]. Uchida *et al*^[67] demonstrated the efficacy of postoperative elective ventilatory support for leakage protection in primary anastomoses of EAs.

Esophageal strictures

Anastomotic strictures are the most common cause of recurrent surgery in children with EA/TEF, and the incidence varies between 30%-40% of cases. The majority of these cases respond to one or two dilatations^[1,2,66,68]. Risk factors that have been implicated in stricture formation include anastomotic tension, anastomotic leakage and GER. With meticulous handling of the esophageal ends, the preservation of the blood supply and careful inclusion of mucosa in each suture of the anastomosis, strictures can be kept to a minimum^[1]. The occurrence of stenosis of the anastomosis is more common in patients with long-gap EA, which is thought to be due to the fact that the repair is under tension^[7,48,59].

The definition of a stricture, however, is not universally accepted as a mild radiological narrowing on a contrast esophagram. Occasionally, this finding may not have any clinical relevance to the physician or to the patient, who may be able to swallow satisfactorily^[2].

Treatment may require successive endoscopic dilatations under general anesthesia^[1,40]. Most strictures respond to dilatation, but it is crucial that reflux is aggressively treated to diminish the impact of acid reflux on recurrent stricture formation^[2]. There is disagreement as to whether dilatation should be performed in a prophylactic or therapeutic fashion. According to Koivusalo *et al*^[69], therapeutic dilatation has the advantages of requiring fewer procedures, shorter hospitalization times and lower costs when compared to prophylactic dilatation.

GER

GER is extremely common among infants following EA repair and may affect between 40% and 65% of patients^[2]. The presence of significant GER is generally believed to be due in part to an intrinsic deficiency in the motor function of the esophagus itself. However, it is likely that GER is exacerbated by the surgical repair and gastrostomy, causing an alteration of the anatomical gastro-esophageal junction and the angle of His^[2]. GER is more common following tension anastomosis^[1,37,70,71]. The symptoms include acute or chronic respiratory problems, regurgitation, recurring vomiting and growth failure^[4]. The diagnosis is made primarily through esophagoscopy,

a 24 h pH probe, intraluminal impedance or a contrast swallow^[2,72].

The treatment of GER can be either clinical or surgical, the latter constituting approximately 28% of cases. The clinical treatment of GER includes dietary modification, adequate positioning of the infant and medication^[5]. The most frequently recommended drug is omeprazole, the effective dose of which is 1.9 mg/kg to 2.5 mg/kg per day, until resolution of the GER and stenosis^[54,73-75].

Surgical treatment consists of Nissen fundoplication, in which the closure of the esophageal hiatus occurs with the approximation of the diaphragm pillars. This is followed by the construction of anti-reflux valves using the gastric fundus to completely envelop the abdominal esophagus^[1,5]. Unfortunately, however, a significant number of infants (> 40%) develop recurrent GER, which may be due in part to the inherent dysmotility of the esophagus^[2].

Long-standing GER is associated with considerable morbidity, resulting in chronic esophageal inflammation and contributing to recurrent pulmonary infections and abnormalities of pulmonary function in a significant number of patients. Moreover, GER is associated with the development of columnar metaplasia that may undergo dysplastic changes, giving rise to esophageal adenocarcinoma through a metaplasia-dysplasia-carcinoma sequence^[37,76-82].

It has been shown that symptoms of GER and histologic findings are poorly correlated. Therefore, long-term endoscopic and pH-metric follow-up of all patients following repair of an EA is warranted. Endoscopic follow-up is recommended for all patients following EA repair irrespective of symptoms. The endoscopic follow-up of children with completely normal esophageal biopsies can be discontinued at the age of 3 years. In patients with mild esophagitis, routine follow-up should be extended to at least 6 years of age^[83-85]. Patients who have undergone anti-reflux surgery should also be followed long-term^[85].

Esophageal dysmotility

Esophageal dysmotility is a very common long-term finding in children with EA/TEF and has been demonstrated in 75% to 100% of patients post-EA/TEF repair. Likewise, in patients who have had some form of esophageal replacement, dysmotility with symptoms such as aspiration, dysphagia, or food bolus obstruction is often reported^[2]. Esophageal dysmotility can be caused by abnormal neural development of the esophagus or may result from complications of EA repair, resulting in deficient peristalsis and uncoordinated contractions of the distal portion of the reconstructed esophagus^[37,86-91]. Esophageal emptying is therefore achieved primarily by gravity. Esophageal and gastric dysmotility can be considered causes of GER^[1,37,86-89]. Scintigraphy and manometry exams are used to diagnose esophageal dysmotility^[86,87,92].

Deformities of the thoracic wall and scoliosis

Chest wall and spinal deformities can be very disfiguring

for patients and may be inadvertently overlooked from a pediatric surgeon's perspective, as these deformities may only become apparent later in life. These anomalies may then be referred to a different specialty, such as orthopedics or plastic surgery. Open thoracotomy can result in significant musculoskeletal morbidity if care is not taken to ensure proper muscle-sparing surgical techniques^[93]. Associated vertebral anomalies can contribute to chest wall or spinal deformities by directly affecting the ribs and vertebral column^[94,95]. A "winged" scapula secondary to neuromuscular injury to the latissimus dorsi muscle has been reported in 24% of patients undergoing a standard posterolateral thoracotomy for EA/TEF repair, with up to 21% of patients exhibiting scoliosis^[2]. Scoliosis is more common in patients who have undergone more than one thoracotomy or division of portions of the serratus anterior and latissimus dorsi muscle groups or their nerve supplies^[2]. Deformities of the thoracic wall are more common in patients who have undergone multiple thoracotomies but can be avoided or diminished with the use of thoracoscopy.

Respiratory disorders

The etiological factors involved in respiratory problems are thought to be retained secretions caused by tracheomalacia, aspiration related to impaired esophageal peristalsis and esophageal stricture and recurrence of TEF or GER. Primary respiratory complications, such as recurrent bronchitis, pneumonias, wheezing illnesses, daily coughing and bronchiectasis are common in patients with repaired EA, but become less frequent with time^[96-102].

Tracheomalacia is due to a structural and functional weakness in the wall of the trachea, resulting in partial and occasionally complete obstruction of the tracheal lumen. This condition results from abnormal tracheal rings, deficiencies in cartilage, and an increase in length of the transverse muscle. Tracheomalacia is a consistent finding in most if not all children with TEF, but is reported to be clinically significant in only 10%-20% of patients and tends to improve with age^[1,7,37]. Approximately half of symptomatic patients require surgical correction, the aortopexy. This repair is reserved for those with near-death episodes or recurrent pneumonia. Success rates of 35% to 88% have been reported^[2].

Bronchoscopy is the gold standard for the diagnosis of tracheomalacia. The severity of tracheomalacia is based on a macroscopic estimation. It is considered severe when the anteroposterior collapse is $\geq 75\%$ with cough or expiration, moderate when the collapse is 50% to 75%, and mild when the collapse is $< 50\%$ ^[96].

CONCLUSION

Improvements have been made in the treatment of EA over the years^[44-49,55]. However, there is no ideal surgical procedure for this congenital anomaly. The search for simpler, less burdensome, more efficient methods for the treatment of EA is closely tied to the difficulty in obtain-

ing sophisticated equipment and qualified personnel for the follow-up of these patients. Long-term outcomes and follow-up data are limited for post-EA/TEF repairs. GER affects these children at the five-year follow-up and gradually declines in children followed for over ten years. Additional long-term morbidities include recurrent respiratory infections, tracheomalacia, dysphagia and choking episodes. Each of these afflictions diminishes over long-term follow-up, but morbidity from esophageal dysmotility may be a life-long problem. Indeed, although EA primarily affects patients during the neonatal period, postoperative complications may lead to common gastroenterological disorders that require long-term follow-up by gastroenterologists. Therefore, determining the risk factors of the complicated evolution of a EA/TEF repair can positively impact the long-term prognosis of these patients, likely avoiding relevant gastroenterological symptoms during adulthood. Knowledge of these risk factors will allow the identification of patients who may benefit from a more intensive follow-up program. Much remains to be understood about EA. Meanwhile, in order to treat patients with individualized care, it is necessary for physicians to stay current regarding treatments for EA.

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Intraductal neoplasm of the intrahepatic bile duct: Clinicopathological study of 24 cases

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Abstract

AIM: To investigate the clinicopathological features of intraductal neoplasm of the intrahepatic bile duct (INiHB).

METHODS: Clinicopathological features of 24 cases of INiHB, which were previously diagnosed as biliary papillomatosis or intraductal growth of intrahepatic biliary neoplasm, were reviewed. Mucin immunohistochemistry was performed for mucin (MUC)1, MUC2, MUC5AC and MUC6. Ki-67, P53 and β -catenin immunoreactivity were also examined. We categorized each tumor as adenoma (low grade), borderline (intermediate grade), and malignant (carcinoma *in situ*, high grade including tumors with microinvasion).

RESULTS: Among 24 cases of INiHB, we identified 24 tumors. Twenty of 24 tumors (83%) were composed of a papillary structure; the same feature observed in intraductal papillary neoplasm of the bile duct (IPNB). In contrast, the remaining four tumors (17%) showed both tubular and papillary structures. In three of the four tumors (75%), macroscopic mucin secretion was limited but microscopic intracellular mucin was evident. Histologically, 16 tumors (67%) were malignant, three (12%) were borderline, and five (21%) were adenoma. Microinvasion was found in four cases (17%). Immunohistochemical analysis revealed that MUC1 was not expressed in the borderline/adenoma group but was

expressed only in malignant lesions ($P = 0.0095$). Ki-67 labeling index (LI) was significantly higher in the malignant group than in the borderline/adenoma group (22.2 ± 15.5 vs 7.5 ± 6.3 , $P < 0.01$). In the 16 malignant cases, expression of MUC5AC showed borderline significant association with high Ki-67 LI ($P = 0.0622$). Nuclear expression of β -catenin was observed in two (8%) of the 24 tumors, and these two tumors also showed MUC1 expression. P53 was negative in all tumors.

CONCLUSION: Some cases of INihB have a tubular structure, and are subcategorized as IPNB with tubular structure. MUC1 expression in INihB correlates positively with degree of malignancy.

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Key words: Intraductal biliary neoplasm; Intraductal papillary neoplasm of the bile duct; Intraductal tubular neoplasm of the bile duct; Intraductal tubulopapillary neoplasm of the bile duct; Mucin expression

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INTRODUCTION

Mucin-producing tumors arising from the bile duct have been reported previously^[1-5], but as a disease category, consensus has not yet been reached. Recently, Shibahara *et al*^[6] and Zen *et al*^[7] have contributed to the development of the concept of papillary tumors in the bile duct that resemble intraductal papillary mucinous neoplasm of the pancreas (IPMN-P) and pancreatic intraepithelial neoplasia. These bile duct tumors show papillary proliferation in the bile duct with mucin secretion, and are considered as intraductal papillary neoplasm of the bile duct (IPN-B), that is, the biliary counterpart of IPMN-P^[8,9]. Recent studies have indicated that IPN-B could be subcategorized according to the results of mucin immunohistochemistry^[6,7], and the similarity of IPNB and IPMN-P has also been described.

In our current study, we conducted histological and immunohistochemical re-examination of 24 cases of intraductal neoplasm of the intrahepatic bile duct (INihB), which were previously reported as biliary papillomatosis or intraductal growth type of intrahepatic biliary neoplasm^[10-13].

MATERIALS AND METHODS

Definition of INihB

We defined INihB as a tumor that (1) was localized in the liver; (2) arose within the intrahepatic bile duct; (3) had a major lesion that was noninfiltrative and showed an intraductal proliferation pattern; and (4) clinicopathologically communicated with the surrounding bile duct.

Tissue samples

We identified 24 cases of INihB from the medical record at Kurume University Hospital and affiliated institutions. These cases were previously diagnosed as intraductal intrahepatic biliary neoplasm or biliary papillomatosis^[13]. Tissue sections of 4 μ m thickness were prepared from paraffin-embedded tissue samples, and stained with hematoxylin and eosin (HE) in the usual manner. The slides were reviewed by the three pathologists (YN, HK and ON).

Degree of malignancy of intrahepatic bile duct

HE-stained sections were reviewed and each tumor was categorized into three groups according to the degree of malignancy by using the criteria in IPMN-P^[8]: adenoma (low grade), borderline (intermediate grade), and malignant (carcinoma *in situ* and high grade). Tumors with microinvasion were categorized as malignant.

Immunohistochemistry

Paraffin-embedded, 4- μ m-thick sections on a coated glass slides were stained by using the BenchMark XT (Ventata Automated Systems, Inc., Tucson, AZ, United States) with the following antibodies: mucin (MUC)1 (mouse monoclonal, Ma695, dilution 1:100; Novocastra, Newcastle, United Kingdom); MUC2 (mouse monoclonal, Ccp58, dilution 1:100; Novocastra); MUC5AC (mouse monoclonal, CLH2, dilution 1:100; Novocastra); MUC6 (mouse monoclonal, CLH5, dilution 1:50; Novocastra); p53 (mouse monoclonal, DO-7, dilution 1:200; Novocastra); β -catenin (mouse monoclonal, β -catenin-1, dilution 1:200; Dako, Glostrup, Denmark); and Ki-67 (mouse monoclonal, MIB-1, dilution 1:100; Dako). This automated system uses the streptavidin-biotin complex method with DAB as a chromogen (Ventana iVIEW DAB Detection Kit).

Regarding the MUC profile, we evaluated cytoplasmic and luminal surface staining by referring to the method of Shibahara *et al*^[6], and the cells were considered positive when either one or both of the two components were stained. The percentage of positively stained neoplastic cells was also calculated and graded as follows: -, < 5% of the neoplastic cells were stained; +, $\geq 5\%$ and < 20% were stained; 2+, $\geq 20\%$ and < 50% were stained, and 3+, $\geq 50\%$ were stained. These evaluations were conducted by three pathologists (YN, HK and ON). Positivity of p53 and β -catenin were defined by distinct and diffuse nuclear staining among the neoplastic cells. Ki-67 staining was counted on a minimum of 1000 tumor cells and Ki-67 labeling index (LI) was calculated as the per-

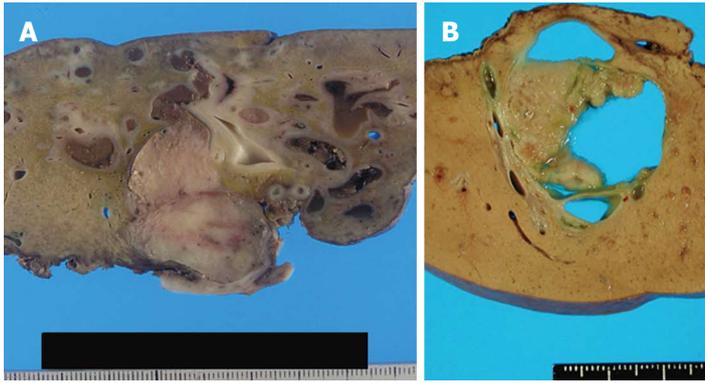


Figure 1 Gross findings. A: Duct-ectatic type. Tumor filled the dilated intrahepatic bile duct. Surrounding bile duct was also dilated; B: Cystic type. Cystic dilatation of intrahepatic bile duct. Papillary tumor was found in the dilated intrahepatic bile duct. Significant retention of mucin was observed.

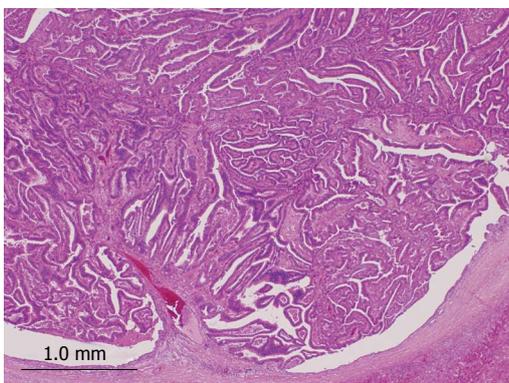


Figure 2 Histopathology of intraductal neoplasm of the intrahepatic bile duct. Tumor shows papillary proliferation within the dilated bile duct (hematoxylin and eosin stain, $\times 20$).

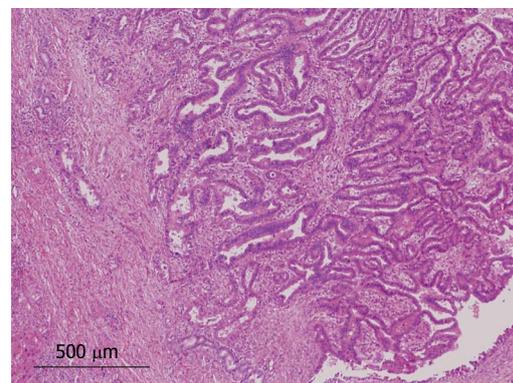


Figure 3 Microinvasion of tumor. Tumor cells infiltrating into the bile duct wall (hematoxylin and eosin stain, $\times 40$).

centage of positively stained cells to total cells.

Statistical analysis

Associations of Ki-67 LI and expression of MUC family (0, 1+ or 2+, 3+) with the degree of malignancy (adenoma, borderline or malignant), and association between Ki-67 LI and MUC profile were examined using the Kruskal-Wallis test and Fisher's exact test. To apply Fisher's exact test, we classified MUC profile into two categories of 0 or 1+ and 2+ or 3+. Association was considered significant when $P < 0.05$. All the statistical analyses were conducted by SAS version 9.12 (SAS Institute Inc., Cary, NC, United States) and R version 2.9.0.

RESULTS

Patients with INihB

We identified 24 tumors in 24 patients. Clinical findings are summarized in Table 1. Median age at the initial diagnosis was 64 years (mean: 63.0 ± 8.1), and the male:female ratio was 1:1. Sixteen tumors (67%) were located in the left lobe. Nineteen tumors (79%) were cystic type associated with mucin hypersecretion, and five tumors (21%) were duct-ectatic type without mucin hypersecretion (Figure 1). Twenty tumors (83%) had papillary struc-

tures in the bile duct as IPNB (Figure 2). The remaining four tumors (17%) presented the following features: the tumor was localized and proliferated in the bile duct, and the tumor showed both tubular and papillary structures. In three of the four tumors (75%), macroscopic mucin secretion was limited but microscopic intracellular mucin was evident. Histologically, 16 tumors (67%) were malignant, three (12%) were borderline, and five (21%) were adenoma. Microinvasion was found in four tumors (21%) (Figure 3), and these were categorized as malignant. There was no ovarian-like stroma in any cases.

Four tumors (17%) were composed of both papillary and tubular structures (Table 2). Histological findings of four patients were similar to those of intraductal tubulopapillary neoplasm (ITPN-P)^[14] or intraductal tubular neoplasm of the pancreas (ITN-P)^[15]. One of these four cases was previously reported as a biliary papillomatosis by Taguchi *et al.*^[13], and the tumor spread from the intrahepatic to extrahepatic bile duct. Another two cases also showed extension of tumor with bile duct dilation. However, the remaining tumor was cystic type and macroscopic mucin hypersecretion was evident. Among tubular structures, a pyloric gland-like structure (Figure 4) was prominent. Three cases were not malignant, but one tumor showed cytological atypia, such as enlarged nuclei with pleomorphism, and was considered as malignant (Figure 5). Atypi-

Table 1 Clinicopathological features of 24 patients with intraductal neoplasm of the intrahepatic bile duct

Age (yr), median (mean ± SD)	64.0 (63.0 ± 8.1)
Gender, <i>n</i> (%)	
Male	12 (50)
Female	12 (50)
Location, <i>n</i> (%)	
Left lobe	16 (67)
Right lobe	8 (33)
Tumor size (cm), median (mean ± SD)	30 (42.2 ± 26.6)
Macroscopic findings of bile duct, <i>n</i> (%)	
Duct-ectatic	5 (21)
Cystic	19 (79)
Macroscopic mucin hypersecretion, <i>n</i> (%)	
Present	19 (79)
Absent	5 (21)
Histological structure pattern, <i>n</i> (%)	
Papillary	20 (83)
Tubular and papillary	4 (17)
Histological grade, <i>n</i> (%)	
Malignant	16 (67)
Borderline	3 (12)
Adenoma	5 (21)
Microinvasion, <i>n</i> (%)	
Present	4 (17)
Absent	20 (83)

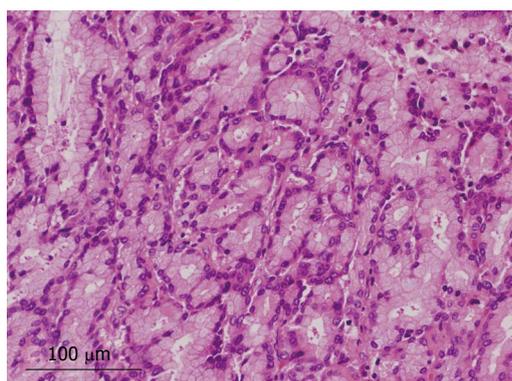


Figure 4 Tubular structure within intraductal neoplasm of the intrahepatic bile duct. Cells with intracellular mucin and mild atypia forming a pyloric gland-like structure (hematoxylin and eosin stain, × 200).

cal cells lining the tubular structure were cuboidal or columnar with little or no cytoplasmic mucin. None of these four cases were associated with tumor invasion.

Immunohistochemical analysis of INihB

Immunohistochemical findings are shown in Table 3. MUC1 was not expressed in the borderline/adenoma group but was expressed in malignant lesions ($P = 0.0095$). No specific pattern of expression was observed for other MUC family members. The Ki-67 LI was significantly higher in the malignant group than in the borderline/adenoma group (22.2 ± 15.5 vs 7.5 ± 6.3 , $P < 0.01$, Figure 6A). In the 16 malignant tumors, there was an association of borderline significance between expression of MUC5AC and higher Ki-67 LI ($P = 0.0622$, Figure 6B). Expression of β -catenin was found in two of the 24 tumors (8%, Figure 7), and these two tumors were also positive

Table 2 Clinicopathological findings of intraductal neoplasm of the intrahepatic bile duct with papillary and tubular structure in four patients

	Case 1	Case 2	Case 3 ¹	Case 4
Age (yr)	77	49	63	52
Gender	Male	Female	Male	Male
Location	Left lobe			
Tumor size (cm)	10	2	6	2.6
Mucin produced macroscopically	Absent			Present
Macroscopic findings	Duct-ectatic type			Cystic
Histopathological grade	Malignant	Borderline	Adenoma	Adenoma
Microinvasion	Absent			
Mucin immunohistochemistry				
MUC1	3+	-	-	1+
MUC2	-	-	-	-
MUC5AC	-	1+	-	-
MUC6	3+	3+	+	3+

¹Has been reported as biliary papillomatosis.

Table 3 Immunohistochemical features of 24 cases of intraductal neoplasm of the intrahepatic bile duct

	Malignant <i>n</i> (16.67%)	Borderline <i>n</i> (3.12%)/adenoma <i>n</i> (5.21%)
MUC1 ¹		
3+ (38%)	9 (56)	0
2+ (0%)	0	0
1+/- (62%)	7 (44)	8 (100)
MUC2		
3+ (13%)	1 (6)	2 (25)
2+ (4%)	1 (6)	0
1+/- (83%)	14 (88)	6 (75)
MUC5AC		
3+ (46%)	7 (44)	4 (50)
2+ (12%)	2 (12)	1 (13)
1+/- (42%)	7 (44)	3 (37)
MUC6		
3+ (46%)	4 (25)	5 (63)
2+ (12%)	4 (25)	1 (22)
1+/- (44%)	8 (50)	2 (25)
Proliferation		
Ki-67 (LI, %, mean ± SD) ²	22.2 ± 15.5	7.5 ± 6.3
Genetic status		
TP53 overexpression	0	0
β -catenin nuclear expression (17%) ³	2 (13)	0

¹MUC1 expression was exhibited only in the malignant group ($P = 0.0095$); ²Ki-67 was significantly higher in malignant cases than in borderline/adenoma patients ($P < 0.01$); ³ β -catenin expression was observed only in the cases with MUC1 expression. LI: Labeling index.

for MUC1. P53 was negative in all 24 tumors.

DISCUSSION

Many studies have been conducted on IPMN-P, and histomorphological criteria for diagnosis have been determined^[8]. Regarding similar lesions in the bile duct, the definition of IPNB^[7] and mucin-producing bile duct tumors^[6] is proposed as a new conceptual framework

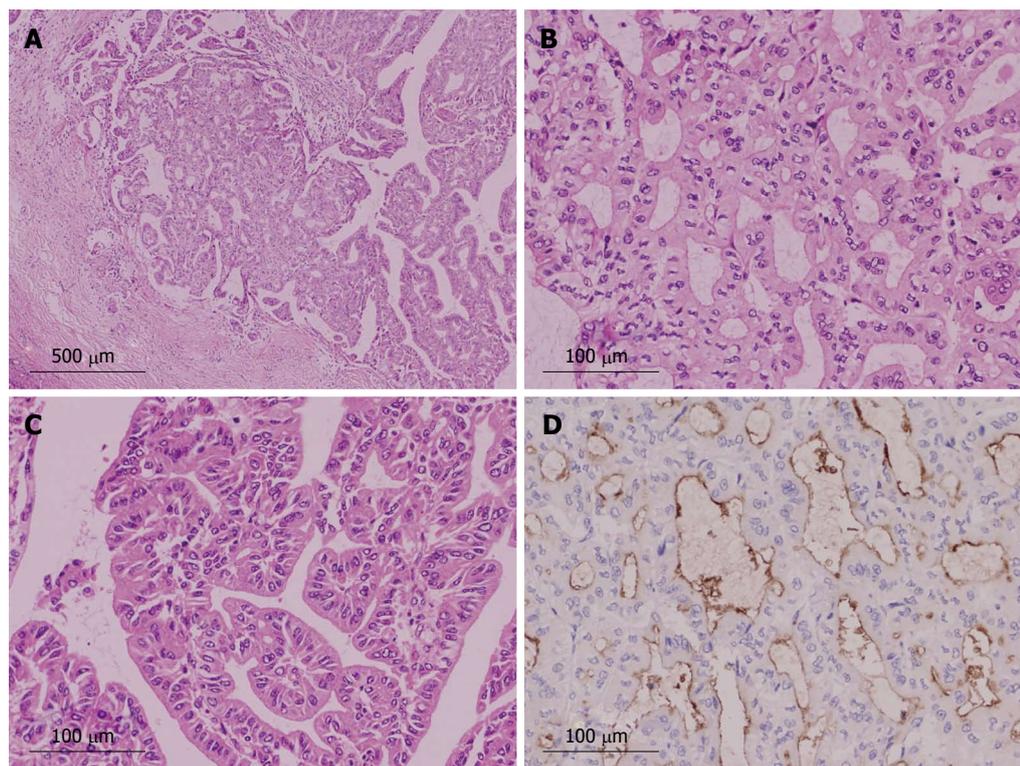


Figure 5 Histology of intraductal neoplasm of the intrahepatic bile duct with papillary and tubular structure (case 1). A: Histological structure was mainly tubular, but papillary structure was also present [hematoxylin and eosin (HE) stain, × 40]; B: Tubular structure (HE stain, × 200); C: Papillary structure (HE stain, × 200); D: Tumor cells were positive for mucin (MUC)1 immunohistochemistry (MUC1 stain, × 200).

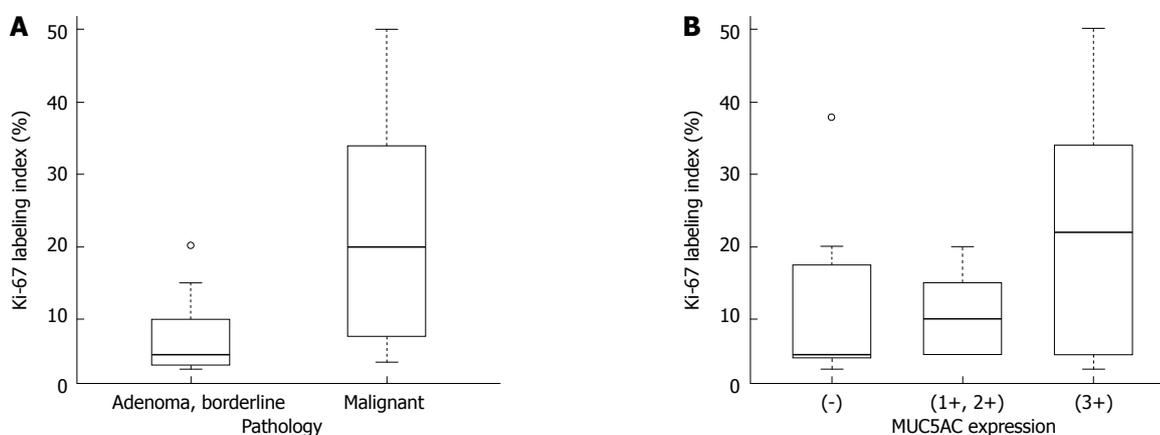


Figure 6 Box plot for Ki-67 labeling index by histological degree of malignancy and MUC5AC expression. A: The Ki-67 labeling index (LI) was significantly higher in the malignant group than in the benign/borderline group; B: There was an association with borderline significance between MUC5AC expression and Ki-67 LI ($P = 0.0622$). MUC: Mucin.

of biliary intraductal neoplasms. These biliary lesions present the same histological features of IPMN-P, and Zen *et al*^[16,17] divided them into four categories, namely, pancreaticobiliary type, intestinal type, gastric type, and oncocytic type. In contrast, Shibahara *et al*^[6] subclassified the lesions into two distinct categories, namely, columnar type and cuboidal type. Columnar type resembles intestinal type, and cuboidal type resembles pancreaticobiliary type or intraductal oncocytic papillary neoplasm, and they hypothesized that these two categories could be the biliary counterparts of IPMN-P.

Among our 24 cases of INihB, 19 cases (79%) were the cystic type that was proposed by Shibahara *et al*^[6], and this type is known as a biliary cystic tumor. Devaney *et al*^[18] reported that ovarian-like stroma was observed in 85% of the adenoma and 28% of the adenocarcinoma tumors. However, none of our cases had ovarian-like stroma. The presence or absence of ovarian-like stroma is an important factor for the classification of IPNB. Recently, Zen *et al*^[17] proposed that biliary cystic tumor could be a cystic variant of IPNB, having bile duct communication and absence of ovarian-like stroma. Based on our

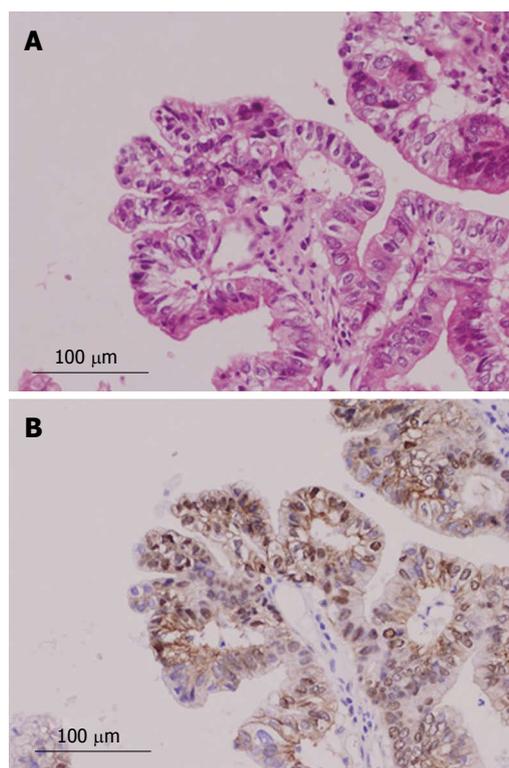


Figure 7 Nuclear expression of β -catenin in pancreaticobiliary type. A: Hematoxylin and eosin stain, $\times 200$; B: β -catenin stain, $\times 200$.

findings, the cystic type reported by Shibahara *et al.*^[6] and biliary cystic variant of IPNB reported by Zen *et al.*^[16] are the most common type among INihB, and they can be recognized by their characteristic macroscopic findings, that is, a cystic change with mucin hypersecretion, even though the tumors have ovarian-like stroma.

In our current study, 20 of 24 INihB tumors (83%) showed purely papillary proliferation that is a morphological feature of IPMN-P, and we diagnosed them as IPNB. In contrast, both papillary and tubular structures were present in the remaining four tumors (17%), and three of these four tumors were duct-ectatic type without macroscopic mucin secretion. These features are common findings in ITN-P or ITPN-P, which are rare diseases^[15,19-24]. These tubulopapillary tumors can be distinctly subcategorized in INihB based on their characteristic macroscopic and histological findings. Intraductal neoplasms with a tubular structure have been reported in the pancreatic area. Albores-Saavedra *et al.*^[19] reported that tumors with a tubular structure included: (1) glands resembling pyloric glands; (2) glands lined by cells with no cytoplasmic mucin and with mild nuclear atypia; and (3) glands lined by pink oncocytic cells. Recently, Yamaguchi *et al.*^[14] proposed the concept of ITPN-P and defined nine diagnostic criteria. Similarly, some authors have described intraductal lesions in which IPNB with formation of the tubular structure is regarded as intraductal tubular neoplasm of the bile duct (ITNB)^[25,26]. In our four cases, we observed morphological similarity with ITN-P or ITPN-P. In particular, Case 1 consisted mainly

of a tubular structure and some papillary patterns, and the neoplastic cells were markedly atypical. In addition, immunohistochemical results were MUC1(+) and MUC5AC(-), which were different from ITN-P. Our case 1 was considered as an ITPN-B subcategory. As discussed above, IPNB contained IPNB with a tubular structure, although small in number, and there might be cases with similar characteristics to ITN-P or ITPN-P.

We also examined Ki-67 and p53 immunoreactivity in the 24 cases of INihB. Ki-67 expression differed significantly between malignant and borderline/adenoma (22.2 ± 15.5 vs 7.5 ± 6.3 , $P < 0.01$), but P53 was negative in all cases. Based on these data, Ki-67 LI correlates with the degree of malignancy in INihB. Shibahara *et al.*^[6] reported significantly high Ki-67 expression and poorer prognosis in the columnar type than in the cuboidal type. In this study, we also investigated the relationship between MUC1/MUC5AC immunostaining and malignant potential. Some studies have reported that the pancreaticobiliary type IPMN-P, which is positive for MUC1, is highly malignant^[8]. Other studies have suggested that MUC1 contributes significantly to tumor growth and metastasis, and that downregulation of MUC1 protein expression decreases the metastatic potential of pancreatic adenocarcinoma^[27]. The association between MUC1 and malignant potential has also been suggested in cholangiocarcinoma^[28]. Although we did not find a significant association between MUC5AC expression and the degree of malignancy or cell proliferation potential, there was a borderline association between the higher expression of MUC5AC and the higher Ki-67 LI in the malignant group. Some authors also have reported that MUC5AC is more strongly expressed in advanced tumors^[17,28]. Thus, we suggest that expression of MUC1 and MUC5AC is related, MUC1 more strongly so than MUC5AC, to the degree of malignancy in INihB.

Nuclear expression of β -catenin was found in 8% of INihB (2/24), and MUC1 was also positive in these cases. β -catenin is a mediator in the canonical Wnt signal transduction pathway^[29] and activation of the Wnt pathway is associated with high proliferation and dedifferentiation in human biliary tumors^[30]. Nuclear accumulation of β -catenin was reported in 15% of intrahepatic cholangiocarcinomas (Sugimachi *et al.*^[31]); 25% of IPN-B, 20% of IPN-B associated with intrahepatic cholangiocarcinoma, and 0% of biliary intraepithelial neoplasms (Itatsu *et al.*^[32]); and 25% of IPN-B (Abraham *et al.*^[33]). The Wnt signaling pathway is frequently activated in biliary neoplasms^[32]. We believe when dealing with INihB one should consider performing immunohistochemical staining for MUC1 and β -catenin, in addition to histomorphological examination, to evaluate malignant potential of the tumor.

In conclusion, we propose the concept of INihB that includes IPNB and IPNB with tubular structure subcategories, that is, ITNB and intraductal tubulopapillary neoplasm of the bile duct. IPNB with tubular structure is rare, and (1) is mostly duct-ectatic without macroscopic mucin hypersecretion; and (2) histologically, has both

papillary and tubular structures. In INihB, MUC1 expression correlates positively with the degree of malignancy and cell proliferation potential. MUC5AC expression also correlates with the degree of malignancy, although the relationship is less robust. Further pathological and molecular studies are necessary to clarify the characteristics of INihB.

COMMENTS

Background

The clinicopathological characteristics of intraductal neoplasm of the intrahepatic bile duct (INihB) remains unclear. In this article, the authors present the clinicopathological features of 24 cases of INihB.

Research frontiers

Some studies have reported that the pancreatobiliary-type intraductal papillary mucinous neoplasm (IPMN-P), which is positive for mucin (MUC)1 expression, is highly malignant, and other studies have suggested that MUC1 contributes significantly to tumor growth and metastasis, and that downregulation of MUC1 protein expression decreases the metastatic potential of pancreatic adenocarcinoma. The association between MUC1 and malignant potential has also been suggested in cholangiocarcinoma. Some authors have reported that MUC5AC is more strongly expressed by advanced tumors. In this study, the authors demonstrated that MUC1 and MUC5AC expression may be related to the malignant potential of INihB.

Innovations and breakthroughs

Recent reports have highlighted the relationship between MUC profile and malignancy potential. In particular, the authors investigated the relationship between MUC1/MUC5AC immunohistochemical staining and Ki-67 labeling index.

Applications

This study offers a better understanding of clinicopathological characteristics of INihB and a potential strategy to predict tumor behavior for better patient care.

Terminology

MUC1/MUC5AC are mucin proteins in the pancreatobiliary/gastric epithelium, respectively. It has been suggested that these proteins play an important role in tumor growth and metastasis in pancreatobiliary disease.

Peer review

Immunohistochemical analysis revealed that Ki-67 expression in the 24 INihBs was significantly high in the malignant group. MUC staining showed that MUC1 was not expressed in the borderline/adenoma group but was expressed only in malignant lesions, and the Ki-67 labeling index was significantly higher in the malignant group than in the borderline/adenoma group. These results may represent a mechanism of MUC protein in tumor growth, that is, malignant potential of the tumor.

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Study of human B7 homolog 1 expression in patients with hepatitis B virus infection

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Abstract

AIM: To further investigate the role of human B7 homolog 1 (B7-H1) in the mechanism of persistent hepatitis B virus (HBV) infection.

METHODS: Peripheral and intra-hepatic B7-H1 expression were compared by flow cytometry and immunohistochemical staining between two distinct groups, one

being chronic HBV tolerance patients (CHB-T) and the other being acute hepatitis B patients (AHB). B7-H1 mRNA expression level was also compared by real time polymerase chain reaction between CHB-T and AHB patients. The location of intra-hepatic B7-H1 and CD40 expression were analyzed by immunofluorescence. The levels of B7-H1 and CD40 expression on cultured myeloid dendritic cells (mDCs) with or without hepatitis B surface antigen (HBsAg) treatment were analyzed dynamically by flow cytometry. Intracellular interferon- γ (IFN- γ) staining and the stimulatory capacity of mDC of cultured mDC with or without HBsAg treatment were also compared by flow cytometry.

RESULTS: Peripheral B7-H1 expression on mDCs was increased significantly in AHB compared to CHB-T patients ($P < 0.05$). In the liver tissues from CHB-T patients, B7-H1 positive cells were almost absent despite a persistently elevated serum HBsAg load. In contrast, there were indeed increased B7-H1-positive cells *in situ* in the liver tissue from AHB. *In vitro* analysis showed the parallel upregulation of B7-H1 and CD40 on CD11c+ mDCs after the onset of stimulation. Addition of recombinant hepatitis B surface antigen (rHBsAg) significantly decreased CD40 expression ($P < 0.05$ at 16 h, 20 h and 24 h time points). B7-H1 expression was also inhibited by rHBsAg, and the inhibition rate of CD40 was greater than that of B7-H1. This preferential inhibition of CD40 expression on mDCs by rHBsAg resulted in the dysfunction of mDCs and T cells in the mixed leucocyte reaction (MLR) system. With rHBsAg pretreatment, in a carboxyfluorescein diacetate succinimidyl ester (CFSE) labeled MLR system at a ratio of 1:5 responder cell-stimulator cell (R/S), the CFSE^{dim} percentage of T cells decreased from 85.1% to 25.4% and decreased from 30.3% to 12.0% at 1:10 R/S. IFN- γ production by CD8+ T cells, in the MLR system, was reduced significantly by HBsAg pretreatment. At ratios of 1:5 R/S, the percentage of IFN- γ and CD8 dual positive T cells decreased from 55.2% \pm 5.3% to 15.1% \pm

3.1% ($P < 0.001$), and decreased from $35.0\% \pm 5.1\%$ to $7.3\% \pm 2.7\%$ at ratios of 1:10 R/S ($P < 0.001$).

CONCLUSION: B7-H1 is not a signature of immune dysfunction, but an inflammation marker. HBsAg regulate immune response by tipping the balance between B7-H1 and CD40.

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Key words: Hepatitis B virus; Hepatitis B; Human B7 homolog 1; Immune tolerance; Co-stimulatory molecule

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INTRODUCTION

Patients with self-limited acute hepatitis B (AHB) can develop appropriate virus-specific immune responses, however these immune responses are insufficient to eliminate the virus in chronic hepatitis B patients and eventually lead to chronic hepatitis B virus (HBV) tolerance (CHB-T). To date, the mechanisms underlying this defect of the HBV specific immune response have not been fully elucidated. T-cell exhaustion, lack of CD4+ T-cell help, induction of T-cell tolerance and viral variation may contribute to HBV persistence^[1-8]. However, it is also possible that the antigen presenting cells (APCs) of hepatitis B patients fail to prime an appropriate T-cell response^[9-13].

Dendritic cells (DCs) are professional APCs that are specialized for the initiation and regulation of T-cell immunity. The efficiency of DCs in activating T cells is determined by many factors. DCs express high levels of positive co-stimulatory molecules, [B7.1 (CD80), B7.2 (CD86), and CD40] which interact with receptors on T cells to mediate T cell activation^[14]. On the other hand, DCs also express negative molecules such as human B7 homolog 1 (B7-H1) and human B7 homolog DC (B7DC), which bind to programmed death 1 (PD1) to deliver a co-inhibitory signal to T cells, thus leading to T-cells tolerance^[14]. Based on these studies, the balance between positive and negative co-stimulatory molecules is viewed as a key factor for determining the outcome of HBV infection^[15,16].

Previous studies have indicated that the B7-H1/PD-1 pathway plays a key role in myeloid dendritic cells (mDCs) dysfunction and T-cell exhaustion when these cells are exposed to high HBV or hepatitis C virus (HCV) antigen loads. In addition, blockade of the PD-1/B7-H1 interaction can restore the allostimulatory ability of mDCs and

the function of HBV and HCV-specific CD8+ T cells with increased proliferation, cytotoxicity and cytokine production^[2,17-19]. Some studies have reported that B7-H1 and PD1, which are expressed in circulating mDCs and HBV specific T cells respectively, were significantly up-regulated in chronic hepatitis B patients. The specificity of this upregulation to HBV or HCV infection was determined by the lack of a significant increase in B7-H1 and PD1 in cytomegalovirus, Epstein-Barr virus and influenza A infected patients^[20-22]. Based on these studies, relatively high levels of B7-H1/PD-1 expression were viewed as the signature of impairments in the HBV- and HCV- specific immune response. Both HBV and HCV might exploit the B7-H1/PD1 pathway to facilitate persistent infection.

On the other hand, recent studies revealed that B7-H1 and PD1 expression are significantly upregulated in the early phase of AHB infection, and successful viral clearance is correlated with a decrease in PD1 expression^[12,23]. These results are in line with reports showing that B7-H1 and PD1 expression is upregulated in liver nonparenchymal cells during the acute phase of inflammation to limit an over-vigorous inflammatory response^[24].

Our clinical observation revealed that chronic HBV infection was composed of 2 different components. On one hand, the CHB-T condition is characterized by active HBV-DNA replication, but shows no serum alanine aminotransferase (ALT) upregulation. The other component is a fluctuating inflammation condition that shows repeated inflammation flare-ups with fluctuating levels of serum ALT and HBV-DNA.

To examine the role of B7-H1 in the mechanism of HBV tolerance, we compared B7-H1 expression between AHB and CHB-T patients.

The results showed that peripheral and intra-hepatic expression of B7-H1 was more significant in AHB than that in CHB-T patients. In the liver tissue of CHB-T subjects, B7-H1 was almost absent despite a persistently elevated hepatitis B surface antigen (HBsAg) load. *In vitro* analysis showed that CD40 and B7-H1 were upregulated synchronously after the onset of stimulation, and CD40 was preferentially inhibited by recombinant hepatitis B surface antigen (rHBsAg), which impaired the allostimulatory capacity of mDCs and interferon- γ (IFN- γ) production by CD8+ T cells in the mixed leucocyte reaction (MLR) system.

Our findings showed that the analysis of B7-H1 expression alone is not sufficient to elucidate the mechanism of HBV immune tolerance. B7-H1 is an inflammatory marker, but not an absolute indicator of HBV-specific immune tolerance. The preferential inhibition of CD40 expression by rHBsAg can be considered as part of a mechanism by which HBV impairs the immune response and results in persistent infection in CHB-T patients.

MATERIALS AND METHODS

Subjects

This study examined 27 adults with HBV infection (18

Table 1 Clinical data of these patients at the time of the first physician consultation

	Male/female	ALT	HBsAg	HBV-DNA load
AHB (<i>n</i> = 15)	10/5	296 ± 110	404 ± 98 ng/mL	(2 - 7) × 10 ⁴ (<i>n</i> = 4), (3 - 6) × 10 ⁵ (<i>n</i> = 5), (1 - 6) × 10 ⁶ (<i>n</i> = 4), (1 - 2) × 10 ⁷ (<i>n</i> = 2)
CHB-T (<i>n</i> = 12)	8/4	Normal (10-40 μ/mL)	363 ± 58 ng/mL	(3 - 7) × 10 ⁵ (<i>n</i> = 6), (1 - 5) × 10 ⁶ (<i>n</i> = 5), (1.1) × 10 ⁷ (<i>n</i> = 1)
AIH (<i>n</i> = 3)	0/3	301 ± 79.3	Negative	Negative
HC (<i>n</i> = 5)	3/2	Normal (10-40 μ/mL)	Negative	Negative

AHB: Acute hepatitis B; CHB: Chronic hepatitis B virus tolerance; AIH: Autoimmune hepatitis; HC: Healthy controls; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen.

men, 9 women) from August 2008 to August 2010 that were hospitalized in our unit. None of the patients were treated with steroids before sampling. Concurrence of HCV and human immunodeficiency virus infections were excluded from enrolled individuals. The study protocol was approved by the Ethics Committee of our unit, and informed consent was obtained from each subject. The patients were assigned to 2 distinct groups based on plasma HBV DNA loads and ALT levels. The first group (*n* = 12) was formed by CHB-T with HBV-DNA replication and HBs-Ag production, but normal ALT levels (normal range: 10-40 U/L). The other group (*n* = 15) was composed of AHB patients with active HBV replication and significantly elevated serum ALT levels. For comparison, 5 uninfected healthy controls (HC) and 3 patients with autoimmune hepatitis (AIH) were enrolled as controls. The clinical data of these patients at the time of the first physician consultation are summarized in Table 1.

Human B7-H1 expression in circulating mDCs

B7-H1 expression on circulating mDCs was measured using 1 mL of fresh heparinized peripheral blood. Cells were lysed with fluorescence-activated cell sorter (FACS) lysing solution (BD Pharmingen) to remove red blood cells and then incubated with antibodies against B7-H1-Phycoerythrin (PE) and CD11c-fluorescein isothiocyanate (FITC) for 20 min at room temperature. After washing twice with phosphate buffered saline (PBS), the cells were analyzed by flow cytometry on a FACSCalibur (BD Biosciences).

Preparation of mDCs and T cells

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood of healthy donors by Ficoll density gradient centrifugation. PBMCs were then plated (5 × 10⁶ cells/well) into a 6-well plate and incubated at 37 °C for 2 h. T-cell-enriched and T-cell-depleted fractions were prepared by adherence to plastic in complete RPMI 1640 medium. The immature dendritic cells (iDCs) were prepared from the T-cell-depleted fraction by culturing cells in the presence of granulocyte macrophage colony-stimulating factor (50 ng/mL) and interleukin 4 (IL-4) (50 U/mL) for 5 d.

MDCs culture and CD40-, and human B7-H1 expression

iDCs were prepared from PBMC and incubated in a 24-well plate in RPMI 1640 medium containing 10%

FCS and treated with or without rHBsAg-adr (1 μg/mL). (r)HBsAg adr was purified from transfected Chinese Hamster Ovary (CHO) cells. Cell lysates of CHO without HBsAg transfection served as a negative control. After 5 d, these cells were stimulated by 20 μg poly (I: C), and then collected at 4 h, 8 h, 16 h, 20 h, and 24 h after stimulation. The cells were incubated with anti-B7-H1-PE and CD40-FITC antibodies for 20 min at room temperature. After washing three times with PBS, the expression of B7-H1 and CD40 was analyzed by flowjo7.6.

RNA preparation and real-time polymerase chain reaction

Total RNA from liver tissues was isolated using RNeasy kits (Qiagen). Reverse transcription of RNA was performed using a SuperScript One-Cycle cDNA kit (Invitrogen). The cDNA served as a template in real-time polymerase chain reaction (PCR). The human B7-H1 primers for RT-PCR were used as follow: 5'-TTTACTGTACGCGTTCCC-3' (sense) and 5'-TGTTCTTATCCTCCATTTC-3' (antisense); human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, 5'-CTGCCCCCTCTGCTGATG-3' (sense) and 5'-TCCACGATACCAAAGTTGTTCATG-3' (antisense). All reactions were performed in triplicate. The B7-H1 mRNA expression of different specimens was normalized to GAPDH. Relative mRNA levels are presented as unit values of 2^{-ΔΔCt}, where Ct is the threshold cycle value defined as the fractional cycle number at which the target fluorescent signal passes a fixed threshold above baseline.

Immunochemical staining

Acetone-fixed liver tissue cryosections (7 μm) were incubated with anti B7-H1 antibodies (Abcam) at 4 °C overnight. After DAB peroxidase staining, positive cells (brown color) were counted in 3 different fields by 2 independent observers. To determine B7-H1, CD40, CD68, macrophage inflammatory protein (MIP3) α, secondary lymphoid tissue chemokine (SLC), CD11c, chemokine (C-C) receptor (CCR) 7 and HBsAg expression, immunofluorescence double staining was performed. Briefly, liver tissues were incubated for 12 h at 4 °C with diluted primary Abs followed by diluted secondary Abs for 1 h at room temperature. The following primary antibodies were used for CD11c, CD40, B7-H1, CD68, SLC, MIP3α, CCR7 and HBs-Ag: mouse antihuman CD40,

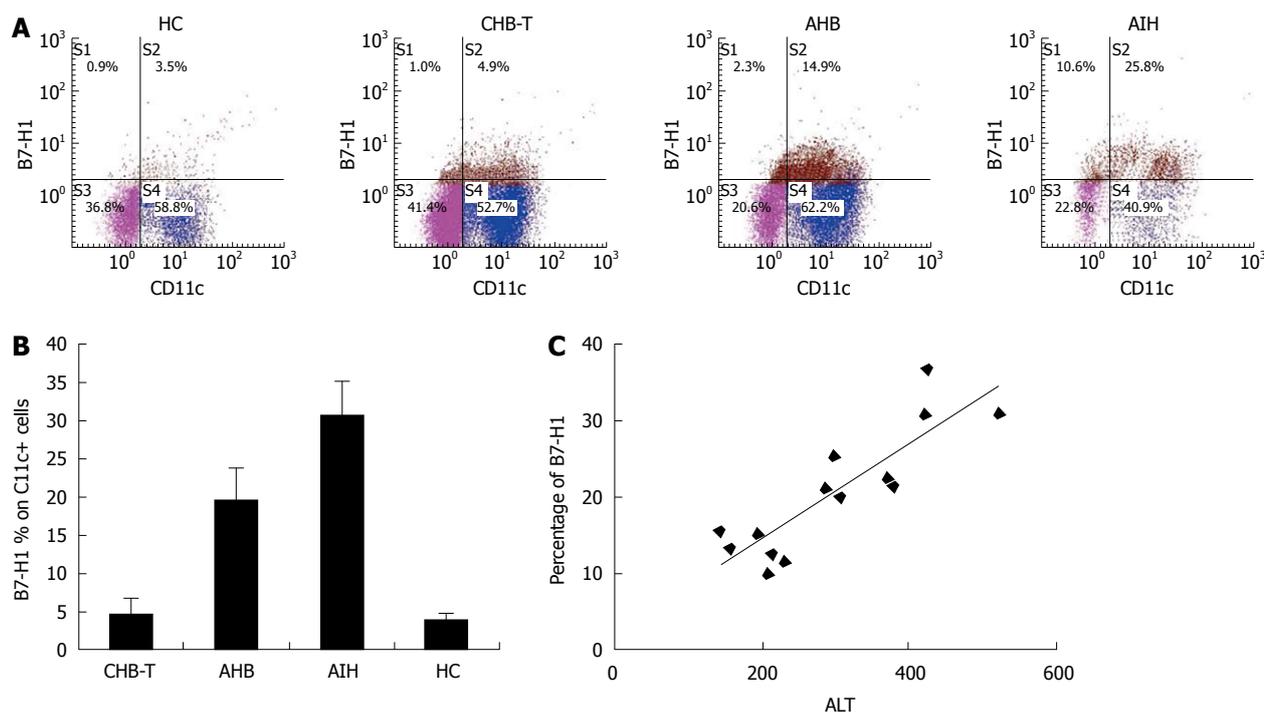


Figure 1 Circulating human B7 homolog 1 expression comparison among acute hepatitis B, chronic hepatitis B virus tolerance and autoimmune hepatitis patients. A: Representative dot plots of double measurements of fifteen independent experiments; B: B7 homolog 1 (B7-H1) expression level on circulating myeloid dendritic cells (mDCs) of acute hepatitis B (AHB) patients was significantly higher than that in chronic hepatitis B virus tolerance (CHB-T) patients ($n = 12$, $P < 0.05$). Autoimmune hepatitis (AIH) patients exhibited the highest levels of B7-H1 expression on circulating mDCs among these groups ($n = 3$, $P < 0.05$); C: In AHB patients, there was significant, positive correlation between B7-H1 expression on mDCs and serum alanine aminotransferase (ALT) levels in AHB patients ($r = 0.809$). HC: Healthy controls.

CD68 and CD11c (diluted 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, United States), and-, goat antihuman B7-H1, MIP3 α , SLC, CCR7 (diluted 1:100; Santa Cruz). As secondary antibodies, a rabbit anti-goat IgG PE-conjugated antibody (diluted 1:100; Santa Cruz), a rat anti-mouse IgG FITC-conjugated antibody, and a rat anti-mouse IgG rhodamine-conjugated antibody were used.

Allogeneic mixed leukocyte reaction

Purified mDCs with or without HBsAg treatment were matured for 24 h in a 96-well flat-bottom culture plate (at 1×10^5 cells/200 μ L) in culture medium containing poly (I:C) (20 μ g/mL; Sigma). On the following day, mDCs were treated with mitomycin C and were added at different concentrations (1/5 and 1/10) into purified T cells from a normal healthy volunteer (1×10^5 cells/200 μ L) and labeled with carboxyfluorescein diacetate succinimidyl ester [CFSE (1 mmol/mL); Molecular Probes, Eugene, OR] for 5 d. T-cells proliferation was assessed by the loss of CFSE analyzed by flow cytometry. Flow cytometric data were analyzed by CellQuest (Becton Dickinson). All measurements were performed in triplicate.

Intracellular IFN- γ staining

Next, IFN- γ staining during these MLRs was measured. T cells were collected after 5 d of mixed culture, phorbol myristate acetate (1 μ g/mL), ionomycin (0.1 mg/mL; T cell receptor-bypassing reagents) and 0.1 mg/mL monomycin

(Sigma-Aldrich) were added into a mixed culture system 4 h before analysis. T cells were then washed in PBS, stained with anti-CD8 (FITC) mAb and then permeabilized and fixed according to the manufacturer's instructions. After a further wash in PBS, cells were stained with anti-IFN- γ -PE mAb at room temperature for 20 min. After 2 additional washes, cells were fixed and acquired immediately on flow cytometry on a FACSCalibur (BD Biosciences).

Statistical analysis

All experimental conditions were pair analyzed against their controls. Data on co-stimulatory molecules, and cytokine and B7-H1 mRNA expression were analyzed by the Student's *t* test. The correlation between ALT levels and B7-H1 expression obtained by FACS was assessed by Pearson's correlation analysis. Data corresponding to the expression of co-stimulatory molecules and cytokine production are expressed as mean \pm SE. A *P* value of < 0.05 was considered statistically significant.

RESULTS

The expression of human B7-H1 on circulating mDCs was significantly upregulated in AHB patients and AIH

The levels of B7-H1 expression were detected on circulating CD11c + DCs from AHB, CHB-T, and AIH patients, and HC (Figure 1A). B7-H1 expression on circulating CD11c + DCs from enrolled patients was generally

increased, in particular in AHB patients, who expressed higher levels of B7-H1 than CHB-T individuals ($P < 0.05$, Figure 1B). In CHB-T patients, B7-H1 expression also increased, but was not significantly different from that in HC. AIH patients exhibited the highest levels of B7-H1 expression on CD11c + DCs among these groups (all $P < 0.05$, Figure 1B). Correlation analysis revealed that there was a significant, positive correlation between B7-H1 expression on circulating CD11c + DCs and serum ALT levels in AHB patients ($r = 0.809$, Figure 1C).

Intra-hepatic human B7-H1 expression is much higher in AHB than in CHB-T patients

Immunohistochemical staining performed to analyze intrahepatic B7-H1 expression showed an increased number of B7-H1-positive cells *in situ* in the livers of AHB patients. In contrast, B7-H1-positive cells were almost completely absent in the livers of CHB-T subjects and healthy donors (Figure 2A). Immunofluorescence double staining revealed that almost all CD11c + DCs, CD123 + DCs and Kupffer cells expressed B7-H1 molecules in the liver tissue from AHB patients (Figure 2B-D), while in liver tissue from CHB-T patients, B7-H1 positive cells were almost completely absent despite extensive HBsAg expression (Figure 2E). These results were in line with data obtained with circulating CD11c + DCs. In addition, in the liver tissue from AHB patients, B7-H1 was always co-expressed with the positive co-stimulatory molecule CD40 (Figure 2F).

Human B7-H1 mRNA expression is upregulated in liver tissue from AHB patients

B7-H1 mRNA expression levels in liver tissues were further compared between AHB and CHB-T patients by real-time PCR. As show in Figure 3, B7-H1 mRNA expression levels were significantly higher in AHB than in CHB-T liver tissue. The highest relative B7-H1 mRNA expression level in AHB liver tissue showed a 12-fold increase over the CHB-T level. The lowest B7-H1 mRNA level showed a 2-fold increase in AHB compared to CHB-T liver tissue (Figure 3).

Inflammatory markers showed significant expression in the liver tissue from AHB patients with significant human B7-H1 expression

Peripheral and intrahepatic B7-H1 expression in AHB patients was far more significant than that of CHB-T. These results suggest that B7-H1 is a marker of AHB. To further confirm this conclusion, serially sectioned liver tissues from AHB patients were examined for the markers of inflammatory response, including DCs, lymphocytes infiltration and chemokines expression by immunofluorescence double staining. Liver tissue with significant B7-H1 expression showed significant infiltration of CD11c+ and CD8+-positive cells. Chemokines such as MIP3 α and SLC were also expressed extensively by CD11c + DCs (Figure 4A and B). Mature mDCs expressing CCR7 and activated CD8+ lymphocytes expressing PD1 were recruited to the liver lobe (Figure 4D). In contrast, in liver

tissue from CHB-T patients with significant HBsAg but no B7-H1 expression, CD11c+ and CD8+ infiltration cells were predominantly localized in fibrous septa rather than in the sinusoidal area (Figure 4E). These results suggest that B7-H1 was preferentially expressed at the site of AHB.

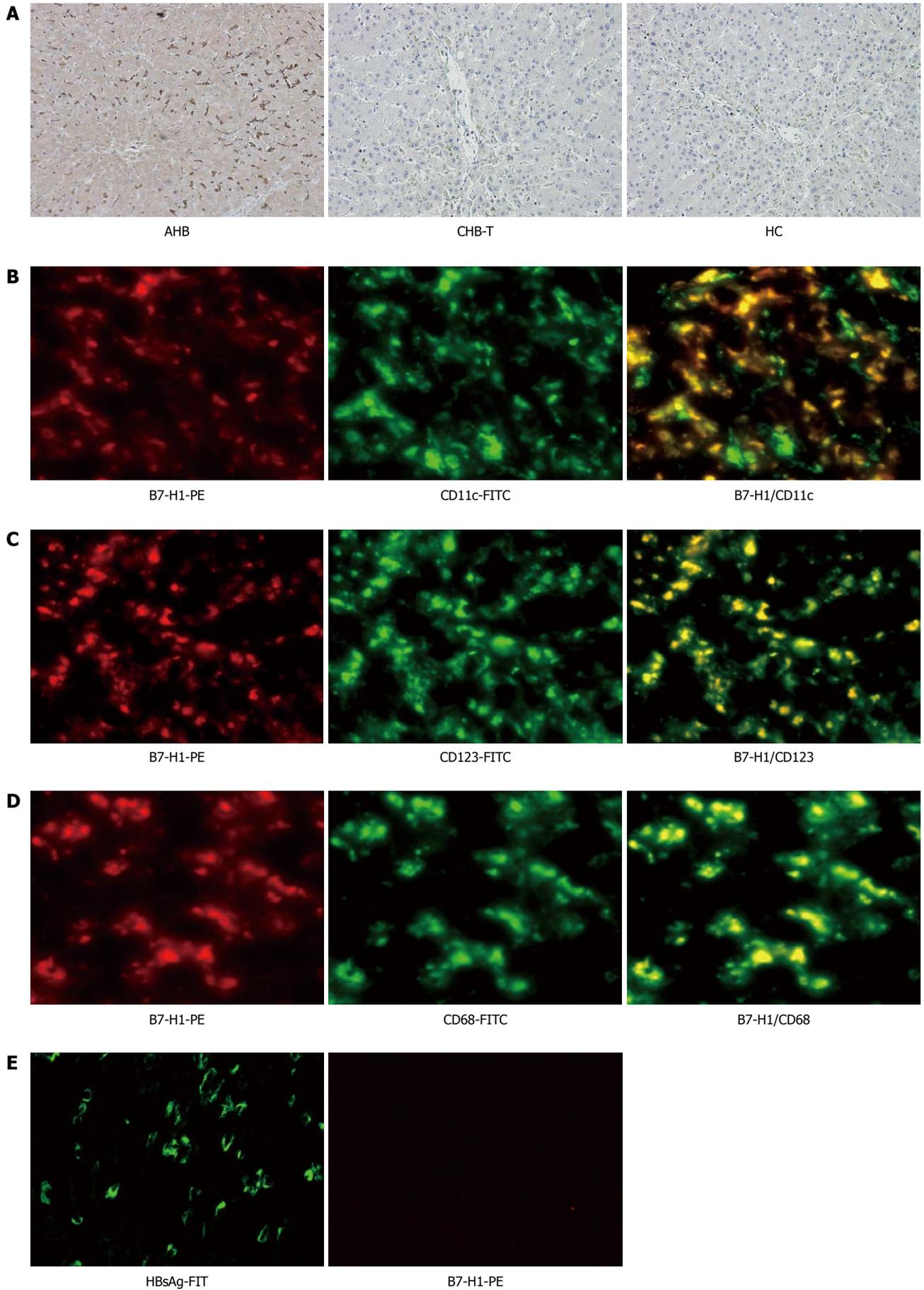
Human B7-H1 expression levels increased during the acute hepatitis phase and decreased with a reduction in inflammation

The correlation between B7-H1 expression and the degree of inflammation in hepatitis was analyzed by detecting B7-H1 expression on circulating mDCs during a follow-up period ranging from 1 mo to 6 mo. In AHB patients, the suppression of HBV replication after anti-viral treatment was accompanied by a decrease in serum HBsAg and ALT levels, and followed by a gradual decrease in B7-H1 expression on circulating mDCs (Figure 5A and B). Two or three mo after the ALT level returned to normal range, the percentage of B7-H1 positive cells decreased within CD11c positive cells from 21.3 ± 5.6 to 6.1 ± 1.4 (Figure 4C). B7-H1 expression levels, as assessed by mean fluorescence intensity (MFI), were also reduced (data not shown). The level of B7-H1 expression increased during the period of acute hepatitis and decreased when inflammation was reduced; supporting the conclusion that B7-H1 is an inflammatory marker. Notably, B7-H1 expression levels remained relatively high (13.2 ± 3.3) when ALT returned to normal range at 16 wk (Figure 5C). Two or three months after the return of serum ALT level to normal, B7-H1 expression further decreased to 6.1 ± 1.4 , approaching healthy control levels (Figure 5B and C). Longitudinal correlation analysis revealed a positive correlation between B7-H1 expression and serum ALT levels in AHB patients (Figure 5D). In CHB-T patients, although serum HBV-DNA and HBS-Ag load remained high, peripheral B7-H1 expression levels did not increase significantly (Figure 5C).

As mentioned above, chronic HBV infection had two different components, namely the CHB-T condition and the chronic active hepatitis status, which was characterized by repeated inflammation flare ups. Detection of peripheral B7-H1 expression in 3 chronic active hepatitis B patients during the inflammatory flare up phase with increasing serum ALT and HBV-DNA level showed that B7-H1 expression on circulating CD11c positive cells increased significantly in all three patients (Figure 6).

High HBsAg loads inhibited the upregulation of CD40 and human B7-H1 on mDCs

The above data showed that the intra and extra-hepatic HBs-Ag load remained persistently high in CHB-T subjects, while B7-H1 and CD40 expression were nearly absent. To further explore the relationship between HBsAg load and B7-H1 and CD40 expression, the effects of HBsAg towards B7-H1 and CD40 expression were analyzed *in vitro*. Population of mDCs with and without HBsAg pretreatment in PBMCs was determined by flow cytometry.



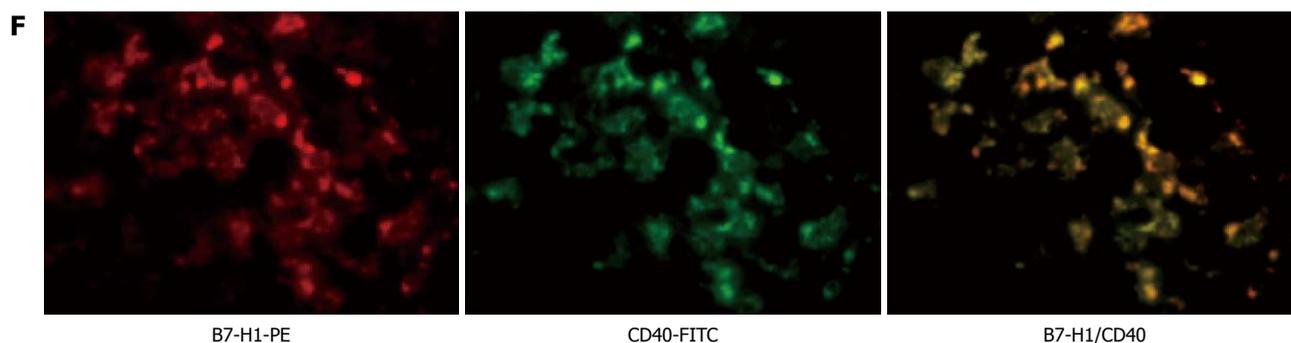


Figure 2 Analysis of intrahepatic human B7 homolog 1 expression among acute hepatitis B and chronic hepatitis B virus tolerance patients. A: Immunohistochemical staining for intrahepatic B7 homolog 1 (B7-H1)-positive cells in acute hepatitis B (AHB), chronic hepatitis B virus tolerance (CHB-T) and healthy controls (HC). Intra-hepatic B7-H1 expression was up-regulated significantly in AHB patients, but was almost absent in both CHB-T and HC subjects. Original magnification $\times 200$; B-D: Co-localization of B7-H1 with CD11c, CD123 and CD68 were shown by immunofluorescence double staining in liver biopsy specimens of AHB patients. B7-H1 (red) is co-localized with CD11c (green) positive mDCs (B), CD123 (green) positive pDCs (C) or CD68 (green) positive Kupffer cells (D). The 2-color merged panels were shown with co-localization visible in yellow. Original magnification $\times 200$; E: Hepatitis B surface antigen (HBsAg) and B7-H1 expression was detected by immunofluorescence stain in the liver tissue of CHB-T patients. Original magnification $\times 200$; F: Colocalization of B7-H1 with CD40 is showed by immunofluorescence double staining in liver biopsy specimens of AHB patients. B7-H1 (red) is colocalized with CD40 (green). The 2-color merged panels were shown with colocalization visible in yellow. Original magnification $\times 200$. PE: Phycoerythrin; FITC: Fluorescein isothiocyanate.

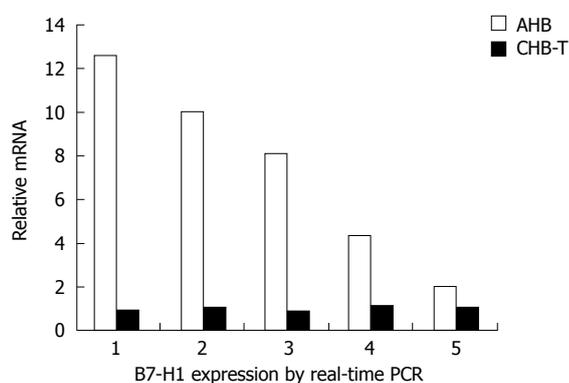


Figure 3 Quantitative real-time reverse transcription-polymerase chain reaction was performed in 5 acute hepatitis B and hepatitis B virus tolerance patients. Acute hepatitis B (AHB) and chronic hepatitis B virus tolerance (CHB-T) patients were matched freely. B7 homolog 1 (B7-H1) mRNA expression level was significantly higher in AHB than in CHB-T liver tissue ($n = 5$, $P < 0.01$). PCR: Polymerase chain reaction.

etry. The forward scatter (FSC) and side scatter (SSC) of mDCs with rHBsAg pretreatment were not altered significantly compared to those without rHBsAg pretreatment (Figure 7A). This result suggests that the vitality of mDCs with or without HBsAg pretreatment is similar.

The kinetics of CD40 and B7-H1 expression on poly (I:C)-stimulated mDCs with or without rHBs-Ag pretreatment were analyzed *in vitro*. The expression of B7-H1 and CD40 on mDCs was upregulated synchronously in response to poly (I:C) stimulation. The expression of CD40 was detected on the surface of mDCs at 1h and gradually increased to peak level at 24 h after the onset of stimulation. During the same period, B7-H1 also increased gradually, reaching the highest expression level after 24 h of stimulation. In addition, when rHBs-Ag was added during the period of DC maturation at a final concentration of $1 \mu\text{g}/\text{mL}$, the up-regulation of CD40 and B7-H1 was inhibited simultaneously (Figure

7B-D). The rate of CD40 and B7-H1 inhibition was calculated using the following equation: inhibition rate = $1 - (\text{MFI from mDCs with HBsAg pretreatment} / \text{MFI from mDCs without HBsAg pretreatment})$. It is noteworthy that the inhibition rate of CD40 was greater than that of B7-H1 (Figure 7E).

The T-cell stimulatory capacity of mDCs pretreated by HBsAg was impaired significantly

Since the inhibition rate of CD40 was greater than that of B7-H1, we further investigated whether the T-cell stimulatory capacity of mDCs pretreated by HBs-Ag was impaired. The mDC was stimulated by poly (I:C) for 20 h with or without HBs-Ag pretreatment, and co-cultured at 1:5 and 1:10 ratios with HLA-mismatched allogeneic T cells labeled with cytoplasmic dye CFSE. After 5 d of incubation, T-cell proliferation was assessed by the dilution of CFSE. In the allo-MLR system pretreated by HBsAg, with 1:5 and 1:10 responder cell-stimulator cell (R/S) ratios, approximately 25.4% or 12.0% of the responder T cells were CFSE^{dim} proliferating blasts, respectively (Figure 8A). In the allo-MLR system without HBsAg pretreatment, the percentage of CFSE^{dim} proliferating T cells was 85.1% in the 1:5 R/S ratio and 30.3% in the 1:10 R/S ratio, respectively (Figure 8B). The results showed that mDCs pretreated by HBsAg were less efficient at inducing T-cell proliferation at ratios of 1:5 and 1:10 compared with mDCs without HBsAg pretreatment.

Intracellular IFN- γ staining

In correlation with T-cell proliferation, the intracellular IFN- γ produced in these MLRs was measured after 5 d of co-culture. In the MLRs system at ratios of 1:5 and 1:10 R/S, without HBsAg pretreatment, the percentage of IFN- γ positive cells in CD8⁺ T cells was greater than $55.2\% \pm 5.3\%$ and $35.0\% \pm 5.1\%$ respectively. In the MLRs system, with HBsAg pretreatment, the percentage of IFN- γ positive cells in CD8⁺ T cells decreased to

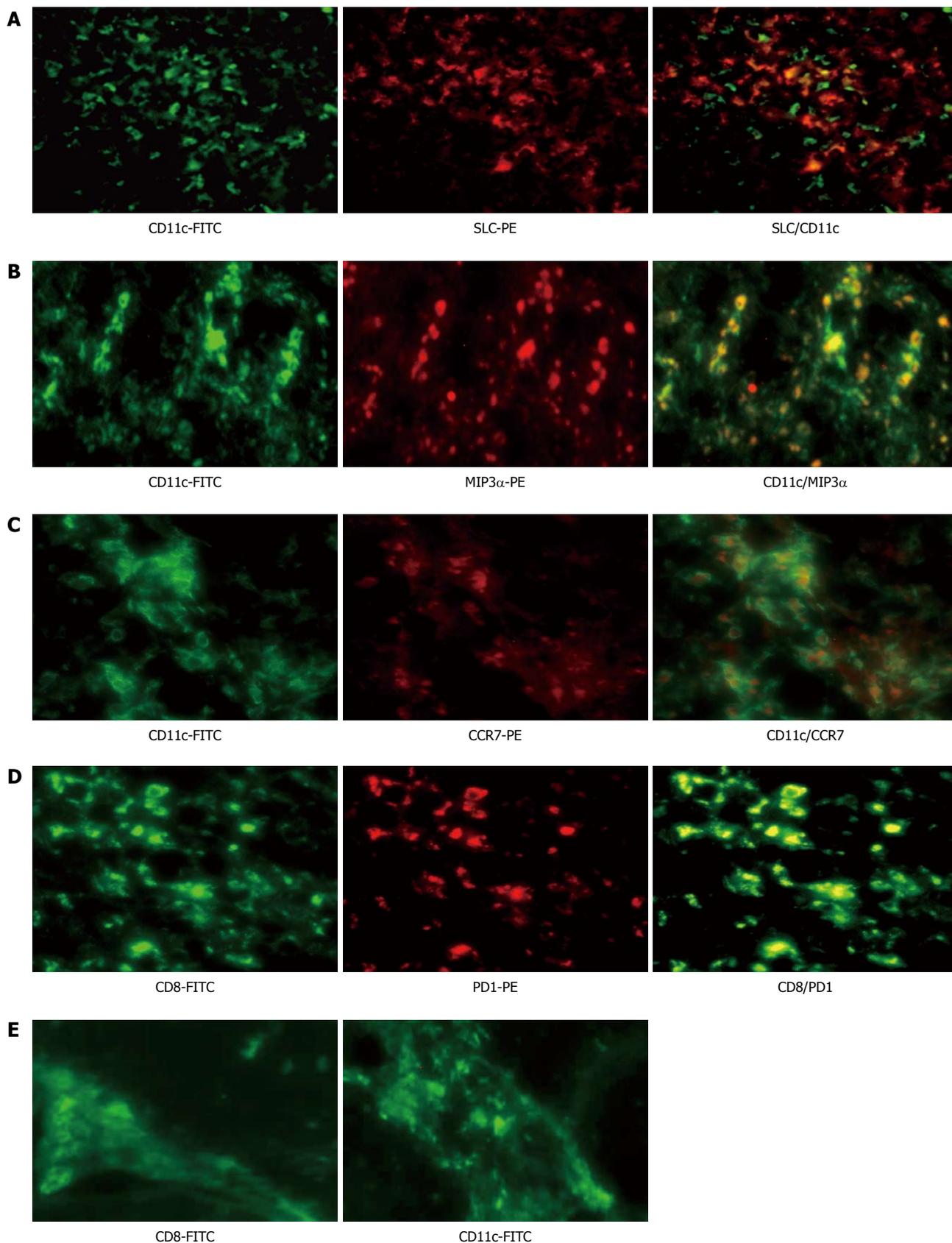


Figure 4 Localization of CD11c and programmed death 1 in liver tissue from acute hepatitis B and chronic hepatitis B virus tolerance patients. A-C: Colocalization of CD11c with SLC, MIP3 α and CCR7 by immunofluorescence double staining in liver biopsy specimens of acute hepatitis B (AHB) patients. CD11c (green) is co-localized with SLC (red) (A), MIP3 α (red) (B) and CCR7 (red) (C). The 2-color merged panels were shown with colocalization visible in yellow. Original magnification $\times 200$; D: Colocalization of CD8 with programmed death 1 (PD1) by immunofluorescence double staining in liver biopsy specimens of AHB patients. CD8 (green) is co-localized with PD1 (red). Original magnification $\times 200$; E: Inflammatory cells, such as CD8 and CD11c positive cells, can be observed mainly in fibrous septa in the liver tissue of chronic hepatitis B virus tolerance patients. FITC: Fluorescein isothiocyanate; PE: Phycoerythrin; SLC: Secondary lymphoid tissue chemokine; MIP3 α : Macrophage inflammatory protein 3 α ; CCR: Chemokine (C-C) receptor.

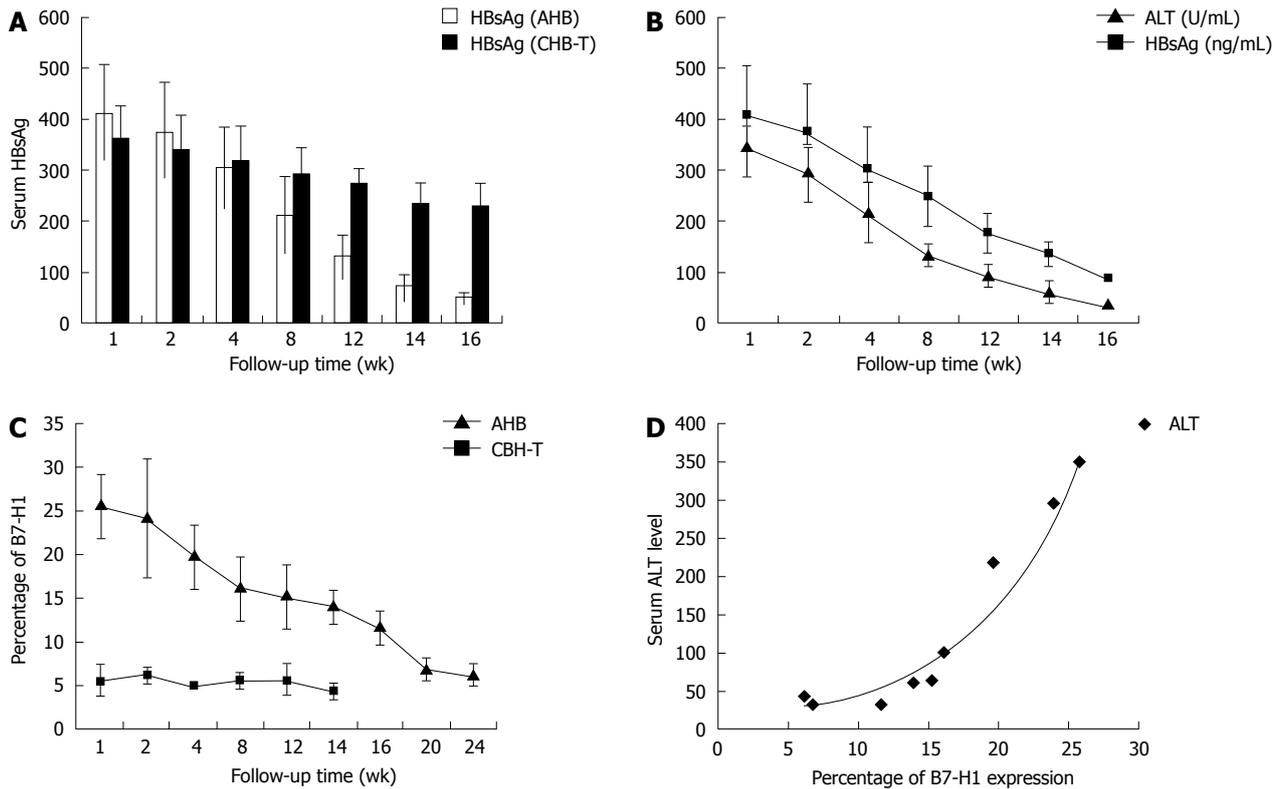


Figure 5 Longitudinal analysis of serum hepatitis B surface antigen, alanine aminotransferase levels and human B7 homolog 1 expression in acute hepatitis B and chronic hepatitis B virus tolerance patients. A: In acute hepatitis B (AHB) patients, serum hepatitis B surface antigen (HBsAg) levels decreased significantly from 404 ± 98.3 ng/mL at the 1st week to less than 30 ng/mL at the 16th week. In chronic hepatitis B virus tolerance (CHB-T) patients, serum HBsAg load remained more than 231 ± 38.6 ng/mL at the 16th week; B: In AHB patients, accompanied by a decrease in serum HBsAg level, serum alanine aminotransferase (ALT) level decreased significantly from 346.5 ± 62.3 at the 1st week to 41.8 ± 82 at the 16th week; C: In AHB patients, followed by a decrease in serum HBsAg and ALT level, the percentage of B7 homolog 1 (B7-H1) positive cells decreased significantly from 25.7 ± 4.0 at the 1st week to 6.3 ± 1.37 at the 24th week. In CHB-T patients, B7-H1 expression levels were not increased significantly during the follow-up period; D: In AHB patients, positive correlation between B7-H1 expression on circulating CD11c + DCs and serum ALT levels was revealed by longitudinal correlation analysis ($r = 0.902$).

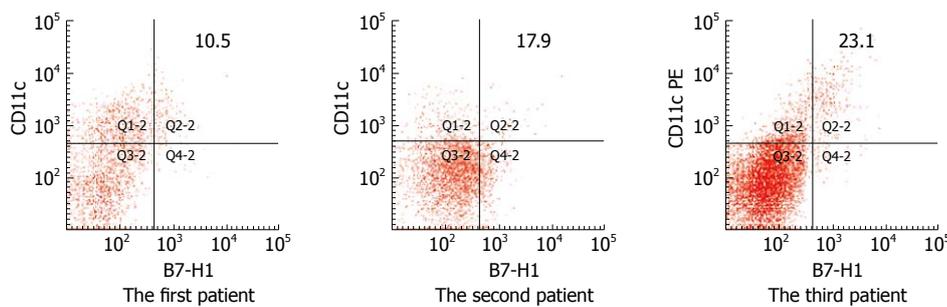


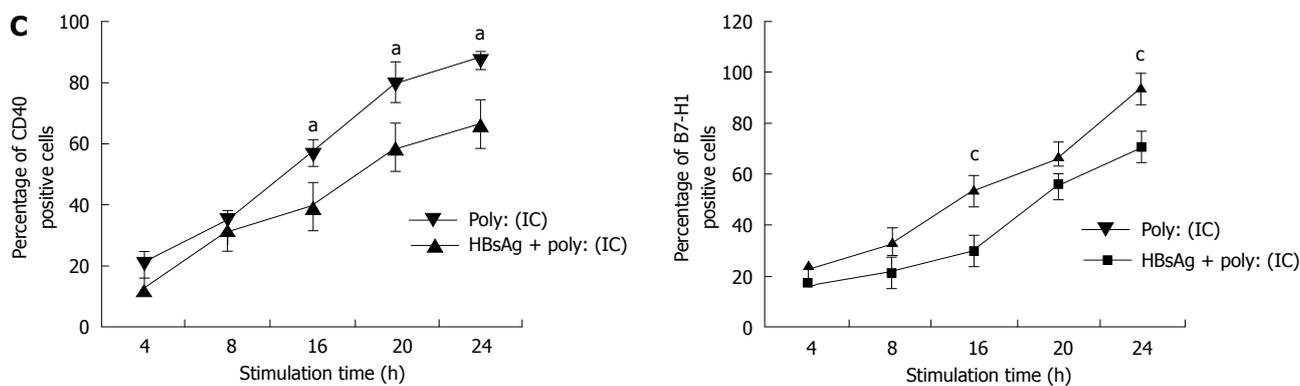
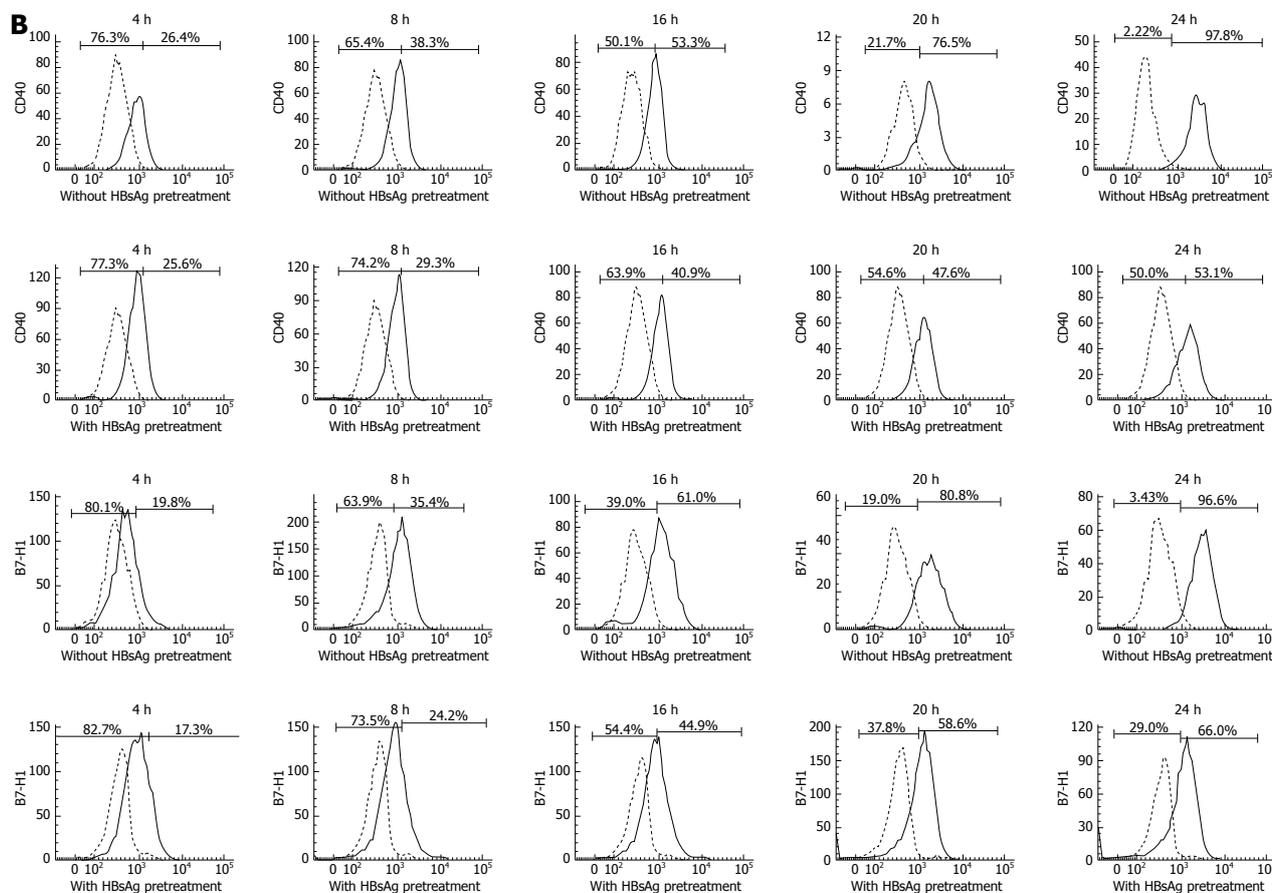
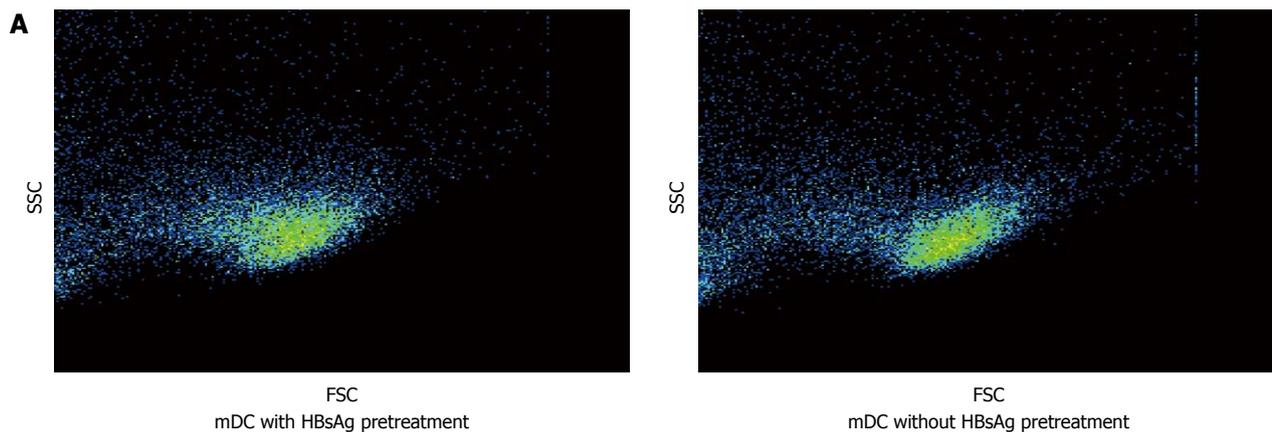
Figure 6 Human B7 homolog 1 expression on circulating CD11c positive cells in three chronic active hepatitis B patients during the inflammatory flare phase. The percentage of B7 homolog 1 (B7-H1) expression on circulating CD11c+ cells increased significantly in all three patients during the inflammatory flare phase. The numbers in the upper right quadrants indicate the percentage of B7-H1 positive CD11c+ dendritic cells. PE: Phycoerythrin.

$15.1\% \pm 3.1\%$ at a 1:5 R/S ratio and $7.3\% \pm 2.7\%$ at a 1:10 R/S ratio, respectively (Figure 9A and B). These results showed that IFN- γ production by CD8+ T cells was reduced significantly in the HBsAg pretreated MLRs system.

DISCUSSION

Recent studies have shown that functional defects in DCs may play a pivotal role in viral persistence during chronic HBV infection. However, the molecular mechanism by

which the impaired mDCs induce HBV-specific T cell immune tolerance remains elusive. Previous studies implicated increased B7-H1/PD-1 signaling in DC malfunction and viral-specific T-cell exhaustion in persistent HBV or HCV infections, which were associated with disease progression. *In vitro* blockade of B7-H1 signaling not only enhanced the mDC mediated allostimulatory capacity, but also up-regulated IL-12 production^[17-22]. Based on these data, B7-H1 was viewed as a signature of impairments in HBV-specific immune response.



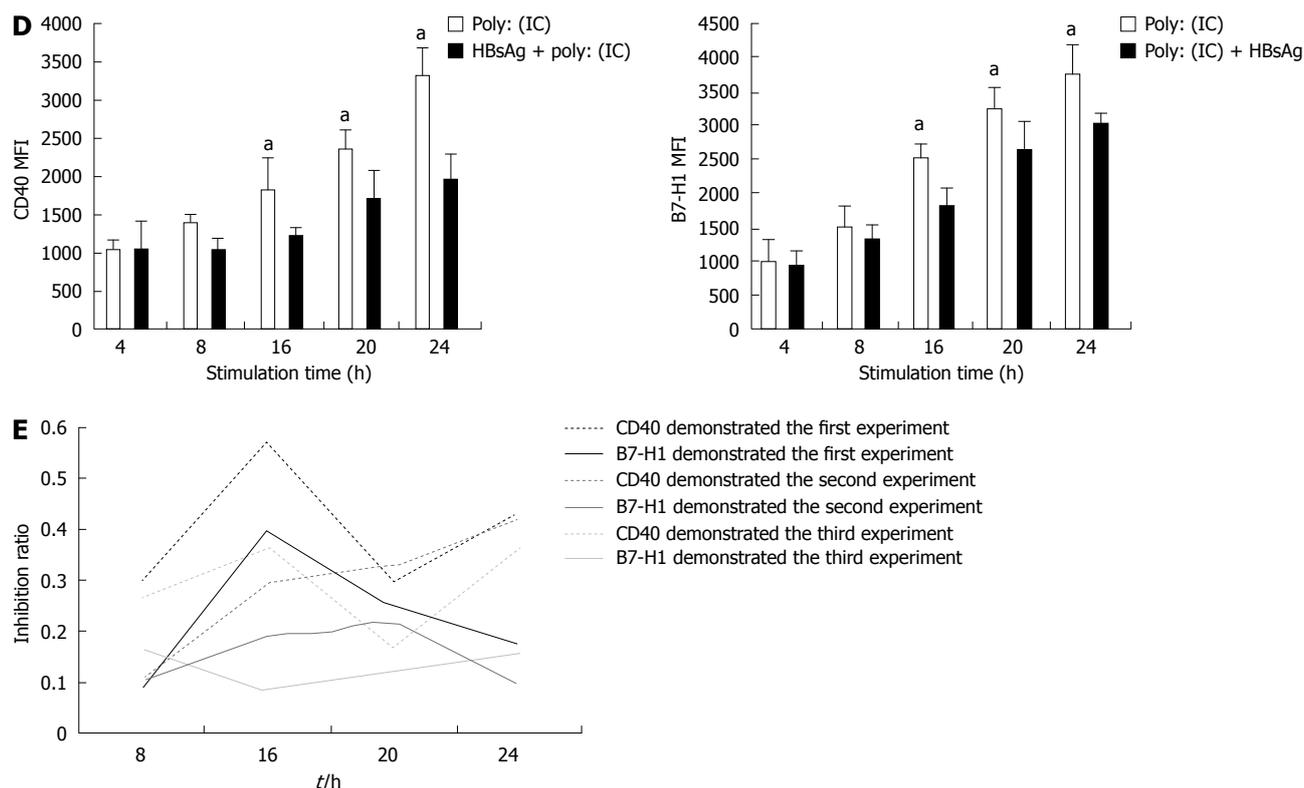


Figure 7 *In vitro* kinetic analysis of CD40 and human B7 homolog 1 expression on myeloid dendritic cells, with or without hepatitis B surface antigen pretreatment, stimulated by poly (I:C). A: The FSC and SSC of myeloid dendritic cells (mDCs) with r hepatitis B surface antigen (HBsAg) pretreatment were not altered significantly compared to those without rHBsAg pretreatment. This result suggests that the vitality of mDCs with or without HBsAg pretreatment is similar; B: Representative plots of CD40 and B7 homolog 1 (B7-H1) expression on mDCs with or without HBsAg pretreatment. Values in the upper right quadrant indicate the percentage of CD40 or B7-H1 positive cells; C: With HBsAg pretreatment, the percentage of CD40 (left) and B7-H1 (right) positive cells within mDCs were decreased significantly at 16 h, 20 h and 24 h time points (for CD40, $^aP < 0.05$ at 16 h, 20 h and 24 h time point; for B7-H1, $^aP < 0.05$ at 16 h and 24 h time point, $n = 10$); D: With HBsAg pretreatment, the MFI of CD40 (left) and B7-H1 (right) were decreased significantly at 16 h and 20 h time points (for CD40 and B7-H1, $^aP < 0.05$ at 16 h, 20 h and 24 h time point, $n = 10$); E: The inhibition rate was calculated using following equation: 1-MFI of CD40 or B7H1 with HBsAg pretreatment / MFI of CD40 or B7H1 without HBsAg pretreatment. The results showed that at 8 h, 16 h, 20 h and 24 h time point, the inhibition rate of CD40 was greater significantly than that of B7H1 ($P < 0.05$ at 8 h time point, $P < 0.01$ at 16 h time point, $P < 0.05$ at 20 h time point, $P < 0.01$ at 24 h time point). MFI: Mean fluorescence intensity; FSC: Forward scatter; SCC: Side scatter.

The present data indicate that B7-H1 is not a signature of HBV immune malfunction, but rather an inflammatory marker of acute hepatitis. Firstly, AIH patients, who did not possess viral etiology, exhibited the highest levels of B7-H1 expression on mDCs among the groups studied. Secondly, B7-H1 expression at protein and mRNA levels was more significant in AHB patients, who were characterized by high levels of serum ALT, than in CHB-T subjects. Thirdly, in AHB patients, despite significant upregulation of intra-hepatic B7-H1 expression, the inflammatory response in liver tissues was vigorous, while in the liver tissues from CHB-T subjects, markers of inflammation were not observed in liver lobe. Only a few inflammatory cells were located in fibrous septa, and B7-H1 molecules were mostly absent despite extensive HBsAg expression. Finally, longitudinal analysis revealed that the level of B7-H1 expression increased during the period of acute hepatitis and decreased when the inflammation was reduced.

Based on our clinical observations, chronic hepatitis B was divided into 2 different components, namely the immune tolerant HBV condition (characterized by active HBV-DNA replication, but with no serum ALT level upregulation) and the chronic active hepatitis condition

(which show repeated inflammation flare ups).

In the present study, B7-H1 expression was also analyzed on circulating mDCs in chronic active hepatitis subjects during the hepatitis flare-up period, and the results showed that B7-H1 expression on circulating mDCs was up-regulated to the same extent as in AHB subjects. These data further demonstrate that B7-H1 is a marker of hepatitis.

The current longitudinal analysis revealed that B7-H1 expression on circulating mDCs remained relatively high when serum ALT returned to normal range on week 16, but decreased to near normal level after serum ALT had remained within normal range for 2 mo or 3 mo, indicating a lag phase between the decrease of serum ALT and the return of B7-H1 expression to normal levels. If the blood sample was acquired within the lag phase, B7-H1 expression was higher than in HC.

In the liver tissue of AHB patients, the majority of B7-H1-positive cells co-expressed CD40, indicating that B7-H1 is always expressed on activated APCs. These results are in line with previous findings showing that B7-H1 was significantly increased by proinflammatory cytokines, including IFN- γ and tumor necrosis factor- α .

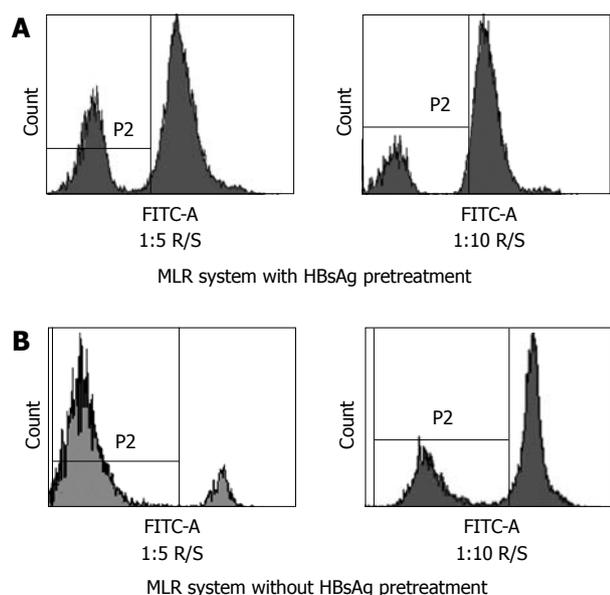


Figure 8 The analysis of the effects of hepatitis B surface antigen on T cell proliferation by carboxyfluorescein diacetate succinimidyl ester labeled mixed leucocyte reaction. A: Myeloid dendritic cells (mDCs) with hepatitis B surface antigen (HBsAg) pretreatment are weak stimulators in the mixed leucocyte reaction (MLR). Approximately 25.4% of T cells are carboxyfluorescein diacetate succinimidyl ester (CFSE)^{dim} in 1:5 R/S ratio and 12.0% in 1:10 R/S ratio respectively; B: mDCs without HBsAg pretreatment are potent stimulators in the MLR. Approximately 85.1% of the T cells are CFSE^{dim} in 1:5 R/S ratio and 30.3% in 1:10 R/S ratio respectively. FITC: Fluorescein isothiocyanate. R/S: Responder cell-stimulator cell.

(TNF- α)^[12,23]. The present results also show the presence of markers of inflammation such as infiltrating inflammatory cells and elevated chemokine expression in the liver tissue from AHB patients, with significant B7-H1 expression. On the other hand, in the liver lobes of CHB-T subjects, both B7-H1 molecules and other inflammatory characters were almost absent. Correlation analysis revealed a positive correlation between B7-H1 expression and liver damage, reflected by the levels of serum ALT, both cross-sectionally and longitudinally. Based on these data, we concluded that B7-H1 expression can be induced by inflammatory microenvironment and that the level of B7-H1 expression can serve as a marker of the degree of inflammation.

In vitro analysis showed that the expression of B7-H1 and CD40 on mDCs was upregulated synchronously in response to poly (I:C) stimulation. This result suggested that a delicate balance between the positive and negative co-stimulate molecules may exist in activated mDCs. The B7-H1/ PD1 interaction may be involved in weakening the positive stimulatory signal, limiting the immune response to avoid an extensive inflammatory response. This concept is in line with several studies supporting the conclusion that the B7-H1/PD-1 pathway may assist the liver in protecting itself from immune-mediated destruction^[24-28]. Zhang *et al.*^[23] reported that the delayed expression of B7-H1 or PD1 lead to fulminant hepatitis. In conclusion, the up-regulation of B7-H1 may contribute to maintaining immune response under the upper limit to

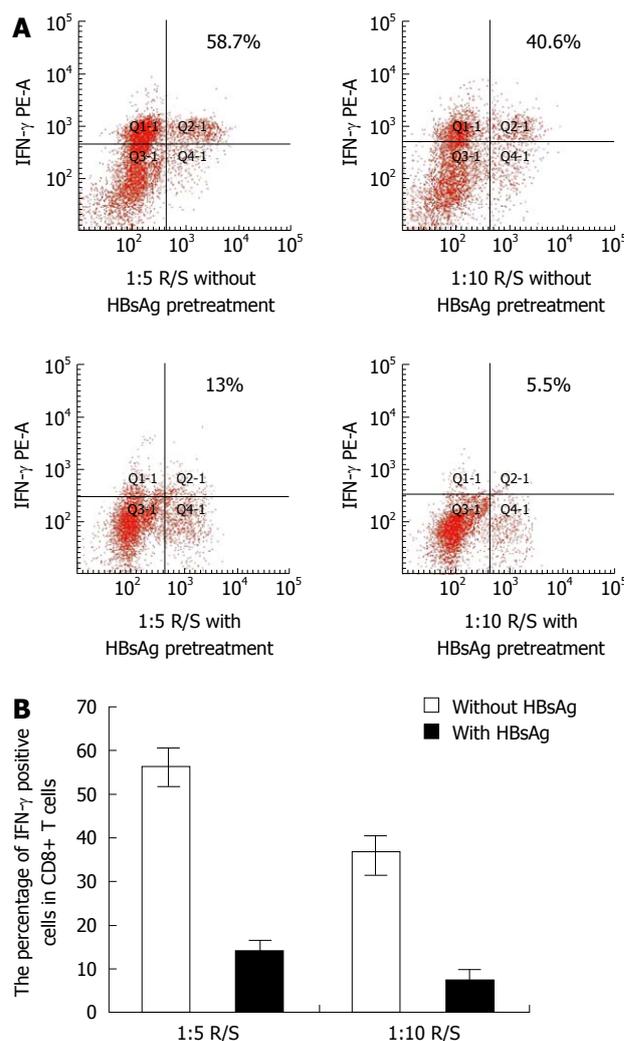


Figure 9 The analysis of the effects of hepatitis B surface antigen on intracellular interferon- γ production. A: Representative plots of intracellular interferon- γ (IFN- γ) produced by CD8+ T cells in mixed leucocyte reaction (MLRs) with or without hepatitis B surface antigen (HBsAg) pretreatment. Values in the upper right quadrant indicate the percentage of IFN- γ positive CD8+ T cells; B: In MLRs system at ratios of 1:5 and 1:10 R/S, IFN- γ production by CD8+T cells was reduced significantly by HBsAg pretreatment (at ratios of 1:5 and 1:10 R/S, $P < 0.001$, $n = 3$). R/S: Responder cell-stimulator cell.

avoid severe immune mediated liver damage during acute HBV infection. In CHB-T patients, immune responses were not elicited and B7-H1 expression was not elevated. These phenomena are consistent with the rule of immune regulation, which states “no activation, no inhibition”.

Previous data revealed that the HBV antigen has immunoregulatory abilities. Studies have reported that exposure to higher level of HBsAg load may lead to mDC dysfunction^[29,30]. Op den Brouw *et al.*^[10] and Loirat *et al.*^[31] reported that both HBsAg and HCsAg have the ability to suppress CD40 or HLA-DR expression and thus contribute to HBV or HCV persistence. Loirat *et al.*^[31] reported that recombinant HBsAg interacts with monocytes through the lipopolysaccharide (LPS) receptor CD14, resulting in diminished LPS-induced monocyte activation. HBsAg was shown to reduce LPS-induced TNF- α production through interference with the activation of

extracellular regulated protein kinases-1,2 and c-Jun NH₂-terminal kinase-1,2^[32,34]. These reports suggest that HBsAg and HBsAg have the ability to weaken the immune response.

The results of the present study revealed that HBsAg load was maintained at a high level in liver tissue as well as in peripheral blood from CHB-T subjects with no significant B7-H1 upregulation. Based on these previous data and the present results, we speculated that B7-H1 expression may also be inhibited by HBsAg, followed by CD40 suppression. To confirm this, the kinetic B7-H1 and CD40 expression in isolated mDCs stimulated by poly (I:C) with or without HBsAg pretreatment were analyzed. The results showed that the upregulation of CD40 and B7-H1 were significantly inhibited by HBsAg. Interestingly, HBsAg displayed a preferential inhibitory effect towards CD40 upregulation. The inhibition rate of CD40 was significantly greater than that of B7-H1, suggesting that HBsAg has the ability to regulate the balance between the CD40 and B7-H1.

Several previous studies have shown the activation of DCs after HBsAg impulsion, but either uric acid or cytokines were added in these investigations to active DCs^[35,36]. Horiike *et al.*^[36] reported that an alum adjuvant was needed in the HBsAg vaccine to active DCs during vaccine therapy. Proof of the low immunogenicity of HBsAg was shown in the research by Reignat *et al.*^[37] and Webster *et al.*^[38] which demonstrated that HBsAg specific CD8⁺ T cells are characterized by an HBV-tolerant phenotype.

Whether the preferential inhibition of CD40 resulted in the impairment of T cell proliferation by mDCs remains unclear. To elucidate this aspect, we compared the T cell stimulatory capacity of mDCs with or without HBsAg pretreatment by MLR. The results showed that T cell proliferation at 1:5 and 1:10 (R/S) ratio was significantly decreased in the HBsAg pretreated MLR system. In addition to T cells proliferation, we examined whether antiviral ability of T cells was affected. Because IFN- γ plays a key role in the control virus infection, the intracellular content of IFN- γ produced during these MLRs was analyzed by flow cytometry. We found that in the HBsAg-pretreated MLR system, IFN- γ production by CD8⁺ T cells decreased significantly. Only 15% of CD8⁺ T cells were IFN- γ positive, while more than 50% were IFN- γ positive in the MLR without HBsAg pretreatment. These results suggested that HBsAg has the ability to regulate the immune response by regulating the balance between CD40 and B7-H1, leading to a reduction in T-cell proliferation and IFN- γ production.

Our results indicated that B7-H1 is an inflammatory marker. It is induced by the inflammatory microenvironment and can serve as a marker of the degree of inflammation. The increase in B7-H1 expression correlated with the expression of positive co-stimulatory molecules to weaken the activation signal for T cells and generate a balance between co-inhibitory and co-stimulatory signal. HBsAg has the ability to tip this balance through preferential inhibition of CD40 expression, leading to a

reduction in T cell proliferation and IFN- γ production. In CHB-T subjects, maintenance of high HBsAg load may impair HBV immunity through the regulation of the balance between CD40 and B7-H1 expression.

Based on our results, we speculated that the outcome of hepatitis B depends on 2 factors. The first is the level of HBV antigen load and HBV replication. The second is the upper limit of the HBV specific immune response set by co-inhibitory molecules such as B7-H1. If the HBV antigen and HBV-DNA are eliminated successfully by the immune response under this upper limit, the hepatitis B patient should recover. The higher the upper limit, the better the chance of HBV elimination.

COMMENTS

Background

Patients with self-limited acute hepatitis B (AHB) can develop appropriate virus-specific immune responses, however these immune responses are insufficient to eliminate the virus in chronic hepatitis B patients and eventually lead to chronic hepatitis B virus (HBV) tolerance. Previous studies have shown that the programmed death 1 and human B7 homology 1 (PD1/B7-H1) interaction impairs HBV-specific immune response and facilitates HBV specific immune tolerance. HBV persistent infection was always attributed to the up-regulation of PD1 and B7-H1 expression. To date, the mechanisms underlying this defect of the HBV specific immune response have not been fully elucidated.

Research frontiers

Studies have indicated that the B7-H1/PD1 pathway plays a key role in myeloid dendritic cells (mDCs) dysfunction and T-cell exhaustion when these cells are exposed to high HBV or hepatitis C virus (HCV) antigen loads. The blockade of the PD1/B7-H1 interaction can restore the allostimulatory ability of mDCs and the function of HBV and HCV-specific CD8⁺ T cells with increased proliferation, cytotoxicity and cytokine production. Some studies have reported that the B7-H1 and PD1 expression were significantly upregulated in chronic hepatitis B patients. The specificity of this upregulation to HBV or HCV infection was determined by the lack of a significant increase in B7-H1 and PD1 in cytomegalovirus, Epstein-Barr virus and influenza A-infected patients. Based on these studies, relatively high levels of B7-H1/PD1 expression were viewed as the signature of impairments in the HBV- and HCV- specific immune response. Both HBV and HCV might exploit the B7-H1/PD1 pathway to facilitate persistent infection. On the other hand, recent studies revealed that B7-H1 and PD1 expression is significantly upregulated in the acute phase of hepatitis B. The upregulation of B7-H1 and PD1 expression protects tissue from severe immune mediated liver damage during AHB. These results are in line with reports showing that B7-H1 and PD1 expression is upregulated in liver nonparenchymal cells during the acute phase of inflammation to limit an over-vigorous inflammatory response.

Innovations and breakthroughs

In this article, the authors reached the conclusion that co-inhibitory molecules, such as B7-H1, are always upregulated during AHB. This phenomenon suggests that co-inhibitory molecules set the upper-limit to immune response to avoid severe liver damage by an over-vigorous immune response. If HBV antigen and HBV-DNA are successfully eliminated by the immune response under this upper limit, the hepatitis B patient should recover. The higher the upper limit, the better the chance of HBV elimination.

Applications

Previous studies attributed malfunction of HBV specific immune response to higher expression levels of PD1 and B7-H1. Based on our data, the authors reached the conclusion that B7-H1 is always upregulated during AHB. Increasing co-inhibitory signals, such as PD1/B7-H1, set the upper-limit to the immune response. If HBV were eliminated under the upper limit of immune response, the hepatitis B patient should recover. This study provides new insight into a co-inhibitory signal pathway function during the clinical course of AHB.

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Due to simultaneous expression of CD40 and B7-H1, the authors supposed that an upper threshold of the immune response set by co-inhibitory molecules

may exist. HBV antigens should be eliminated efficiently before the immune response reached the upper threshold level, otherwise acute HBV infection may turn into a chronic infectious state. The design of this study is reasonable; the results and conclusion of this paper are reliable; and the notion about an "upper threshold of HBV specific immune response" is somewhat attractive.

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Lentiviral vector-mediated down-regulation of IL-17A receptor in hepatic stellate cells results in decreased secretion of IL-6

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Abstract

AIM: To investigate the mechanism of interleukin (IL)-6 secretion through blocking the IL-17A/IL-17A receptor (IL-17RA) signaling pathway with a short hairpin RNA (shRNA) in hepatic stellate cells (HSCs) *in vitro*.

METHODS: HSCs were derived from the livers of adult male Sprague-Dawley rats. IL-6 expression was evaluated using real-time quantitative polymerase chain reaction and enzyme linked immunosorbent assay. The phosphorylation activity of p38 mitogen activated protein kinases (MAPK) and extracellular regulated protein kinases (ERK) 1/2 upon induction by IL-17A and suppression by IL-17RA shRNA were examined using Western blotting.

RESULTS: IL-6 expression induced by IL-17A was significantly increased compared to control in HSCs ($P < 0.01$ in a dose-dependent manner). Suppression of IL-17RA using lentiviral-mediated shRNA inhibited IL-6 expression induced by IL-17A compared to group with only IL-17A treatment (1.44 ± 0.17 vs 4.07 ± 0.43 , $P < 0.01$). IL-17A induced rapid phosphorylation of p38 MAPK and ERK1/2 after 5 min exposure, and showed the strongest levels of phosphorylation of p38 MAPK and ERK1/2 at 15 min in IL-17A-treated HSCs. IL-6 mRNA expression induced by IL-17A (100 ng/mL) for 3 h exposure was inhibited by preincubation with specific inhibitors of p38 MAPK (SB-203580) and ERK1/2 (PD-98059) compared to groups without inhibitors preincubation (1.67 ± 0.24 , 2.01 ± 0.10 vs 4.08 ± 0.59 , $P < 0.01$). Moreover, lentiviral-mediated IL-17RA shRNA 1 inhibited IL-17A-induced IL-6 mRNA expression compared to random shRNA in HSCs (1.44 ± 0.17 vs 3.98 ± 0.68 , $P < 0.01$). Lentiviral-mediated IL-17RA shRNA 1 inhibited phosphorylation of p38 MAPK and ERK1/2 induced by 15 min IL-17A (100 ng/mL) exposure.

CONCLUSION: Down-regulation of the IL-17RA receptor by shRNA decreased IL-6 expression induced by IL-17A *via* p38 MAPK and ERK1/2 phosphorylation in HSCs. Suppression of IL-17RA expression may be a strategy to reduce the inflammatory response induced by IL-17A in the liver.

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Key words: Interleukin 17A; Interleukin 6; Hepatic stellate cells; Liver fibrosis

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INTRODUCTION

Hepatic stellate cells (HSCs), also known as fat-storing cells, are a major cell type involved in liver fibrosis. Activation and proliferation of HSCs are associated with liver injury^[1]. Activated HSCs accumulate excess extracellular matrix and produce a variety of pro-inflammatory cytokines, including macrophage inflammatory protein-2, monocyte chemo-attractant protein-1, interleukin (IL)-6, IL-8, and transforming growth factor- β 1 (TGF- β 1)^[2-3]. These pro-inflammatory cytokines can eventually lead to liver fibrosis.

IL-17A is the founding member of the IL-17 cytokine family, which now includes six major isoforms, IL-17A, -B, -C, -D, -E, and -F^[4]. The original "IL-17" has been designated IL-17A and is considered a T cell (Th17 cells)-specific cytokine^[5]. The IL-17A receptor (IL-17RA) is expressed on many cell types in the human body^[6]. IL-17A exerts pro-inflammatory, pro-apoptotic, and pro-mitogenic effects *via* binding to IL-17RA^[6,7].

In this study, we constructed a highly efficient lentiviral short hairpin RNA (shRNA) targeting IL-17RA to study the level of IL-6 secretion in the absence of IL-17RA in activated HSCs and the underlying mechanism(s). Our results showed that the silencing effect of IL-17RA shRNA eliminated IL-6 secretion in activated HSCs. We suggest that secretion of IL-6 involves the mitogen activated protein kinases (MAPK) signaling pathway through phosphorylation of p38 MAPK and extracellular regulated protein kinases (ERK) 1/2. Our finding may provide a novel interventional therapy against hepatic inflammatory responses and hepatic fibrosis.

MATERIALS AND METHODS

Reagents

Recombinant murine IL-17A was purchased from Pe-protech (Princeton Business Park, NJ). Anti-IL-17R (sc-1902, dilution factor 1:200) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PD-98509 (inhibitor of MEK1) and SB-203580 (inhibitor of p38 MAPK) were obtained from Sigma-Aldrich (Saint Louis, MO). The p38 MAPK antibody (dilution factor 1:200), phospho-p38 MAPK rabbit mAb (dilution factor 1:200), ERK1/2 MAPK rabbit mAb (dilution factor 1:250), and phospho-ERK1/2 MAPK rabbit mAb (dilution factor 1:250) were obtained from Cell Signaling Technology (Beverly, MA).

shRNA design and plasmid constructs

Candidate sequences targeting rat IL-17RA mRNA (GenBank Accession NM 001107883) were designed using the Dharmacon siDESIGN Center procedure. Two complementary single-strand oligonucleotides containing the target sequences were synthesized chemically and annealed. The double-stranded oligonucleotides were inserted between *Age*I and *Eco*RI restriction sites in the pGCL-green fluorescent protein (GFP) small interfering RNA (siRNA) vector that contains a cytomegaviruses-driven enhanced green fluorescent protein reporter gene. The ligated plasmid was transformed into *Escherichia coli* DH5 α competent cells for pGCSIL/IL-17RA shRNA plasmid amplification.

shRNA lentivirus transduction

Plasmids containing the IL-17RA shRNA lentivirus were transfected into 293T cells using the ViraPower packaging mix (pGCSIL/IL-17RA shRNA plasmids, pHelper 1.0, and pHelper 2.0) and Lipofectamine 2000 (Invitrogen). After 48 h, the harvested viral supernatant was used to infect HSCs at a multiplicity of infection of 10 for 24 h. Cells with the GFP label were harvested after 48 h, and total RNA was extracted. The interference efficiency of IL-17RA shRNA was determined using real-time quantitative polymerase chain reaction (qPCR), and the most efficient silencing sequence of IL-17RA shRNA was selected for subsequent studies.

Isolation and culture of rat HSCs

Adult male Sprague-Dawley rats (body weight, 400-500 g) were used for HSC isolation as described previously^[8]. The liver tissues were digested with collagenase IV (0.5 g/L) and deoxyribonuclease I (0.03 g/L) before fractionation on a discontinuous gradient of iodixanol. HSCs were harvested from the 11.5% medium interface, washed, and seeded in tissue culture plates. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, United States) with 10% fetal bovine serum (Sijiqing Bio. Co. Hangzhou, China), 100 U/mL penicillin, and 100 μ g/mL streptomycin. Culture medium was changed every third day. All experiments were performed with cells from passage numbers 3-6.

Identification of primary HSCs and activated HSCs

The harvested primary HSCs were studied at days 1, 2, and 5 after isolation. Primary d1 HSCs showed intrinsic fluorescence of lipid droplet and desmin expression (Boster, Wuhan, China) under a fluorescence microscope. Nuclei cells were stained with DAPI. After the first sub-culture passage, activated HSC purity was assessed using α -smooth muscle actin (α -SMA, dilution factor 1:100; Boster) and immunocytochemical staining.

IL-6 secretion

Cells cultured in 6-well plates were exposed to different concentrations of IL-17A for 24 h. The amount of IL-6

Table 1 Interleukin-17A receptor short hairpin RNA and random short hairpin RNA sequences

	Target sequence	Double strand DNA oligo sequence
IL-17RA shRNA 1	CAAGTCCAAGACCATCTTA	5'-ccggcaCAAGTCCAAGACCATCTTAttcaagagaTAAGATGGTCTTGGACTTGtgttttg-3' 5'-aattcaaaaacaCAAGTCCAAGACCATCTTAtctcttgaaTAAGATGGTCTTGGACTTGtg-3'
IL-17RA shRNA 2	CCAGCGATCCAATGTCACA	5'-ccggcaCCAGCGATCCAATGTCACAttcaagagaITGTGACATTGGATCGCTGGtgttttg-3' 5'-aattcaaaaacaCCAGCGATCCAATGTCACAtctcttgaaITGTGACATTGGATCGCTGGtg-3'
IL-17RA shRNA 3	CAGCAGCCATGAACATGAT	5'-ccggcaCAGCAGCCATGAACATGATtcaagagaATCATGTTTCATGGCTGCTGtgttttg-3' 5'-aattcaaaaacaCAGCAGCCATGAACATGATtctcttgaaATCATGTTTCATGGCTGCTGtg-3'
IL-17RA shRNA 4	GGAAGAAAGTGGAGTGGTA	5'-ccgggaGGAAGAAAGTGGAGTGGTAttcaagagaTACCACTCCACTTTCTTCtcttttg-3' 5'-aattcaaaaagaGGAAGAAAGTGGAGTGGTAtctcttgaaTACCACTCCACTTTCTTCtc-3'
Random shRNA	TTCTCCGAACGTGTCACGT	5'-ccggTTCTCCGAACGTGTCACGTtcaagagaACGTGACACGTTCCGGAGAAAttttg-3' 5'-aattcaaaaTTCTCCGAACGTGTCACGTtctcttgaaACGTGACACGTTCCGGAGAA-3'

shRNA: Short hairpin RNA; IL-17RA: Interleukin-17A receptor.

in supernatants was determined using enzyme linked immunosorbent assay kits purchased from R and D Systems (Minneapolis, MN).

Western blotting analysis

Cells exposed to IL-17A in the presence or absence of inhibitors for the indicated time period were extracted with lysis buffer containing a phosphatase inhibitor cocktail and a protease inhibitor cocktail. The protein concentration was measured using the Bradford assay. Proteins from each sample were loaded equally and separated on a 6% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. Proteins were electrophoretically transferred onto polyvinylidene fluoride membranes, which were then incubated with primary antibodies at 4 °C overnight. On the following day, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at 37 °C for 2 h and then signals were detected using chemiluminescence (ECL Plus, GE Healthcare). Glyceraldehyde-3-phosphate dehydrogenase [(GAPDH); dilution factor 1:2000, Santa Cruz Biotechnology] was used as a loading control.

RNA extraction and gene expression analysis

Real-time qPCR was used to examine the expression of IL-17RA and IL-6 in HSCs. Total RNAs were isolated from HSCs using the Trizol reagent following the manufacturer's protocol and reverse-transcribed using a cDNA synthesis kit (Promega). SYBR Green detection was used and the values were normalized using GAPDH. Real-time qPCR was performed with a DNA Engine (ABi 7500) using SYBR GREENER qPCR UNIVERSAL. Primer sequences were: IL-17RA (forward) 5'-TG-GCGGTTCTCCTTCAGTC-3' and IL-17RA (reverse) 5'-CGGTGTAGTCATCTTCATCTCC-3', IL-6 (forward) 5'-CGTTTCTACCTGGAGTTTGTG-3' and IL-6 (reverse) 5'-ATTAGGAGAGCATTGGAAGTTGG-3', and GAPDH (forward) 5'-TTCAACGGCACAGT-CAAGG-3' and GAPDH (reverse) 5'-CTCAGCACCAG-CATCACC-3'. Relative expression levels of each primer set were normalized to GAPDH expression.

Statistical analysis

Data were expressed as mean \pm SD. The statistical signifi-

cance of changes was determined using the *t*-test. *P* values < 0.05 were considered to indicate statistical significance.

RESULTS

IL-17RA shRNA lentiviral construction and transduction

The sequences of IL-17RA shRNAs 1, 2, 3, and 4 and random shRNA are shown in Table 1. The recombinant plasmid of pGCSIL/IL-17RA shRNA 1 was confirmed by sequence analysis (Figure 1A) and titers were approximately 1×10^9 TU/mL. The interference efficiency of IL-17RA shRNAs 1, 2, 3, and 4 are shown in Figure 1B. The relative expression of IL-17RA mRNA for IL-17RA shRNAs 1, 2, 3, and 4 were 0.253 ± 0.011 , 0.643 ± 0.022 , 0.673 ± 0.051 , and 0.444 ± 0.043 , respectively. IL-17RA shRNA 1 exhibited a significant silencing effect, with 74.7% interference efficiency. Figure 1C shows HSC morphology with successfully transduced GFP-labeled IL-17RA shRNA 1 using a fluorescence or light microscope ($\times 200$) after 72 h.

Isolation and culture of rat HSCs

Primary HSC culture after isolation on days 1, 2, and 5 are shown in Figure 2A. The cultured HSCs were assessed for intrinsic fluorescence of lipid droplet and desmin expression (Boster; Figure 2B). The purity of activated HSCs was confirmed using immunocytochemical staining for α -smooth muscle actin (Boster; Figure 2C); the purity reached > 98%.

IL-17A induces IL-6 expression

HSCs were incubated with increasing concentrations of IL-17A for 24 h, and the levels of IL-6 secretion in the supernatant were measured by ELISA. As shown in Figure 3A, IL-17A-induced IL-6 secretion increased in a dose-dependent manner. The expression level of IL-6 mRNA by IL-17A was quantified using real-time qPCR (Figure 3B). A rapid increase in IL-17A-induced IL-6 mRNA expression reached a maximum level at 3 h of IL-17A exposure. However, the level of IL-6 mRNA expression decreased gradually after 3 h exposure.

IL-17A induces activation of MAPKs

In various cells, the MAPK family has been shown to

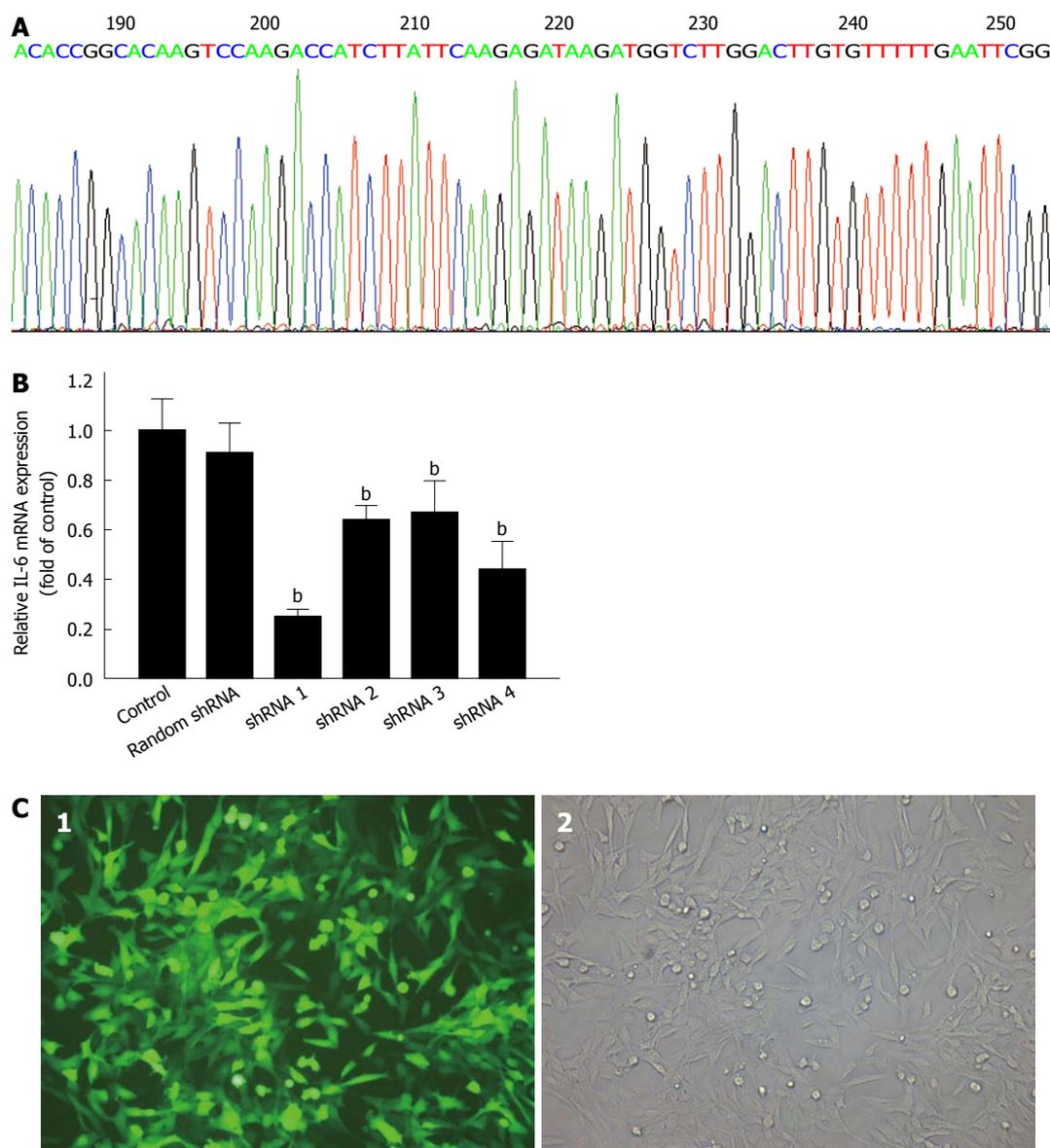


Figure 1 Short hairpin RNA design, interference efficiency assay, and lentivirus transduction. A: DNA sequence analysis of pGCSIL/Interleukin-17A receptor (IL-17RA) short hairpin RNA (shRNA) 1; B: Lentiviral-mediated IL-17RA shRNA 1, 2, 3, and 4 (shRNA 1, 2, 3, and 4) inhibited IL-17RA mRNA expression in hepatic stellate cells (HSCs). HSCs infected with lentivirus at a multiplicity of infection of 10 for 72 h, and IL-17RA mRNA expression was quantified by polymerase chain reaction. IL-17RA shRNA 1 was the most efficient silencing tool for IL-17RA. ^b $P < 0.01$ vs control; C: Cell morphology of HSCs under fluorescence (1) and light (2) microscope after 72 h transduction.

play an important role in regulating gene expression in response to inflammatory mediators^[7]. To investigate whether IL-17A induced activation of p38 MAPK and ERK1/2 in HSCs, phosphorylation of p38 MAPK and ERK1/2 in HSCs were evaluated after IL-17A stimulation. IL-17A induced rapid phosphorylation of p38 MAPK and ERK1/2 after 5 min, and showed the strongest levels of phosphorylation of p38 MAPK and ERK1/2 at 15 min (Figure 3C). However, IL-17A did not affect the expression of total p38 MAPK or ERK1/2. This result indicated that IL-17A enhanced p38 MAPK and ERK1/2 phosphorylation in HSCs.

MAPK inhibitors suppress IL-6 expression

To further assess the phosphorylation of p38 and ERK1/2

induced by IL-17A, two specific inhibitors, SB-203580 (p38 MAPK) and PD-98059 (ERK1/2), were used. As shown in Figure 3D, SB-203580 inhibited p38 MAPK phosphorylation and PD-98059 blocked phosphorylation of ERK1/2 induced by IL-17A in HSCs. SB-203580 and PD-98059 were used to further evaluate the roles of p38 MAPK and ERK1/2 in IL-6 mRNA expression induced by IL-17A in HSCs. Both inhibitors reduced IL-17A-induced IL-6 mRNA expression significantly (Figure 3E).

shRNA suppresses IL-17RA and IL-6 expression

Western blotting and real-time qPCR were performed to study the silencing effect of lentiviral-mediated shRNA on IL-17RA-induced IL-6 expression in HSCs. The protein levels of IL-17RA in shRNA 1-treated HSCs were

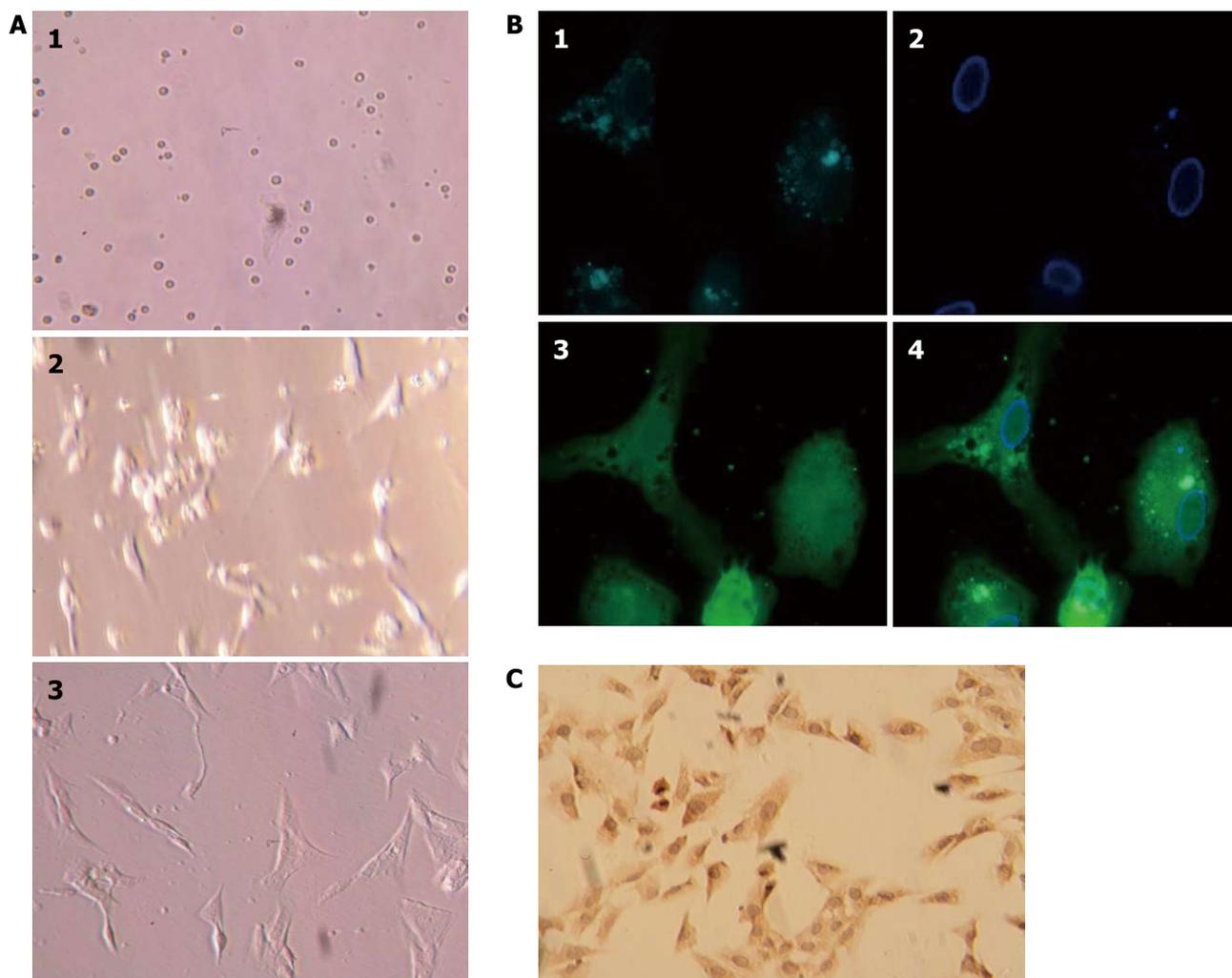


Figure 2 Isolation, culture, and identification of primary rat hepatic stellate cells. A: Primary rat hepatic stellate cells (HSCs) on days 1, 2, and 5 after isolation and plated in culture; B: HSCs isolated from Sprague-Dawley rats showed characteristic multiple lipid droplets with a rapid fade of the blue-green autofluorescence at 328 nm. 1: Lipid droplets; 2: Cell nucleus; 3: Cell body. 4: Merged images; C: HSC culture after the first passage and stained with anti- α -smooth muscle actin antibody.

reduced, compared with random shRNA and control (Figure 4A). HSCs treated with IL-17A alone or IL-17A with random shRNA showed increased IL-6 mRNA expression, whereas, IL-6 mRNA expression in HSCs pretreated with IL-17RA shRNA 1 was decreased significantly (Figure 4B).

shRNA suppresses phosphorylation of p38 and ERK1/2

IL-17A induced IL-6 expression *via* p38 MAPK and ERK1/2 phosphorylation. In Figure 4C, we show that HSCs treated with IL-17A transduced with shRNA 1 exhibited reduced protein levels of phosphorylation of p38 MAPK and ERK1/2, compared with IL-17A alone and IL-17A transduced with random shRNA group. Phosphorylation of p38 MAPK and ERK1/2 was blocked in the group treated with lentiviral-mediated IL-17RA shRNA 1, whereas there was almost no change in the IL-17A alone or IL-17A transduced with random shRNA groups.

DISCUSSION

IL-17A is a pro-inflammatory cytokine secreted by a subset of T helper cells, named Th17 cells. IL-17A signals exert miscellaneous effects through binding to the IL-17A receptor, which is expressed on a variety of cells and tissues^[9,10]. The IL-17A pathway plays important roles in the human inflammatory and autoimmune diseases and tumor progression^[11,12]. Clinical studies have shown that high levels of IL-17A and other cytokines related to the IL-17A pathway are seen in sera or tissues of patients with diseases such as psoriasis, multiple sclerosis, systemic sclerosis, ankylosing spondylitis, and juvenile idiopathic arthritis^[13-17]. The IL-17RA is found on hepatocytes, Kupffer cells, HSCs, biliary epithelial cells, and sinusoidal endothelial cells^[18]. Thus, IL-17A function in liver diseases has attracted much attention. Previous studies indicated that IL-17A is elevated in various liver diseases, including liver autoimmunity and inflammatory diseases, alcoholic liver disease (ALD), and hepatocellular carcinoma^[19].

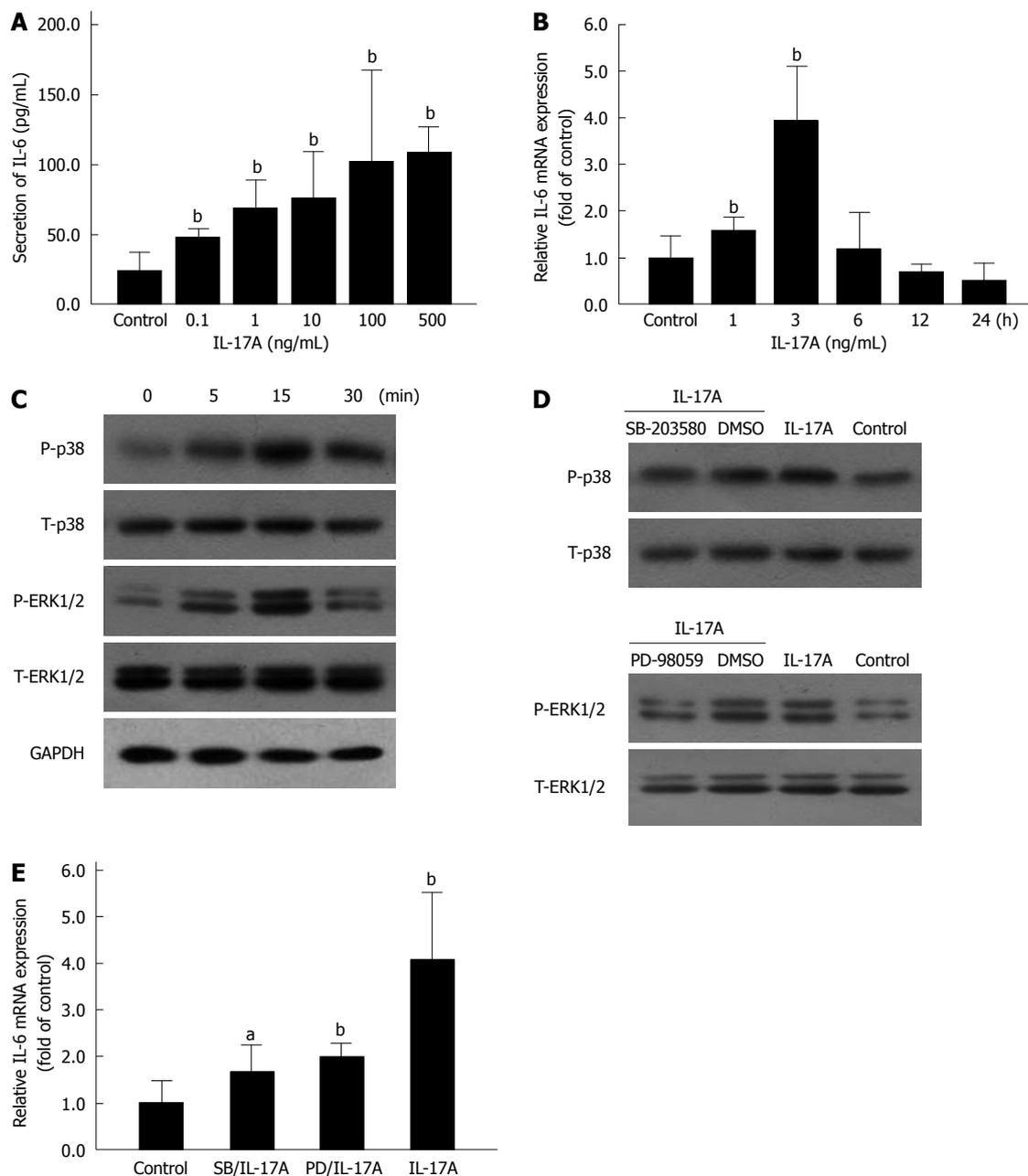


Figure 3 Mitogen activated protein kinases pathway involved in interleukin 17A induced interleukin 6 expression. A: Secretion of interleukin (IL)-6 in hepatic stellate cells (HSCs) induced by interleukin 17A (IL-17A) was determined using enzyme-linked immunosorbent assay. ^b*P* < 0.01 vs control; B: IL-6 mRNA expression in HSCs induced by IL-17A (100 ng/mL) was measured by polymerase chain reaction. ^b*P* < 0.01 vs control; C: Phosphorylation of p38 MAPK and ERK1/2 induced by IL-17A (100 ng/mL) in HSCs was detected using Western blotting; D: Phosphorylation of p38 MAPK and ERK1/2 induced by IL-17A (100 ng/mL) for 15 min was blocked by preincubation with MAPKs inhibitors, SB-203580 (1 μ mol/L in DMSO, 30 min) and PD-98059 (10 μ mol/L in DMSO, 1 h); E: IL-6 mRNA expression induced by IL-17A (100 ng/mL) for 3 h exposure was inhibited by preincubation with SB-203580 (SB) and PD-98059 (PD) for the same time period as above. ^a*P* < 0.05, ^b*P* < 0.01 vs control. T: Total; P: Phosphorylation; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; MAPK: Mitogen activated protein kinases; ERK: Extracellular regulated protein kinases.

Studies based on acute hepatic injury (AHI) and hepatitis C virus (HCV) showed that patients with AHI and HCV have significantly higher serum IL-17A levels than controls^[20-22]. Furthermore, percentages of circulating Th17 cells and Th17-associated cytokines, including IL-17A, IL-6, and IL-23, were increased markedly in peripheral blood and in liver tissues of chronic hepatitis B patients compared with controls^[23,24]. Recent studies indicate that patients with ALD had significantly higher plasma levels of IL-17A compared with healthy subjects. Moreover, the

number of liver Th17 cells was correlated positively with hepatocellular damage and the severity of the disease in a cohort study of patients with ALD^[25].

HSCs are the major source of extracellular matrix in liver fibrosis^[26] and participate in modulating liver inflammation during liver fibrogenesis^[27]. IL-17RA is expressed on HSCs, and thus blockade of IL-17A/IL-17RA signal transduction in HSCs may be a strategy to interfere with hepatic inflammatory processes in chronic liver diseases. In our study, we sought to investigate the secretion of

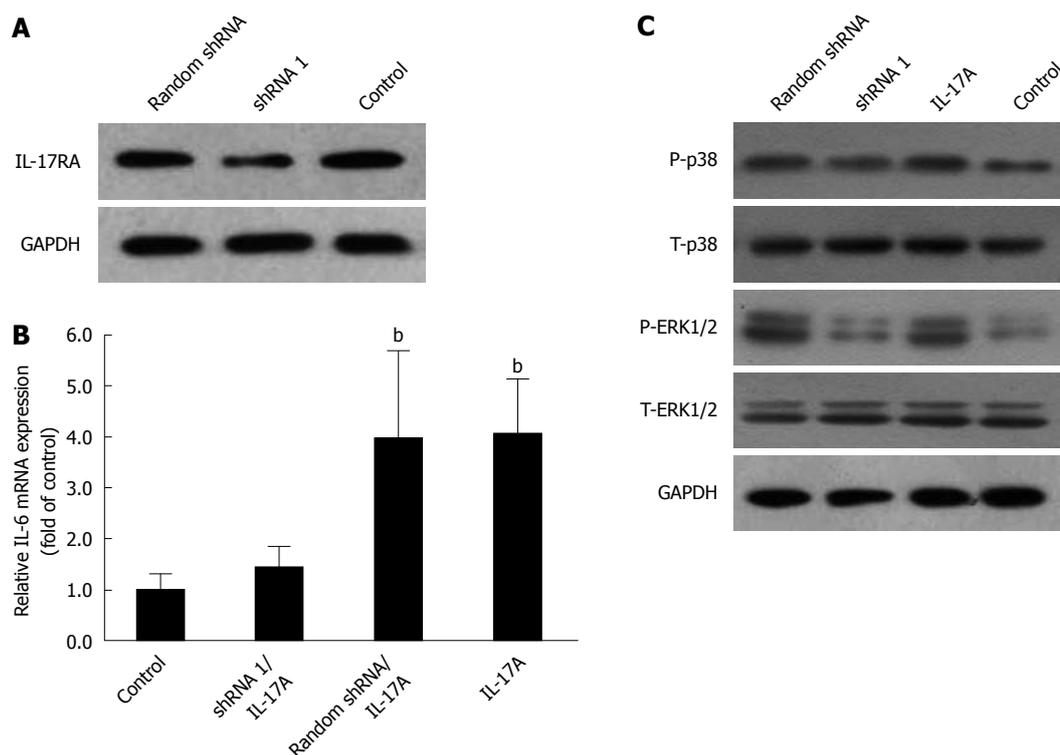


Figure 4 Lentiviral-mediated interleukin 17A receptor short hairpin RNA arrested interleukin 6 expression, partly through suppressing phosphorylation of p38 mitogen activated protein kinases and extracellular regulated protein kinases 1/2. A: Lentiviral-mediated interleukin (IL)-17A receptor short hairpin RNA (shRNA) 1 [at multiplicity of infection (MOI) = 10 for 72 h] inhibited the expression of IL-17RA in hepatic stellate cells (HSCs); B: Lentiviral-mediated IL-17RA shRNA 1 inhibited IL-17A-induced IL-6 mRNA expression in HSCs. HSCs were infected with IL-17RA shRNA 1 or random shRNA lentivirus at MOI = 10 for 72 h, and followed by 3 h exposure to IL-17A (100 ng/mL). ^a*P* < 0.01 vs control; C: Lentiviral-mediated IL-17RA shRNA 1 inhibited phosphorylation of p38 MAPK and ERK1/2. HSCs were transduced with IL-17RA shRNA 1 or random shRNA lentiviral at MOI = 10 for 72 h, and followed by 15 min exposure to IL-17A (100 ng/mL). T: Total, P: Phosphorylation; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

IL-6, one of the major cytokines in various liver injuries^[28-30], stimulated by IL-17A in HSCs. Our data show that IL-17A induces a large amount of IL-6 secretion (68.96 ± 8.30 pg/mL) even at a low concentration of IL-17A treatment (1 ng/mL). With increasing concentrations of IL-17A, the secretion of IL-6 also increased. Surprisingly, a higher concentration of IL-17A (500 ng/mL) induced only moderate expression of IL-6 (109.16 ± 6.91 pg/mL). This may suggest that there is saturation of binding between IL-17A and IL-17RA at a particular concentration or that there may be a negative feedback response on IL-6 expression on IL-17A induction. Our data further show that the IL-6 mRNA expression detected in real-time qPCR reached a maximum level after 3 h exposure to IL-17A and declined thereafter. These results are based on data obtained from the expression of IL-6 protein in HSCs supernatants and indicate that IL-17A induces IL-6 expression *in vitro*. We made a lentivirus-mediated IL-17RA shRNA construct to repress endogenous IL-17RA in HSCs. As a result, we found that IL-6 mRNA expression was notably reduced. Our results further suggest that the MAPK pathway in IL-6 expression is induced by IL-17A in HSCs. We showed that the phosphorylation of p38 MAPK and ERK1/2 is increased after 15 min exposure to IL-17A, and lentivirus-mediated IL-17RA shRNA decreased the phosphorylation of p38 MAPK and ERK1/2. Moreover, use of specific inhibi-

tors further revealed the importance of the MAPK pathway in IL-17A-induced IL-6 mRNA expression in HSCs. The imidazole compound SB-203580, a specific inhibitor of p38 MAPK, caused a significant decrease in IL-17A-induced IL-6 mRNA expression. Additionally, PD-98059, a specific inhibitor of MEK1 that is the directly upstream protein kinase of ERK1/2, promotes marked suppression of IL-17A induced IL-6 mRNA expression. Thus, we conclude that the p38 MAPK and ERK1/2 pathways are involved in IL-6 mRNA expression induced by IL-17A in HSCs. Hot *et al*^[31] indicated that IL-17A stimulated IL-6 secretion by inducing activation of all three MAPKs (ERK, p38, JNK). Inhibition of IL-17RA expression *via* siRNA lead to near complete abrogation of IL-6 expression mediated by IL-17A.

IL-6 is an important cytokine in regulating different inflammatory responses in liver diseases and is a marker in the diagnosis of symptoms of liver cirrhosis^[32-34]. Previous studies have described that IL-6, in conjunction with TGF- β , promotes Th17 cell differentiation and drives IL-17A production^[35,36]. On the other hand, IL-17A can also stimulate IL-6 expression^[37] and IL-6 is a key downstream target gene for IL-17A in non-immune cells, such as fibroblasts^[38]. IL-17A triggers a positive feedback loop of IL-6 signaling and forms the "IL-6 amplifier"^[37,38]. IL-17A plays a pivotal role in immune and non-immune tissues^[37]. The IL-6 amplifier exerts its ef-

fects through activation and phosphorylation of both NF- κ B and STAT3 proteins. It is also known that HSCs are involved in extracellular matrix degradation and maintenance, imbalances in which lead to liver fibrosis, as a result of unmatched levels of metalloproteinases, inhibitors of fibrillary collagen, and tissue inhibitors of metalloproteinase-1 and -2^[2]. Thus, it is important to examine the role of the IL-17A/IL-6 pathway in HSCs. Zhao *et al.*^[39] indicated that IL-17A induced IL-6 expression *via* the MAPK signaling pathway in hepatocytes, which, in turn, may further stimulate Th17 cells and forms a positive feedback loop. They concluded that Th17 cells and the IL-17A signaling pathway played an important role in targets for AIH. Yan *et al.*^[40] demonstrated that acute Con A-induced liver injury was reduced in mice treated with an adenovirus vector encoding a soluble IL-17R immunoglobulin (Ig)G fusion (AdIL-17R:Fc) that neutralized the interaction between IL-17A and IL-17RA. This supported the key role of IL-17A/IL-17RA signaling in Con A-induced hepatitis. Blockade of the IL-17A/IL-17RA signaling pathway may provide a novel therapeutic target in human autoimmune-related hepatitis. Genovese *et al.*^[41] also reported that IL-17A neutralization can achieve positive results in rheumatoid arthritis.

Consistent with previous reports in other cells, our data indicate that IL-17A induces IL-6 expression in HSCs. Suppression of IL-17RA with lentivirus-mediated IL-17RA shRNA inhibited IL-6 expression and blocked IL-17A-triggered positive-feedback loop of IL-6 signaling. Additionally, phosphorylation of p38 MAPK and ERK1/2, induced by IL-17A, was blocked in the presence of IL-17RA shRNA. Negative regulation of MAPK signaling using two specific inhibitors (SB-203580 and PD-98059) for p38 MAPK and ERK1/2, respectively, significantly attenuated IL-6 mRNA expression. These results indicated that blocking IL-17RA expression may be an alternative strategy to reduce the inflammatory response induced by IL-17A in the liver. Further, this study allows us to further understand the function of IL-17RA in HSCs. We would like to examine more potential target substrates that may be affected by the IL-17A/IL-17RA signaling pathway in the future and to further examine the underlying mechanism of HSCs in liver fibrosis.

ACKNOWLEDGMENTS

The study protocol and animal care were approved by the Animal Care and Use Committee of Wenzhou Medical College.

COMMENTS

Background

Liver fibrosis results from chronic damage to the liver in accompany with repeated chronic liver inflammation and evenly evolves into liver cirrhosis. Following chronic injury, Hepatic stellate cells (HSCs) activate, migrate and accumulate at the sites of tissue repair, acquiring contractile, proinflammatory, and fibrogenic properties. Activated HSCs secrete large amounts of extracellular matrix (ECM) and regulate ECM degradation. HSCs are also involved in modulating liver inflammation in liver fibrogenesis. But the mechanism involved in the secretion of interleukin (IL) 6 (which is a crucial cytokine in regulating different inflammatory

responses in liver diseases and is a marker to diagnose symptoms of liver cirrhosis) mediated by IL-17 stimulation in HSCs is not understood totally.

Research frontiers

IL-17A is a pro-inflammatory cytokine secreted by a new subset T help cells named Th17 cells. IL-17A signals exert miscellaneous effects through binding to the IL-17A receptor (IL-17RA) which is expressed in a variety of cells and tissues. In recently years, Clinical studies have shown that high levels of IL-17A and other cytokines related to IL-17A pathway exhibit various liver diseases including liver autoimmunity and inflammatory diseases, viral hepatitis, alcoholic liver disease and hepatocellular carcinoma. The research hotspot is how to reduce liver injury by modify liver inflammatory response, and then inhibit or postpone liver fibrosis and cirrhosis.

Innovations and breakthroughs

The present study indicated that IL-17A induces IL-6 secretion *via* p38 MAPK and ERK1/2 pathway by binding to IL-17RA in HSCs. Suppression of IL-17RA using lentiviral-mediated IL-17RA shRNA inhibits IL-6 secretion.

Applications

The study results suggest that IL-17 could stimulate IL-6 secretion *via* p38 MARK and ERK1/2 pathway in HSCs. Downregulation of IL-17RA receptor by Lentiviral vector-mediated IL-17RA shRNA decreases IL-6 expression induced by IL-17A *via* p38 MAPK and ERK1/2 Phosphorylation in HSCs. Suppression of IL-17RA expression may be an optional strategy to impair the inflammation response induced by IL-17A in the liver. This suggests that modulating IL-17RA expression might be a strategy to interfere with hepatic inflammatory processes in various liver diseases.

Terminology

HSCs are the main ECM-producing cells in the injured liver. In the normal liver, HSCs reside in the space of Disse and are the major storage sites of vitamin A. Following chronic injury, HSCs activate or transdifferentiate into myofibroblast-like cells, acquiring contractile, proinflammatory, and fibrogenic properties. Activated HSCs migrate and accumulate at the sites of tissue repair, secreting large amounts of ECM and regulating ECM degradation. If the hepatic injury persists, then eventually the liver regeneration fails, and hepatocytes are substituted with abundant ECM, including fibrillar collagen. As fibrotic liver diseases advance, disease progression from collagen bands to bridging fibrosis to frank cirrhosis occurs.

Peer review

The manuscript is addressing clearly the objective of the study. The results sustain the conclusion obtained and the experiments are performed properly. The novelty is enough to be an interesting paper.

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ERCP for the treatment of bile leak after partial hepatectomy and fenestration for symptomatic polycystic liver disease

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Abstract

AIM: To describe endoscopic treatment of bile leaks in these patients and to identify risk factors in these patients which can predict the development of bile leaks.

METHODS: Retrospective case-control study examining consecutive patients who underwent partial hepatectomy for polycystic liver disease (PLD) and developed a postoperative bile leak managed endoscopically over a ten year period. Each case was matched with two controls with PLD who did not develop a postoperative bile leak.

RESULTS: Ten cases underwent partial hepatectomy with fenestration for symptoms including abdominal distention, pain and nausea. Endoscopic retrograde cholangiopancreatography (ERCP) showed anatomic abnormalities in 1 case. A biliary sphincterotomy was performed in 4 cases. A plastic biliary stent was placed

with the proximal end at the site of the leak in 9 cases; in 1 case two stents were placed. The overall success rate of ERCP to manage the leak was 90%. There were no significant differences in age, gender, comorbidities, duration of symptoms, history of previous surgery or type of surgery performed between cases and controls.

CONCLUSION: ERCP with stent placement is safe and effective for management of post-hepatectomy bile leak in patients with PLD.

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Key words: Polycystic liver; Hepatectomy; Bile leak; Endoscopic retrograde cholangiopancreatography

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INTRODUCTION

Polycystic liver disease (PLD) is a rare inherited disorder characterized by multiple cysts in the liver, often associated with autosomal dominant polycystic kidney disease (ADPKD)^[1,2]. The cysts develop as progressive dilation of biliary microhamartomas^[3,4] which communicate with the hepatic sinusoids and the portal tree. Mature cysts

do not communicate with the biliary tree but are lined by biliary epithelium^[5]. These cysts grow slowly but can become large and produce massive hepatomegaly. Most patients with PLD are asymptomatic until significant hepatomegaly develops. The commonest symptoms are abdominal pain, dyspnea from diaphragmatic compression, and lower extremity edema. Medical therapy is largely ineffective for the management of these symptoms and therefore, surgical treatment aimed at reduction in hepatic volume is the treatment of choice. These modalities include surgical fenestration or unroofing of cysts, partial hepatectomy or a combination.

Bile leaks are a common complication of surgical management of PLD and occur in 6%^[6] to 15%^[7] of patients. Management of bile leaks can be conservative, surgical, or by the use of endoscopic retrograde cholangiopancreatography (ERCP) with placement of a biliary stent with or without a biliary sphincterotomy. Although ERCP has been reported to be used successfully to manage bile leaks in patients without PLD following hepatectomy^[8-12] there are no reports on endoscopic management of biliary leaks in patients with PLD following surgery. Hence, we hope to define the use of ERCP in these patients.

In this report, we describe endoscopic treatment of bile leaks following surgical management of PLD. We also sought to determine if there were patient or procedural factors in patients with PLD which can predict the development of bile leaks.

MATERIALS AND METHODS

IRB consent was obtained from the Mayo Clinic Institutional Review Board prior to initiation of the study. We performed a retrospective case control study. All patients with PLD undergoing surgical treatment between January 1, 1998 and September 30, 2008 were identified. These patients were referred for surgery because they were symptomatic from the PLD, with the commonest symptoms being abdominal pain and early satiety. Patients were included only if they underwent fenestration and/or hepatectomy or wedge resection to address these symptoms. PLD was defined as having at least 20 simple cysts larger than 1 cm in diameter in the liver excluding any infectious, parasitic or traumatic causes. Only patients with research authorization were then further studied. Cases were defined as patients having a clinical diagnosis of bile leaks as identified by review of the medical record. Controls were defined as patients who underwent a similar surgery for PLD, matched by date of surgery within 4 months before or after the surgery of the case, without development of a post-operative bile leak. Two controls were chosen for each case. Demographic data including age and gender were abstracted. Charlson score^[13] was assessed for each patient as also the duration of disease at the time of surgery. Surgical procedural details including type of surgery performed, hepatic lobe(s) removed, and concomitant surgeries performed such as cholecystectomy were noted. The exact classification of hepatic

resection was limited because of anatomic distortion in the livers of patients with PLD^[7]. The need for ERCP was decided by the surgeon in consultation with the performing endoscopist based upon clinical symptoms and presence and/or volume of ongoing bile leakage from surgical or percutaneous drains. Endoscopic procedural details including time between surgery and ERCP, the leak site identified, performance of a sphincterotomy or placement of a biliary stent, and development of complications were identified. The outcome of ERCP was assessed from chart review and resolution of symptoms, removal of drains, follow-up non-invasive imaging studies or by follow-up ERCP.

Statistical analysis

Descriptive statistics were used to describe the case patient characteristics and ERCP outcomes. For comparative statistics for the case control portion of the study, a standard unpaired *t*-test or Mann Whitney *U* test was used to compare parametric and nonparametric continuous variables respectively, and the Fischer's exact test was used to compare dichotomous variables. A *P* value ≤ 0.05 was considered statistically significant.

RESULTS

Case series description

10 patients developed a post-operative bile leak after surgery for symptomatic PLD treatment between between January 1, 1998 and September 30, 2008 and were treated endoscopically (Table 1). Of these 10 patients, all were women, with a mean age of 48.3 ± 9.7 years. The median duration of symptoms prior to the index surgery was 12 years (range 2-30 years), and 3 had had previous fenestration or alcohol sclerosis of cysts. All patients underwent partial hepatectomy with fenestration for symptoms including abdominal distention, pain and nausea. The exact classification of hepatic resection was limited because of anatomic distortion in the livers of patients with PLD^[7]. Half had right hepatectomy and half had left hepatectomy. All the surgeries were performed by one highly experienced surgeon (David M Nagorney). All were open laparotomy and at least 1 percutaneous drain was placed. Seven patients had concomitant cholecystectomy.

Bile leak was diagnosed and ERCP performed after a mean of 17^[12] postoperative days. Bile leak was suspected when persistent bilious drainage was noted from the percutaneous drains. Computed tomography confirmation was obtained in two cases. ERCP showed the exact site of leak in 5 (50%) cases and in three more cases a leak of contrast was noted to communicate with the surgical drain but the exact site could not be determined. In two patients, no leak was seen. Leaks occurred from the common hepatic duct and the right intrahepatic ducts in one patient each and the other three arose from the left intrahepatic ducts. None of the patients had a previous sphincterotomy; biliary sphincterotomy was performed in 4 (40%) cases. A 10 French plastic biliary stent was placed

Table 1 Characteristics of 10 patients with polycystic liver disease who underwent endoscopic retrograde cholangiopancreatography management of post-hepatectomy bile leaks

Patient	Duration of diagnosis prior to surgery (yr)	Surgery performed in addition to fenestration	Cholecystectomy performed?	Time to ERCP (d)	Leak site	Sphincterotomy performed?	Outcome
1	30	Extended left hepatectomy ¹	Yes	8	Not specified ²	No	Success on repeat ERCP
2	2	Extended right hepatectomy	Yes	16	Common hepatic duct	No	Died 10 d after ERCP
3	17	Right hepatectomy	Yes	21	Left intrahepatic	No	Success clinically
4	6	Extended right hepatectomy	No	28	Left intrahepatic	No	Success clinically
5	8	Right hepatectomy	Yes	7	No leak seen	No	Success clinically
6	4	Left hepatectomy	Yes	20	Not specified	No	Success clinically
7	4	Right hepatectomy	Yes	15	Left intrahepatic	Yes	Success on repeat ERCP
8	26	Left hepatectomy	No	43	Not specified	Yes	Success clinically
9	30	Extended left hepatectomy	Yes	4	No leak seen	Yes	Success on repeat ERCP
10	20	Left hepatectomy	No	6	Right intrahepatic	Yes	Success on repeat ERCP

¹Extended included removal of segments 4A and/or 4B of the liver; ²Leak noted into percutaneous drain, but no specific site identified. ERCP: Endoscopic retrograde cholangiopancreatography.

Table 2 Risk factors for bile leak development

	Cases (n = 10)	Controls (n = 20)	Effect	P value
Age (yr) (mean ± SD)	48.3 ± 9.7	52.2 ± 9.2	-3.9	0.30
Duration of symptoms (yr) (mean ± SD)	14.7 ± 11.3	13.4 ± 7.6	1.3	0.71
Previous surgery (Y/N) n (%)	3 (30)	4 (20)	OR: 1.71, 95% CI: 0.3-9.78	0.66
Charlson score median (range)	1.5 (0-4)	1.5 (1-5)	No difference	0.60
Wedge resection n (%)	2 (20)	10 (50)	OR: 0.25, 95% CI: 0.04-1.48	0.24
Post-op drains median (range)	1 (1-2)	1 (1-2)	No difference	0.67
Concomitant cholecystectomy n (%)	7 (70)	17 (85)	OR: 0.41, 95% CI: 0.07-2.56	0.37

OR: Odds ratio; CI: Confidence interval; Y/N: Yes/no.

with the proximal end at the site of the leak when identified or into the common bile duct in 9 (90%) cases; in 1 (10%) case two stents were placed. None of the patients developed post-ERCP complications. A repeat ERCP was performed in 5 patients after a mean (range) of 26 (8-47) d, 4 of which had resolved the leak. The one patient who in who the leak had not resolved underwent a second ERCP 8 d later for worsening jaundice and suffered fatal fulminant hepatic failure 3 d later. One more patient (20% total) died of liver failure after clinical resolution of bile leak but prior to repeat ERCP. Three cases were lost to follow up in terms of removal of the biliary stent but clinically did well with regards to the bile leak, and in one case the stent passed spontaneously without complication. Overall, 9 of the 10 patients (90%) were deemed to have successful treatment of the bile leak by ERCP management. The patient who died of hepatic decompensation with an ongoing leak was counted as a failure of therapy though

death was not directly related to the bile leak or treatment.

Risk factors for bile leak development

In order to determine if there were any patient or procedural factors predictive of developing a post-operative bile leak, the above 10 cases were matched retrospectively 1:2 to control patients with PLD who underwent similar surgical procedures within 4 mo of the matched case patient, but who did not develop a post-operative bile leak. All the controls were female and all but three underwent fenestration along with hepatectomy; one patient had only fenestration performed and two had fenestration with wedge resections. When comparing cases to controls, there was no significant difference in age (48.3 ± 9.7 years *vs* 52.2 ± 9.2 years, *P* = 0.30), duration of symptoms (14.7 ± 11.3 years *vs* 13.4 ± 7.6 years, *P* = 0.70), history of previous surgical resection [odds ratio (OR): 1.71, 95% confidence interval (CI): 0.30-9.78, *P* = 0.66], Charlson score [median 1.5 (range: 0-4) *vs* 1.5 (range: 1-5), *P* = 0.61], performance of wedge resection (OR: 0.25, 95% CI: 0.04-1.48, *P* = 0.24), number of post-operative drains [median 1 (range: 1-2) *vs* 1 (range: 1-2), *P* = 0.67] or concomitant cholecystectomy (OR: 0.42, 95% CI: 0.07-2.56, *P* = 0.37) (Table 2). All the surgeries were performed by a single highly experienced surgeon (David M Nagorney). Thus, no factors could be identified that would predict development of a bile leak in patients with PLD undergoing surgical fenestration of hepatectomy.

DISCUSSION

PLD is a disease where multiple cysts develop in the liver and grow with age. When these cysts are significantly enlarged, they cause symptoms including abdominal pain, shortness of breath, and early satiety with resultant adverse affect on quality of life. Surgical manage-

ment of symptomatic cysts includes fenestration of the cysts, wedge resection of the most affected areas of the liver (for limited cystic involvement) or hepatectomy to remove the most enlarged areas. Bile leaks are the commonest complication from hepatectomy and it can be surmised that patients with PLD who have distorted anatomy because of the presence of cysts might be at an even higher risk for this complication. In fact, the risk of biliary complications in patients with PLD undergoing surgery is as high as 15%^[7]. The incidence of biliary complications following hepatectomy in patients without PLD is 4.8% to 8.1%^[14].

Traditionally, management of post-hepatectomy bile leaks in patients with PLD has been exploratory laparotomy or conservative management. However, a laparotomy significantly increases morbidity and costs. Conservative management results in a much longer recovery process^[14] and is not always successful^[8]. Nagano *et al.*^[14] reported that patients who underwent biliary drainage with a nasobiliary tube had markedly shorter healing times compared to those who did not (30 d *vs* 179 d). However, nasobiliary tubes are more cumbersome for patients and technically more difficult to place than indwelling biliary stents. Endoscopic therapy has been used successfully to treat biliary leaks that occur after cholecystectomy^[15] and also in patients without PLD following liver surgery^[16]. However, there are no data to support its use in patients with PLD where theoretically, bile leaks may be more difficult to treat given the altered anatomy. Thus, our aim was to publish experience with ERCP in the treatment of post-hepatectomy bile leaks.

Our findings show that ERCP may be an effective therapy for post-hepatectomy bile leaks in patients with PLD. Nine of 10 patients had successful healing of bile leaks. The patient who died with a persistent leak died of hepatic failure, not as a result of complications from the leak.

Bile leaks were treated with and without endoscopic biliary sphincterotomy based upon endoscopist preference. A 10 French biliary stent was used in all cases and the proximal end was placed in the branch of the hepatic duct where the leak was seen to originate. No complications including pancreatitis, perforation or bleeding occurred after ERCP.

The case-control comparison was performed to determine if patient or procedural factors could predict the risk of post-operative bile leak. Previous studies in patients without PLD have determined that age, gender, cut surface area and other operative factors, presence of hepatitis B or C, baseline laboratory studies and the presence of cirrhosis can affect the risk of development of a bile leak^[14]. In our cohort of patients with PLD, all the cases and controls were women, of similar ages, had no other liver disease or cirrhosis. PLD causes symptoms by mechanical effect and therefore laboratory studies were mostly normal. There is a risk of hepatic decompensation following large hepatectomy because the remaining liver volume may be insufficient though synthetic function

is not affected at baseline. There was also no significant difference between cases and controls in duration of disease, whether right or left hepatectomy was performed, and whether a wedge resection was performed.

There are several limitations to the study. Because of its retrospective nature, there is inherent selection bias. Also, the numbers of patients are small. This is not unusual given that the prevalence of symptomatic PLD is low. The case-control comparison may not be sufficiently powered to detect a difference in risk factors but over 10 years, only 10 patients were identified with clinically important bile leaks. Also, operative details were not prospectively collected and hence not available for review in all cases. Certain operative details such as difficulty of resection, amount of blood loss, and intra-operative damage and repair of bile ducts could have impacted the risk of developing a bile leak.

In summary, we conclude that ERCP with placement of an internal biliary stent is a safe and effective treatment for post-hepatectomy bile leaks in patients with PLD. ERCP should be considered as a therapeutic option when bile leak is suspected clinically due to persistent biliary drainage from percutaneous tubes in these patients. No patient factors can be identified as risk factors to predict development of bile leaks in these patients. Further prospective studies could be performed to assess risk factors.

COMMENTS

Background

Polycystic liver disease (PLD) is a rare inherited disorder characterized by multiple cysts in the liver, and surgical treatment aimed at reduction in hepatic volume is the treatment of choice. Bile leaks are a common complication of surgical management of PLD. Here, the authors describe the use of endoscopic retrograde cholangiopancreatography (ERCP) to manage bile leaks in these patients.

Research frontiers

In the area of management of bile leaks in polycystic liver disease, the research hot spot is in determining the safest and most effective modality of treatment, whether medical, surgical or endoscopic.

Innovations and breakthroughs

Previously, endoscopic therapy had been used successfully to treat biliary leaks that occur after cholecystectomy, and also in patients without PLD following liver surgery. The innovation described in this report is in the successful use of endoscopic therapy in a novel patient population.

Applications

The study demonstrates that ERCP may be an effective therapy for post-hepatectomy bile leaks in patients with PLD.

Terminology

PLD is a disease where multiple cysts develop in the liver and grow with age. When these cysts are significantly enlarged, they cause symptoms including abdominal pain, shortness of breath, and early satiety with resultant adverse effect on quality of life.

Peer review

Although the endoscopic treatment in patients with biliary leaks after hepatectomy is a well recognized procedure, the authors report their experience, as case series, concerning a subset of patient with hepatic cysts.

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Physical activity, obesity and gastroesophageal reflux disease in the general population

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Abstract

AIM: To clarify the association between physical activity and gastroesophageal reflux disease (GERD) in non-obese and obese people.

METHODS: A Swedish population-based cross-sectional survey was conducted. Participants aged 40-79 years were randomly selected from the Swedish Registry of the Total Population. Data on physical activity, GERD, body mass index (BMI) and the covariates age, gender, comorbidity, education, sleeping problems, and tobacco smoking were obtained using validated questionnaires. GERD was self-reported and defined as heartburn or

regurgitation at least once weekly, and having at least moderate problems from such symptoms. Frequency of physical activity was categorized into three groups: (1) "high" (several times/week); (2) "intermediate" (approximately once weekly); and (3) "low" (1-3 times/month or less). Analyses were stratified for participants with "normal weight" (BMI < 25 kg/m²), "overweight" (BMI 25 to ≤ 30 kg/m²) and "obese" (BMI > 30 kg/m²). Multivariate logistic regression was used to calculate odds ratios (ORs) with 95% confidence intervals (CIs), adjusted for potential confounding by covariates.

RESULTS: Of 6969 eligible and randomly selected individuals, 4910 (70.5%) participated. High frequency of physical activity was reported by 2463 (50%) participants, GERD was identified in 472 (10%) participants, and obesity was found in 680 (14%). There were 226 (5%) individuals with missing information about BMI. Normal weight, overweight and obese participants were similar regarding distribution of gender and tobacco smoking status, while obese participants were on average slightly older, had fewer years of education, more comorbidity, slightly more sleeping problems, lower frequency of physical activity, and higher occurrence of GERD. Among the 2146 normal-weight participants, crude point estimates indicated a decreased risk of GERD among individuals with high frequency of physical activity (OR: 0.59, 95% CI: 0.39-0.89), compared to low frequency of physical activity. However, after adjustment for potential confounding factors, neither intermediate (OR: 1.30, 95% CI: 0.75-2.26) nor high (OR: 0.99, 95% CI: 0.62-1.60) frequency of physical activity was followed by decreased risk of GERD. Sleeping problems and high comorbidity were identified as potential confounders. Among the 1859 overweight participants, crude point estimates indicated no increased or decreased risk of GERD among individuals with intermediate or high frequency of physical activity, compared to low frequency. After adjustment for confounding, neither intermediate (OR: 0.75, 95% CI: 0.46-1.22) nor high frequency of physical activity were

followed by increased or decreased risk of GERD compared to low frequency among nonobese participants. Sleeping problems and high comorbidity were identified as potential confounders for overweight participants. In obese individuals, crude ORs were similar to the adjusted ORs and no particular confounding factors were identified. Intermediate frequency of physical activity was associated with a decreased occurrence of GERD compared to low frequency of physical activity (adjusted OR: 0.41, 95% CI: 0.22-0.77).

CONCLUSION: Intermediate frequency of physical activity might decrease the risk of GERD among obese individuals, while no influence of physical activity on GERD was found in non-obese people.

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Key words: Physical exercise; Gastroesophageal reflux disease; Population-based study; Risk factor; Body mass index; Obesity

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is a public health concern defined by troublesome and frequent symptoms of heartburn or regurgitation^[1,2], affecting up to 20% of the adult population in the western world^[3]. GERD is associated with a negative impact on health-related quality of life (HRQL), increased risk of esophageal adenocarcinoma, and great costs for patients and society^[3-8]. Established risk factors for GERD are overweight, tobacco smoking, low socioeconomic status, and heredity^[9-15], while the potential role of physical activity is complex and intriguing. Intense physical activity is known to trigger reflux symptoms^[16], but physical activity has many positive effects on health in general, and some data indicate that high physical activity might also prevent GERD in a long-term perspective^[17,18]. Two population-based studies have assessed associations between physical activity and reflux; our twin study from Sweden indicated that high physical activity at work was related to an increased risk of GERD, while recreational physical activity decreased this risk^[17], and our nested case-control study from Norway has sug-

gested a protective effect of physical activity on the risk of GERD^[18]. Few studies have addressed this issue and the available results are partly conflicting, thus, more research is required before any preventive effect of physical activity on GERD can be established. Obesity is strongly linked with both physical activity and GERD, therefore, for this study, we aimed to test the hypothesis that the association between frequency of physical activity and GERD differs between non-obese and obese people^[11]. The occurrence of GERD and frequency of physical activity might change with time, therefore, we conducted a cross-sectional study assessing both variables at the same time, enabling us to investigate whether a higher frequency of physical activity is associated with an increased risk of GERD in an unselected general population.

MATERIALS AND METHODS

Design

A population-based, cross-sectional study was performed between April and June 2008 in Sweden. Participants aged 40-79 years were randomly selected from the Swedish Registry of the Total Population; a registry that contains information of all Swedish residents regarding vital status, gender, age, place of residence, with a maximum of 2 wk delay. Eligible individuals received a postal questionnaire assessing physical activity, body weight, height, and GERD, together with socio-demographic variables, concurrent disease, and lifestyle factors. By completing and returning the questionnaires, participants consented to their data being used for research purposes. Up to two reminder letters were sent to non-responders. Participants were not offered any inducement for participation.

Study variables

Physical activity: The questionnaire assessed physical activity by asking "How often do you perform a physically demanding activity lasting at least 30 min? For example running, cycling, swimming". Frequency of physical activity was categorized into three groups: (1) "high" (several times per week); (2) "intermediate" (approximately once per week); and (3) "low" (1-3 times per month, or less often).

GERD: Information regarding GERD was collected on a 5-point Likert scale for frequency and symptom severity. In line with the implementation of the Montreal definition of GERD^[19], individuals were categorized as having GERD or not (yes or no). Participants were categorized as having GERD if they reported heartburn or regurgitation occurring at least once a week, and having at least moderate problems from such symptoms. Participants reporting use of medications for heartburn or regurgitation at least once weekly were also included in the group fulfilling the criteria for GERD, irrespective of symptom severity.

Covariates: The socio-demographic information included

Table 1 Characteristics of 4910 study participants, randomly selected from the Swedish general population, and stratified into normal weight *n* (%)¹

	BMI < 25 (kg/m ²)	BMI 25 to ≤ 30 (kg/m ²)	BMI > 30 (kg/m ²)
Gender	2146 (46)	1859 (40)	680 (15)
Male	885 (41)	1110 (60)	340 (50)
Female	1261 (59)	749 (40)	340 (50)
Age (yr) (mean ± SD)	58 ± 10	57 ± 11	59 ± 10
Years of formal education			
≤ 9	628 (29)	682 (37)	295 (43)
10-12	798 (37)	550 (30)	204 (30)
> 12	653 (30)	585 (31)	163 (24)
No. of diseases			
None	1226 (57)	810 (44)	225 (33)
1	546 (25)	553 (30)	193 (28)
2	228 (11)	269 (14)	118 (17)
At least 3	146 (7)	227 (12)	144 (21)
Sleeping problems			
Not at all	1295 (60)	1159 (62)	383 (56)
A little	545 (25)	467 (25)	168 (25)
Quite a bit	214 (10)	163 (9)	89 (13)
Very much	69 (3)	52 (3)	33 (5)
Current tobacco smoking			
No	1762 (82)	1576 (85)	584 (86)
Yes	384 (18)	282 (15)	96 (14)
Physical activity ²			
Low	446 (21)	495 (27)	219 (32)
Intermediate	432 (20)	473 (25)	176 (26)
High	1236 (58)	869 (47)	265 (39)
Gastroesophageal reflux disease ³			
No	2009 (94)	1668 (90)	569 (84)
Yes	137 (6)	190 (10)	111 (16)

¹Two hundred and twenty-six persons had missing information about body mass index. Percentages not adding up to 100 are explained by missing data in some variables; ²Low: 1-3 times per month or less often; Intermediate: approximately once per week; and High: several times per week; ³Definition of GERD^[19]: heartburn or regurgitation occurring at least once a week and at least having intermediate problems from heartburn or regurgitation. Persons reporting use of medications for heartburn or regurgitation at least once weekly were also included in the group fulfilling the criteria for GERD, irrespective of their severity of the problem. GERD: Gastroesophageal reflux disease.

gender, age, years of formal education, and marital status. The questionnaire further assessed diseases confirmed by a physician (yes or no), including angina, heart failure, atrial fibrillation, myocardial infarction, hypertension, stroke, chronic obstructive pulmonary disease, asthma, diabetes, rheumatoid arthritis, osteoarthritis, kidney failure requiring dialysis, chronic pain, depression under treatment, and cancer. Data on sleeping problems during the past week were assessed with the validated EORTC QLQ-C30^[20] questionnaire. Tobacco smoking was assessed by asking if the person had been smoking during the past 3 mo (yes or no). For calculation of body mass index (BMI), the questionnaire assessed adult height and current weight, and participants were categorized as normal weight (BMI < 25 kg/m²), overweight (BMI 25 to ≤ 30 kg/m²) or obese (BMI > 30 kg/m²).

Statistical analysis

Sample characteristics were described by standard de-

scriptive statistics. In order to examine the associations between frequency of physical activity and presence of GERD, odds ratios (ORs) with 95% confidence intervals (CIs) were calculated using logistic regression with multivariate adjustment for potential confounders. Analyses were stratified for participants with normal weight (BMI < 25 kg/m²), overweight (BMI 25 to ≤ 30 kg/m²), or obese (BMI > 30 kg/m²). Potential confounders were: (1) gender (male or female); (2) age (as a continuous variable); (3) education level (< 9 years, 10-12 years, or > 12 years); (4) number of concurrent diseases (0, 1, 2 or > 2); (5) sleeping problems (“none”, “a little”, “quite a bit” or “very much”); and (6) current tobacco smoking (yes or no). A weighting factor was applied to ensure that the characteristics of the study sample conformed to independently estimated national distributions by age, gender and region. The statistical package STATA 12 for Windows (STATA Corp, College Station, Texas, United States) and SAS (SAS Institute, Cary, NC, United States) were used for the analyses.

RESULTS

Study participants

Of 6969 eligible individuals, 4910 (70.5%) participated. Some characteristics of the participants are presented in Table 1. High physical activity was reported by 2463 (50%) of all participants, GERD was identified in 472 (10%) participants, and obesity was found in 680 (14%) of the participants. Missing information about BMI was found in 226 individuals. Normal weight, overweight and obese participants were similar regarding distribution of gender and tobacco smoking status, while obese participants were on average slightly older, had fewer years of education, a greater number of comorbidities, slightly more sleeping problems, lower frequency of physical activity, and higher occurrence of GERD.

Frequency of physical activity and GERD in normal weight participants

Among normal weight participants (BMI < 25 kg/m²), crude point estimates indicated a decreased risk of GERD among individuals with high frequency of physical activity (OR: 0.59, 95% CI: 0.39-0.89), compared to low frequency of physical activity. After adjustment for confounding variables, neither intermediate (OR: 1.30, 95% CI: 0.75-2.26) nor high (OR: 0.99, 95% CI: 0.62-1.60) frequency of physical activity was followed by a decreased risk of GERD compared to low frequency of physical activity among normal weight participants (Table 2). Sleeping problems and a high number of comorbidities were identified as potential confounders, since they had a major impact in the multivariable model.

Frequency of physical activity and GERD in overweight participants

Among overweight participants (BMI 25 to ≤ 30 kg/m²), crude point estimates indicated no increased or decreased

Table 2 Association between frequency of physical exercise and occurrence of gastroesophageal reflux disease

	Normal weight (BMI < 25 kg/m ²)			Risk of GERD ¹			Overweight (BMI 25 to ≤ 30 kg/m ²)		Obese (BMI > 30 kg/m ²)	
	OR (95% CI) ²	<i>n</i> = 2146 (46%) P value	<i>n</i> (%)	OR (95% CI) ²	<i>n</i> = 1859 (40%) P value	<i>n</i> (%)	OR (95% CI) ²	<i>n</i> = 680 (15%) P value		
Physical activity ³										
Low	Reference		495 (27)	Reference		219 (33)	Reference			
Intermediate	1.30 (0.75-2.26)	0.35	869 (47)	0.74 (0.46-1.22)	0.24	176 (27)	0.41 (0.22-0.77)	0.01		
High	0.99 (0.62-1.60)	0.98	473 (25)	1.34 (0.90-2.00)	0.15	265 (40)	0.83 (0.50-1.35)	0.45		

¹Definition of GERD^[9]: Self-reported heartburn or regurgitation occurring at least once a week and at least having moderate problems from heartburn or regurgitation. Persons reporting use of medications for heartburn or regurgitation at least once weekly were also included in the group fulfilling the criteria for GERD, irrespective of their severity of the problem; ²Adjusted for gender, age, education level, number of diseases, sleeping problems and tobacco smoking; ³Low = 1-3 times per month or less often, Intermediate = approximately once per week, and High = several times per week. Association between frequency of physical exercise and occurrence of gastroesophageal reflux disease in normal weight people in a random sample of 4910 people from the Swedish general population (226 participants had missing information on BMI). Presented as OR: with 95% CI. GERD: Gastroesophageal reflux disease; BMI: Body mass index; CI: Confidence interval; OR: Odds ratio.

risk of GERD among individuals with intermediate or high frequency of physical activity, compared to low frequency of physical activity. After adjustment for confounding variables, neither intermediate (OR: 0.75, 95% CI: 0.46-1.22) nor high frequency of physical activity were followed by an increased or decreased risk of GERD compared to low frequency of physical activity among overweight participants (Table 2). Identified potential confounders were sleeping problems and a high number of comorbidities, because they had a major impact in the multivariate model.

Frequency of physical activity and GERD in obese participants

In obese individuals (BMI > 30 kg/m²), crude ORs were similar to the adjusted ORs and no particular confounding factors were identified. Intermediate frequency of physical activity was associated with a decreased occurrence of GERD compared to low frequency of physical activity (adjusted OR: 0.41, 95% CI: 0.22-0.77). A non-significantly decreased risk of GERD was found among obese individuals with high frequency of physical activity compared to low frequency of physical activity (adjusted OR: 0.83, 95% CI: 0.50-1.35).

DISCUSSION

This study indicated that intermediate frequency of physical activity was associated with a lower occurrence of GERD among obese individuals, while no such association was found among normal weight or overweight individuals.

The strengths of the present study include the population-based design with random selection of participants, the high participation rate, and the large sample size. Moreover, symptoms of GERD were measured with a well-validated questionnaire^[21], fulfilling the consensus criteria for GERD^[1]. Furthermore, it was possible to adjust the results for several potential confounding factors. Limitations include an inherent uncertainty about the accuracy of self-reported data and lack of validation of the

assessment of frequency of physical activity, BMI, as well as information about previous surgical interventions for GERD. Also, because this was a cross-sectional study, it is not possible to know if participants with a self-detected association between reflux and physical exercise may have changed their behavior, resulting in reverse causality.

The decreased risk of GERD in people participating in physical activity is in line with previous population-based studies assessing an association between physical activity and GERD within the general population^[17,18]. However, none of the previous studies conducted stratified analyses for BMI categories; meaning that the decreased risk of GERD limited to obese individuals is a first-time observation.

A potential biological mechanism underlying increased risk of reflux among obese persons is through increased extrinsic gastric compression by surrounding adipose tissue and anatomical disruption of the gastroesophageal junction^[8,22]. This is also thought to be the mechanism when physical activity triggers reflux symptoms^[16]. It has also been argued that physical exercise might cause GERD by decreasing the gastrointestinal blood flow and changing the esophagogastric motor function. On the other hand, physical activity might strengthen striated muscles in the diaphragmatic crurae and thereby reinforce the antireflux barrier^[16,23-25]. Furthermore, both intensity and type of physical exercise might pose different risks for GERD^[16,23,26]. Should the present results be confirmed in future research, the findings from this study might be important for the prevention and treatment of GERD and its complications.

In conclusion, this large population-based study indicates decreased occurrence of GERD in obese people who report intermediate frequency of physical activity, while no influence of frequency of physical activity on GERD was identified in non-obese people.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) is a public health concern defined by

troublesome and frequent symptoms of heartburn or regurgitation, affecting up to 20% of the adult population in the western world. Established risk factors for GERD are overweight, tobacco smoking, low socioeconomic status, and heredity, while the potential role of physical activity is complex and intriguing.

Research frontiers

GERD is very common and bothersome for people experiencing this disease. All types of risk factors and possible treatments and interventions to reduce symptoms are of relevance.

Innovations and breakthroughs

Two population-based studies have assessed associations between physical activity and reflux; the twin study from Sweden indicated that high physical activity at work was related to an increased risk of GERD, while recreational physical activity decreased this risk, and the nested case-control study based on data from Norway suggested a protective effect of physical activity on the risk of GERD.

Applications

To summarize the actual application values, the implications for further application and modification, or the perspectives of future application of the outcome of the study. Further studies are necessary to determine the direction of causation between physical exercise and GERD in obese patients.

Terminology

GERD is defined by troublesome and frequent symptoms of heartburn or regurgitation. Body mass index (BMI), is calculated by dividing height in meters by the square root of weight in kilograms.

Peer review

The study was well-designed and the results add to current knowledge about GERD and its relieving and aggravating factors.

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Irritable bowel syndrome: Physicians' awareness and patients' experience

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Abstract

AIM: To study if and how physicians use the irritable bowel syndrome (IBS) diagnostic criteria and to assess treatment strategies in IBS patients.

METHODS: A questionnaire was sent to 191 physicians regarding IBS criteria, diagnostic methods and treatment. Furthermore, 94 patients who were diagnosed with IBS underwent telephone interview.

RESULTS: A total of 80/191 (41.9%) physicians responded to the survey. Overall, 13 patients were diag-

nosed monthly with IBS by specialists in gastroenterology (SGs) and 2.5 patients by general practitioners (GPs). All the SGs knew of the criteria to diagnose IBS, as did 46/70 (65.7%) GPs. Seventy-nine percent used the patient's history, 38% used a physical examination, and 38% exclusion of other diseases to diagnose IBS. Only 18/80 (22.5%) physicians used specific IBS criteria. Of the patients interviewed, 59/94 (62.8%) knew they had experienced IBS. Two out of five patients knew IBS and had seen a physician because of IBS symptoms. Half of those received a diagnosis of IBS. A total of 13% were satisfied with treatment. IBS affected daily activities in 43% of cases.

CONCLUSION: Half of the patients with IBS who consulted a physician received a diagnosis. Awareness and knowledge of diagnostic criteria for IBS differ between SGs and GPs.

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Key words: Irritable bowel syndrome; Questionnaire study; Diagnostic criteria; Manning criteria; Rome criteria; Physician knowledge

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INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder. The prevalence of IBS is estimated to range from 3% to 28% depending on the country studied^[1-4]. The prevalence of IBS in the western countries is estimated to be 10%-15%^[2]. However, ascertaining prevalence is based on various approaches in studies using different diagnostic criteria.

The criteria that have been used to identify IBS patients are the Manning criteria^[5], Rome I^[6], Rome II^[7] and the most recent Rome III criteria^[8,9]. The Rome criteria are more refined than the Manning criteria and include the duration of symptoms as part of the definition of IBS^[10]. Studies have also shown that the Manning criteria are relatively sensitive but lack specificity^[11] (Table 1).

It has been questioned whether the Rome criteria are sensitive enough to diagnose patients in general practice. The current lack of interest in these criteria, especially among general practitioners (GPs), is unlikely to change unless they can be considerably improved^[12]. The challenges and uncertainties for diagnosis of IBS have been listed as follows^[13,14]: (1) there is currently no consistent biological marker of IBS, leaving clinicians to rely on patient symptoms alone to make the diagnosis; (2) symptoms of IBS are often difficult to quantify objectively; (3) symptoms can vary among individuals with IBS; and (4) many organic conditions can masquerade as IBS.

With these uncertainties, many physicians approach IBS as a diagnosis of exclusion^[14]. A recent study concluded that: (1) the best practise diagnostic guidelines have not been uniformly adopted in IBS, particularly among primary care providers; (2) most community providers believe IBS is a diagnosis of exclusion (this belief is associated with increased diagnostic resource use); and (3) despite the dissemination of guidelines regarding diagnostic testing in IBS, there remains extreme variation in beliefs among both experts and non-experts^[14].

Patients diagnosed with IBS exhibit a higher use of outpatient visits, inpatient stays, outpatient prescriptions, and number of hospitalizations than those not diagnosed with IBS^[15-17]. A recent study showed that knowledge and use of the Rome criteria or their positive predictive values for IBS did not correlate with reduced use of diagnostic tests^[18]. The cost for outpatient visits, drugs and diagnostic testing has been shown to be 51% higher for IBS patients than for others^[15-17]. IBS patients have been shown to lose time from work more often than others and are less productive while at work^[19]. This may reflect the morbidity in this relatively benign disorder, although up to 70% of IBS patients in the United States do not consult a health care provider regarding their symptoms^[20]. IBS patients are often reluctant to consult a physician, often because they think their symptoms do not warrant a visit to a physician or are afraid that they have a serious life-threatening illness^[2,19]. United States family practitioners have problems with IBS patients, which include difficulties in satisfying patients and difficulties in

making a strategy decision and finding the time required, and their lack of knowledge could interfere with patient care^[21]. No specific treatment options for IBS are available. In clinical practice, the decision to treat is up to the discretion of the physician^[19]. While some physicians recommend lifestyle modification or trials with over-the-counter products, others recommend antispasmodics and antidepressants.

In our study, we aimed to analyze IBS from the physicians' and patients' point of view. The specific aims of this study were: First, physician study, to assess if and how physicians [GPs and specialists in gastroenterology (SGs)]: (1) use the diagnostic criteria to identify IBS; (2) diagnose patients with IBS, and which symptoms of IBS they identify; and (3) which treatment they recommend; and Second, patient study, to assess how patients with IBS are diagnosed and treated by physicians, as well as studying the ideas that patients have about IBS. The results of a parallel study based on the same database but focusing on functional dyspepsia, stability of IBS and heartburn have been published^[22-24].

MATERIALS AND METHODS

Patient study

Participants and setting: In 1996, an epidemiological study of gastrointestinal diseases was performed in Iceland^[25] among 2000 inhabitants aged 18-75 years. The individuals were randomly selected from the National Registry. Equal distribution of sex and age was secured in each age group. In 2006, we attempted to contact all the individuals from 1996, as well as adding 300 new individuals in the 18-27 years age group, who were also randomly selected from the National Registry of Iceland. A questionnaire was mailed to individuals at baseline and the study questionnaire and an explanatory letter mailed to all eligible individuals. Reminder letters were mailed at 2 wk, 4 wk and 7 wk, using the Total Method of Dillman^[26]. Individuals who indicated at any point that they did not want to participate in the study were not contacted further.

Questionnaire: The Bowel Disease Questionnaire (BDQ)^[27,28] was translated from English into Icelandic and modified for this study. The questionnaire was translated by two gastroenterologists and a pharmacist. A specialist in the Icelandic language at the University of Iceland made linguistic modifications. The questionnaire was piloted within a small group of IBS patients diagnosed by a gastroenterologist. The questionnaire was designed as a self-report instrument to measure symptoms experienced over the previous year and to collect the participants' past medical history^[29].

The Icelandic version of the BDQ questionnaire addressed 47 gastrointestinal symptoms and 32 items that measured past illness, health care use, and sociodemographic and psychosomatic symptoms, together with a valid measure of non-gastrointestinal somatic complaints, the Somatic Symptom Checklist (SSC)^[30]. The SSC consisted of 12 non-gastrointestinal and five gastrointestinal

Table 1 Manning, Rome I, II and III criteria for irritable bowel syndrome^[5-7,9]

Manning
Pain eased after BM
Looser stools at onset of pain
More frequent BM at onset of pain
Abdominal distension
Mucus throughout rectum
Feeling of incomplete emptying
Rome I criteria
3 mo or more of continuous or recurrent symptoms
Abdominal pain or discomfort
Relieved with defecation; and/or
Associated with a change in frequency of stool; and/or
Associated with a change in consistency of stool; and
Two or more of the following, at least 25% of occasions or days
Altered stool frequency (> 3 BMs/d or < 3/wk)
Altered stool form (lumpy/hard or loose/watery stool),
Altered stool passage (straining, urgency, tenesmus)
Passage of mucus
Bloating or feeling of abdominal distension
Rome II criteria
At least 12 wk (which need not be consecutive)
In the preceding 12 mo, of abdominal discomfort or pain that has two out of three features
Relieved with defecation; and/or
Onset associated with a change in frequency of stool, and/or
Onset associated with a change in form (appearance) of stool
Rome III criteria
Recurrent abdominal pain or discomfort at least 3 d/mo
In the last 3 mo association with two or more of the following:
Improvement with defecation
Onset associated with a change in frequency of stool
Onset associated with a change in form (appearance) of stool

BM: Bowel movement.

symptoms or illnesses. Individuals were instructed to indicate, on a 5-point scale, how often each symptom appeared and how bothersome it was. There were only a few changes in the 2006 questionnaire, which addressed 51 gastrointestinal symptoms and 33 items that measured past illness, health care use, and sociodemographic and psychosomatic symptoms. The 2006 questionnaire furthermore addressed 17 items to identify heartburn and symptoms related to heartburn.

Telephone survey: In the questionnaire, patients were asked to write down their telephone number and give their permission to participate in a telephone survey. Subjects who were diagnosed with IBS based on the Manning criteria and/or the Rome III criteria and had written down their telephone number were called and interviewed.

Physician study

In Iceland (population approximately 300 000), there are 177 physicians working in general practice and 17 SGs (three physicians who were involved in carrying out this study were excluded). A questionnaire was sent to these 191 physicians regarding awareness and application of the three sets of criteria used to diagnose IBS (Table 1), as well as diagnostic methods and treatment of this dis-

Table 2 Awareness of the disorder, diagnosis and treatment in interviewer-diagnosed patients *n* (%)

	All patients (<i>n</i> = 94)
Diagnosed with IBS	20 (22.2)
Knowledge of IBS	37 (39.4)
Seen a physician because of IBS symptoms	37 (39.4)
Satisfied with treatment for IBS	12 (12.8)
IBS affects daily activities	40 (42.6)
Think they will be cured of IBS	29 (30.9)
Think they will always suffer from IBS	27 (28.7)
Takes medication for IBS	11 (11.7)
Uses untraditional medication	15 (16.0)
Thinks dietary modification is important for the treatment of IBS	52 (55.3)

IBS: Irritable bowel syndrome.

order. We assessed the knowledge of validated symptom-based criteria for IBS.

Statistical analysis

Tables were constructed for frequency and percentage. Categorical data were analyzed using the χ^2 test. The type I error protection rate was set at 0.05. The exact *P* values are listed in the tables and text. All the research data were imported into SPSS software.

Ethics

The National Bioethics Committee of Iceland and The Icelandic Data Protection Authority (Personuvernd) gave their permission for the research.

RESULTS

Patient study

A total of 94 patients underwent telephone interview (29.8% male, 70.2% female) with a mean age of 47 years. All these had IBS according to the Manning criteria and 56.0% according to the Rome III criteria (the Rome III criteria being more refined and stricter than the Manning criteria). When patients were asked if they had experienced IBS (self-assessed), 62.8% reported yes and 21.3% said they had received an IBS diagnosis from a physician; 60% of these had a Rome-III-based diagnosis, and 100% had a Manning-based diagnosis.

Table 2 shows the awareness of IBS. Two out of five patients had heard of IBS and the same number had seen a physician because of IBS symptoms, but only half of those had received a diagnosis of IBS. Only 12/94 (12.8%) IBS patients were satisfied with the treatment they had been given. IBS did affect daily activities in approximately 43% of the cases (Table 2). One third of the IBS patients thought they would be cured of IBS but a similar proportion thought they would always suffer from IBS (Table 2). IBS patients were found to use more non-traditional medication than prescribed drugs. More than half of patients believed that dietary modification was important for treatment of IBS (Table 2).

Three out of five IBS patients were diagnosed by a

Table 3 Diagnosis of patients with irritable bowel syndrome (%)

	All patients <i>n</i> = 80 ¹	SG <i>n</i> = 9	GP <i>n</i> = 70
Patients history	79	78	80
Physical examination	38	22	41
Exclusion of other diseases	38	44	35
IBS criteria	22	33	19
Gastrointestinal endoscopy	7	22	6

¹One physician did not list his profession; IBS: Irritable bowel syndrome; SG: Specialists in gastroenterology; GP: General practitioners.

gastroenterologist and two out of five by a GP. Most IBS patients reported abdominal pain (73.7%), bloating (21.1%), constipation (5.3%) and diarrhea (10.5%) as the symptom that led to the diagnosis. More than half (57.9%) of the IBS patients who received management for their IBS symptoms were satisfied.

Physician study

An anonymous questionnaire was sent to a total of 191 physicians in Iceland in the fields of primary care, or to SGs (excluding three physicians involved in carrying out this study). A total of 80 physicians (41.9%) replied (83% male, 17% female) and completed the questionnaire. Of those who answered, 70/175 were GPs and 9/15 were SGs.

On average, 13 patients were estimated to be diagnosed with IBS monthly by SGs and 2.5 by GPs. Physicians reported how they diagnosed patients with IBS (Table 3). Two thirds of all the physicians knew that special diagnostic criteria exist for defining and diagnosing IBS (Figure 1).

When physicians were asked if they knew of the IBS diagnostic criteria, 71% said yes (64% of GPs, 100% of SGs). Despite the fact that 64% of GPs claimed they knew that diagnostic criteria existed, only 10% had heard of the Manning criteria, 27% of Rome I, and 17% of Rome II (Figure 1).

Physicians stated that abnormal bowel movements such as diarrhea and constipation, abdominal pain and bloating were the most commonly reported symptoms of IBS (Table 4).

Physicians reported in most cases that they would give advice on diet and education about IBS as a treatment for IBS symptoms. Both GPs and SGs gave their patients mebeverine in most cases. Psyllium was frequently used by SGs and chlordiazepoxide, and clidinium was in some cases used by both GPs and SGs (Table 5).

DISCUSSION

Most physicians have used the method of exclusion when diagnosing patients with IBS. Most community providers also believe IBS is a diagnosis of exclusion rather than using positive criteria to support the diagnosis^[14]. This approach-or lack of one-has therefore been time consuming and costly for the health care system. The importance of a precise diagnostic tool to diagnose IBS

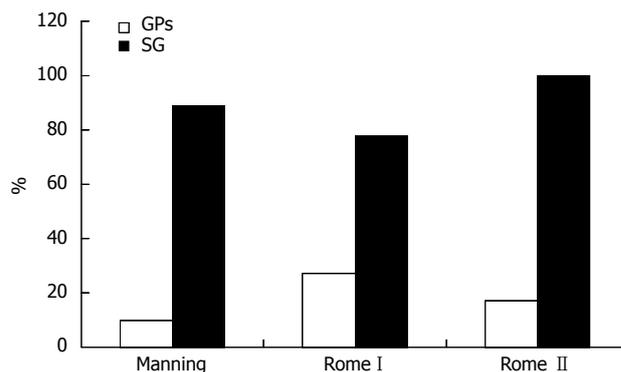


Figure 1 Number of physicians knowing about each set of diagnostic criteria. SG: Specialists in gastroenterology; GP: General practitioners.

Table 4 Most common irritable bowel syndrome symptoms (%)

	GPs	SGs
Abnormal bowel movements	61	100
Abdominal pain	86	67
Bloating	20	56
Gas	9	11
Passage of mucus	5	0
Incomplete evacuation with defecation	5	11

SG: Specialists in gastroenterology; GP: General practitioners.

Table 5 Treatment of irritable bowel syndrome (%)

	GPs	SGs
Medication		
Mebeverine	89	86
Husk	31	43
Chlordiazepoxide and clidinium	29	14
Antidepressants	7	14
Other medicines	9	14
Lifestyle		
Food	98	86
Relaxation	14	14
Exercise	16	14
Education about IBS	90	86
Do not know/ something else	27	14

IBS: Irritable bowel syndrome; SG: Specialists in gastroenterology; GP: General practitioners.

is therefore very important. In recent years, the development of diagnostic criteria for IBS has been ongoing, leading to the recent introduction of the Rome III criteria. There is no doubt that diagnostic criteria constitute a useful and important tool to help physicians make a positive diagnosis of IBS without resorting simply to excluding other diseases. This study has revealed the proportion of Icelandic physicians in two fields of medicine who are aware of the criteria for diagnosing the disease. The study has addressed not only the question of how informed physicians are of the criteria for diagnosing IBS, but also the importance of consensus about the diagnosis of the disease. This study has also addressed the IBS patients' perspective, how many sought physicians, and how they

experienced the disease.

According to the results of this study, most IBS patients were seen by GPs, and this is most likely also the case in other countries, underlining the importance of awareness and knowledge of IBS on the part of the GPs. Although 64% of all GPs reported that they were aware of the fact that special criteria to identify IBS existed, most of them (81%) did not know the criteria and therefore did not rely on them in practice. Most of them seem to make a positive diagnosis of IBS without the use of endoscopy. A United States study showed that only 30% of family practitioners knew that the Manning, Rome and Rome II criteria are used to diagnose IBS, which is in line with the results of the current study^[21]. GPs are more likely than hospital specialists to perceive functional gastrointestinal disorders as having a psychological basis, are far less likely to be familiar with diagnostic criteria, and are more likely to use other methods to make such diagnoses^[31]. However, physicians are aware of and use the most common IBS symptoms such as abnormal bowel movements, abdominal pain and bloating in their diagnostic approach, and these were the most common symptoms IBS subjects in the present study.

In the current study, physicians reported in most cases that they gave advice on diet and education on IBS as a treatment of IBS symptoms; this finding underlines the importance of providing reliable and useful information on IBS to patients, as well as the fact that there are no specific treatment options for IBS that are useful for all patients.

It is of interest that among interviewer-diagnosed IBS patients, only one out of five was diagnosed with IBS, even though more than half of the IBS patients saw a physician because of their symptoms. These results were irrespective of whether the patients fulfilled the Manning or Rome III criteria for IBS. This was also interesting because the majority of IBS patients reported that IBS affected daily activities. This emphasises the question of whether IBS patients reveal to the physicians the low quality of life caused by IBS. It is also conceivable that physicians do not recognize IBS as a disorder that leads to impaired quality of life. The absence of positive diagnosis of IBS might lead to lack of relevant treatment for specific symptoms of IBS such as abdominal pain. There is a need for a simple, practical and reliable diagnostic tool to be used in everyday clinical practice to diagnose IBS more accurately; a tool that will encourage physicians to be able to make a reliable diagnosis and to provide effective treatment^[32,33].

The limitation of this study was the relatively low response rate in the physician study, which raises the question as to whether the level of awareness and knowledge of diagnostic criteria might be even lower than the result obtained. The strengths of the study, however, were that all physicians in Iceland in the relevant fields of general practice and gastroenterology were invited to participate, and the fact that all IBS patients who were contacted by telephone participated in the survey.

In conclusion, in this study, only half of the IBS patients who saw a physician received a diagnosis of IBS. Knowledge of IBS is limited among IBS patients. This study suggests that few physicians use IBS criteria and that awareness and knowledge of diagnostic criteria for IBS differed between SGs and GPs. One out of four physicians used a diagnosis of exclusion.

More widespread knowledge and use of the diagnostic criteria among physicians can be expected to support a more accurate diagnosis of IBS.

COMMENTS

Background

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder. The prevalence of IBS in western countries is estimated to be 10%-15%. The criteria that have been used to identify IBS patients are the Manning criteria and Rome I, II and III criteria. It has been questioned whether the Rome criteria are sensitive enough to diagnose patients in general practice. Many physicians approach IBS as a diagnosis of exclusion. Patients diagnosed with IBS have a higher number of outpatient visits, inpatient stays, outpatient prescriptions, and hospitalizations than those not diagnosed with IBS. IBS patients are often reluctant to consult a physician, often because they think their symptoms do not warrant a visit to a physician, or they are afraid that they have a serious life-threatening illness. United States family practitioners have problems with IBS patients, which include difficulties in satisfying patients and treatment decision making, and finding the time required, and their lack of knowledge could interfere with patient care.

Research frontiers

The prevalence of IBS in the general population is high and physicians often lack the tools to diagnose and treat IBS. It is important for IBS patients as well as the physicians to understand each other and the IBS symptoms, and to improve knowledge of IBS. The aim of the present study was to analyze IBS from the physicians' and IBS patients' points of view. The specific aims of this study were: First, physician study, to assess if and how physicians [general practitioners (GPs), specialists in gastroenterology (SGs)]: (1) use the diagnostic criteria to identify IBS; (2) diagnose patients with IBS, and which symptoms of IBS they identify; and (3) which treatment they recommend; and Second, patient study, to assess how patients with IBS based on criteria are diagnosed and treated by physicians and which treatment they receive, as well as studying the ideas that patients have about IBS.

Innovations and breakthroughs

The prevalence of IBS is high in Iceland. The awareness of IBS is low among patients with IBS and two out of five of those saw physicians because of IBS symptoms. Only half of the IBS patients who saw a physician received a diagnosis of IBS. Knowledge of IBS is limited among patients. This study suggests that few physicians use IBS criteria and that awareness and knowledge of the diagnostic criteria for IBS differed between SGs and GPs. One out of four physicians used a diagnosis of exclusion.

Applications

IBS patient and physician points of view are important for understanding IBS. It is important for the physicians to understand IBS patients and to know that many who seek medical care will not receive a diagnosis. This study creates a database for further studies and hopefully stimulates studies in other countries. International awareness and knowledge of IBS diagnosis and treatment can contribute towards better understanding of IBS.

Peer review

The prevalence of IBS in Western countries is estimated to be 10%-15% and is associated with extensive health care expenses and diminished quality of life. Studies that examine IBS from the patients and physicians point of view are important and there is a need to document secular trends and compare various countries.

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Assessment of the validity of the clinical pathway for colon endoscopic submucosal dissection

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Abstract

AIM: To determine the effective hospitalization period as the clinical pathway to prepare patients for endoscopic submucosal dissection (ESD).

METHODS: This is a retrospective observational study which included 189 patients consecutively treated by ESD at the National Cancer Center Hospital from May 2007 to March 2009. Patients were divided into 2 groups; patients in group A were discharged in 5 d and patients in group B included those who stayed longer than 5 d. The following data were collected for both groups: mean hospitalization period, tumor site, median tumor size, post-ESD rectal bleeding requiring urgent endoscopy, perforation during or after ESD, abdominal pain, fever above 38 °C, and blood test results positive

for inflammatory markers before and after ESD. Each parameter was compared after data collection.

RESULTS: A total of 83% (156/189) of all patients could be discharged from the hospital on day 3 post-ESD. Complications were observed in 12.1% (23/189) of patients. Perforation occurred in 3.7% (7/189) of patients. All the perforations occurred during the ESD procedure and they were managed with endoscopic clipping. The incidence of post-operative bleeding was 2.6% (5/189); all the cases involved rectal bleeding. We divided the subjects into 2 groups: tumor diameter ≥ 4 cm and < 4 cm; there was no significant difference between the 2 groups ($P = 0.93$, χ^2 test with Yates correction). The incidence of abdominal pain was 3.7% (7/189). All the cases occurred on the day of the procedure or the next day. The median white blood cell count was 6800 ± 2280 (cells/ μ L; \pm SD) for group A, and 7700 ± 2775 (cells/ μ L; \pm SD) for group B, showing a statistically significant difference ($P = 0.023$, t -test). The mean C-reactive protein values the day after ESD were 0.4 ± 1.3 mg/dL and 0.5 ± 1.3 mg/dL for groups A and B, respectively, with no significant difference between the 2 groups ($P = 0.54$, t -test).

CONCLUSION: One-day admission is sufficient in the absence of complications during ESD or early post-operative bleeding.

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Key words: Clinical pathway; Colon; Complication; Endoscopic submucosal dissection; Hospitalization period; Rectum

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INTRODUCTION

Conventional laparotomy is the standard treatment for early colon cancer. Subsequently, endoscopic mucosal resection (EMR) was developed for small polyps^[1]. Analysis of surgically resected specimens revealed that in cases of early colon cancer with a depth of invasion of < 1000 μm into the submucosal layer (SM 1), no lymphatic invasion, no vascular involvement, or without a poorly differentiated adenocarcinoma component, curative resection can be obtained by endoscopic treatment^[2,3].

Endoscopic submucosal dissection (ESD) is an advanced technique, compared with EMR, by which higher *en-bloc* resection and lower rates of tumor recurrence are achieved when treating large tumors > 20 mm in diameter^[4-11].

In our institution, gastric ESD has been performed since 1996, and in 2002, a clinical pathway (CP) was introduced to standardize this form of intervention. This CP included a set period of hospitalization to prepare patients and to determine any sign of post-procedure complications. The efficacy of the CP in gastric ESD was then reported^[12]. A similar CP was introduced for colon ESD, which involves a 5 d hospital admission, including a 1 d pre-procedure for bowel preparation. In this study, we examined the appropriateness of this hospitalization period as the CP to prepare patients for ESD and to determine any sign of post-procedure complications.

MATERIALS AND METHODS

In our institution, colon ESD was introduced in 2007, and the CP was implemented in May 2007. All 189 consecutive patients who had colon ESD from May 2007 to March 2009 were included in this study. All used data were recorded in the ESD database.

Patients were divided into 2 groups: group A included patients who were discharged in 5 d and group B included patients who stayed longer than 5 d. The following data were collected for both groups: mean hospitalization period, tumor site, median tumor size, post-ESD rectal bleeding requiring urgent endoscopy, perforation during or after ESD, abdominal pain, fever above 38 °C, and blood test results positive for inflammatory markers before and after ESD.

Perforation during colon ESD was diagnosed when the abdominal cavity could be observed owing to injury of the muscle layer. Cases with no perforation, but with a deep separation of the submucosal layer, enabling the

endoscopist to observe the muscle layer directly were recorded as “exposure of the muscle layer”. Late-onset bleeding was defined as the occurrence of rectal bleeding after ESD, if confirmed by urgent endoscopy. Abdominal pain was defined as the presence of tenderness following examination by a physician or by patient request for analgesia. Late-onset perforation was defined as the finding of free air on abdominal computed tomography or plain X-ray, performed owing to the complaint of abdominal pain. All complications were defined in advance and recorded in the ESD database.

Patients are admitted 1 d before the procedure at noon, and receive a low-fiber diet for lunch and dinner. For bowel preparation on the day of ESD, patients drink 3000 mL of intestinal lavage fluid [polyethylene glycol; (PEG)] over a period of 2 h in the morning. Then, the ward nurse checks their stools. If the bowel preparation is poor, patients will drink an additional 500 mL to 1000 mL of PEG. Otherwise, no food or drink is allowed on the day of the procedure or the following day. The procedure starts in the afternoon after achieving successful bowel preparation. We provide prophylactic antibiotic (cefmetazole 1.0 g, intravenously) just before the procedure.

In general, the ESD procedure is performed using a bipolar needle knife (Xeon Medical Co., Tokyo, Japan), insulation-tipped (IT) knife (Olympus Co., Tokyo, Japan), HemoStat-Y (bipolar forceps for hemostasis, PENTAX, Tokyo, Japan), water jet scope (Olympus Co.), distal attachment (short ST hood, Fujifilm Co., Tokyo, Japan)^[13], and a CO₂ insufflation system (Olympus Co.) for all patients. The high-frequency wave device used is ICC200 (ERBE, Tübingen, Germany); to set the output power, an Endo Cut 50 W/Forced 40 W bipolar needle knife/IT knife is used; a bipolar 25 W is used for the HemoStat-Y.

Conscious sedation is performed to allow positional changes to patients during the procedure. Sedation with midazolam and pentazocine is usually started with 2 mg and 15 mg doses intravenously, respectively, and if required, additional dosing will be provided perioperatively based on the operator’s assessment. Hyoscine butylbromide (Buscopan) (10 mg, intravenously) is administered immediately before the procedure and another 10 mg can be given later if needed.

The morning after the ESD, routine peripheral blood and biochemistry tests are performed. Providing there are no signs or symptoms of complications, patients will start to drink water on day 1 and have meals (rice porridge) on day 2. Patients can only walk to the restroom as they should maintain bed rest the whole day of the ESD procedure. On the next day, they can walk within the hospital ward. If there is no concern with the clinical progression, patients are allowed home on day 3. Patients are instructed to refrain from ingesting alcohol or performing exercise during the first week after hospital discharge.

The ESD procedure is performed by 6 endoscopists, all of whom began performing colon ESD after first experiencing gastric ESD cases.

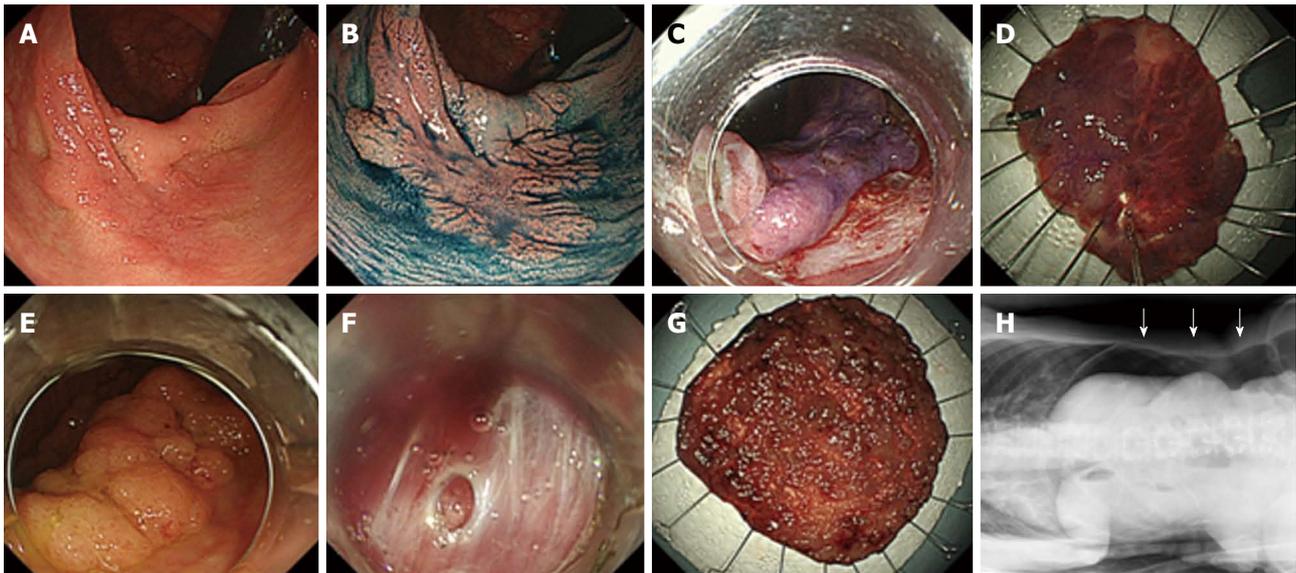


Figure 1 Case without complications and with perforation. A: Tumor located in the ascending colon; B: Image of dye spraying (indigo carmine). The macroscopic type is 0-IIa (LST-NG). The size is 35 mm; C: Treatment by endoscopic submucosal dissection (ESD); D: *En-bloc* resection was performed; E: Tumor located in the cecum. The macroscopic type is 0-IIa (LST-G). The size is 65 mm; F: Perforation occurred during ESD. It was closed by endoscopic clipping; G: *En-bloc* resection was performed by ESD; H: Prominent free air was observed in the abdominal cavity with the patient lying on the left side. The free air is indicated in the Figure by an arrow. LST-NG: Laterally spreading tumors-non-granular; LST-G: Laterally spreading tumors-granular.

Table 1 Results of the patients reviewed

	Group A	Group B	Total or average
Number	156	33	189
Average hospitalization period (d)	4.94	6.67	5.81
Location of lesion			
Colon (%)	108 (69.2)	23 (70.0)	131 (69.0)
Rectum (%)	48 (30.8)	10 (30.3)	58 (31)
Median size of lesion (mm)	34.5	35	35
Hemorrhage (%)	1 (0.6)	4 (12.1)	5 (2.6)
Perforation (%)	1 (0.6)	6 (18.2)	7 (3.7)
Abdominal pain (%)	2 (1.3)	5 (15.2)	7 (3.7)
Fever > 38.0 °C (%)	2 (1.3)	2 (6.1)	4 (2.1)
WBC (cells/ μ L; median)	6800 ^a	7700	7000
Hemoglobin level change pre-/post-ESD > 2.0 (%)	5 (3.2)	1 (3.0)	6 (3.2)
CRP (mg/dL; mean)	0.4 ^b	0.5	0.4

^a $P = 0.023$, ^b $P = 0.54$ vs group B. ESD: Endoscopic submucosal dissection; CRP: C-reactive protein; WBC: White blood cell.

RESULTS

Case presentations

Case without complications: This is the case of a 74-year-old male patient. The tumor was of the macroscopic type, grade 0-IIa laterally spreading tumors-non-granular (LST-NG) with a diameter of 35 mm, located in the ascending colon. *En-bloc* resection was achieved by ESD. The total length of hospital stay was 5 d. Histological examination revealed a well-differentiated adenocarcinoma, low-grade atypia with no lymphatic-vascular invasion, and the lateral and horizontal margins were negative. Curative resection was achieved (Figure 1).

Case with perforation: This is the case of a 58-year-old

female patient. The tumor was of the macroscopic type, grade 0-IIa laterally spreading tumors-granular (LST-G) with a diameter of 65 mm, located in the cecum. *En-bloc* resection was achieved by ESD. A small perforation occurred during the ESD, which was closed by endoscopic clipping immediately after submucosal dissection around the perforation site. Abdominal X-ray showed a small amount of free air, but no abdominal pain was reported or high-grade fever (suggesting peritonitis) observed, so the patient was managed conservatively and stayed for a total of 10 d in the hospital. Histological examination revealed a well-differentiated adenocarcinoma, low-grade atypia with no lymphatic-vascular invasion, and the lateral and horizontal margins were negative. Curative resection was achieved and no surgical treatment was necessary (Figure 1).

Of all the patients, 83% (156/189) could be discharged from the hospital on day 3 post-ESD (group A). On the other hand, the remaining 17% (33/189) of patients required prolonged hospitalization (group B) (Table 1). Complications were observed in 12.1% (23/189) of patients. Perforation was the most commonly observed complication, occurring in 3.7% (7/189) of patients. All the perforations occurred during the ESD procedure and none were of late-onset. They were managed with endoscopic clipping and no patient required surgical intervention. Six out of 7 patients with perforations (86%) were required to stay for more than 5 d.

The incidence of post-operative bleeding was 2.6% (5/189); all the cases involved rectal bleeding. Five cases required hemostatic intervention and 3 of them were in-patient admissions. The period of hospitalization needed to be prolonged for 4 out of the 5 (80%) cases. Two patients had to be re-admitted to undergo emergency

endoscopy due to bleeding which occurred after hospital discharge (post-discharge days 4 and 6); however, bleeding did not recur after that.

To analyze the rates of late-onset bleeding and tumor size, we divided the subjects into 2 groups: one with a tumor diameter < 4 cm (118 patients) and the other with a tumor diameter \geq 4 cm (71 patients). The incidence of post-ESD bleeding was compared. The rates were 5.6% (4/71) for a tumor diameter < 4 cm and 4.2% (5/118) for a tumor diameter \geq 4 cm. There was no significant difference between the 2 groups ($P = 0.93$, χ^2 test with Yates correction).

The incidence of abdominal pain was 3.7% (7/189). All the cases occurred on the day of the procedure or the next day. Of all the patients who had abdominal pain, 70% (5/7) stayed for more than 3 d post-procedure, based on the attending physician's assessment. The most common causes of delayed discharge from the hospital were late-onset bleeding and social reasons (7 patients each). Other complications were as follows: perforation (6 patients), exposure of the muscle layer (6 patients), abdominal pain (5 patients), fever (2 patients), and increased inflammatory reaction (1 patient).

Serum inflammatory markers were also assessed. On the day after ESD, the median white blood cell (WBC) count was 6800 ± 2280 (cells/ μ L; \pm SD) for group A, and 7700 ± 2775 (cells/ μ L; \pm SD) for group B, showing a statistically significant difference ($P = 0.023$, t -test). The mean C-reactive protein (CRP) values the day after ESD were 0.4 ± 1.3 and 0.5 ± 1.3 mg/dL for groups A and B, respectively, with no significant difference between the 2 groups ($P = 0.54$, t -test).

DISCUSSION

The introduction of the CP for colon ESD was demonstrated to be useful for maintaining the safety of ESD and post-procedure care^[12,14-16]. Seventy-nine percent of the patients were discharged on day 3 post-procedure; they had no complications or adverse events requiring medical attention. Three percent had complications, but they did not need to stay any longer. One percent of patients were readmitted 1 week post-procedure due to bleeding.

Looking at the breakdown of the 17% of patients with CP deviation (those who stayed for more than 5 d), it was observed that most cases were due to social reasons. Taking the above into consideration, we conclude that, in the absence of complications during ESD or early post-operative bleeding, the period of admission can be safely shortened to 1 d. However, we have to consider patients' circumstances and traveling requirements. Patients certainly need to be educated before ESD on appropriate ways of responding if symptoms of complications (particularly post-operative bleeding) occur. They may need to be advised to stay in a hotel nearby if they live far away from the endoscopy center. We have no local evidence that inpatient preparation is better than outpatient prepara-

tion. However, to avoid failure of the procedure, and patient dissatisfaction, we have included 1 d hospital stays for these reasons within our CP, particularly since the cost is very low here in Japan. On the other hand, reports from the United States and the United Kingdom have shown no differences between inpatient and outpatient preparation, and the latter situation may even be preferable^[17]. Therefore, a 1 d admission for bowel preparation may not be necessary under all conditions. Omitting this admission would minimize the cost of the procedure.

As mentioned previously, the indications for colon ESD are 0-Is+IIa (LST-G) exceeding 30 mm, LST-NG exceeding 20 mm, IIc and non-lifting sign positive intramucosal lesions, and residual recurrent lesions that cannot be resected by EMR^[18]. This is because the rate of SM invasion of LST-NG lesions is comparatively high, and 27% of them are multifocal invasions, making it difficult to identify the region of invasion before the procedure. Thus, accurate pathological evaluation by reliable *en-bloc* resection is necessary^[3]. In LST-G, 84% of cases of SM invasion are in the macro-nodular area, and if the same area can be resected *en-bloc*, endoscopic piecemeal mucosal resection (EPMR) is also allowed. However, with a 0-Is+IIa (LST-G) exceeding 30 mm, if EPMR is eventually performed, there is the possibility that the pathological assessment of the macro-nodular component will be inaccurate; such lesions are also treated by ESD as a relative indication.

The bowel preparation for colon ESD at our institution consists of domperidone (10 mg) and mosapride citrate hydrate (15 mg) administered with 3000 mL to a maximum of 4000 mL of PEG. This is a more rigorous bowel preparation than that used for conventional colonoscopy. This is to ensure a good field of view during ESD and to prevent diffuse peritonitis due to the discharge of fecal fluid in case perforation occurs^[19].

Currently, there are no fixed guidelines for antibiotics that can be administered prophylactically in colon ESD. In the field of gastroenterological surgery, there is evidence that prophylactic administration of antibiotics is useful in the prevention of wound infection, and broad-spectrum antibiotics are commonly used immediately before surgery. In the field of therapeutic endoscopy of the colon, Ishikawa *et al.*^[20] reported that if the high risk of infectious endocarditis and bacteremia are considered, the administration of antibiotics depended on the type of treatment procedure. This report was on conventional snare polypectomy and hot biopsy. With colon ESD, the risk of perforation is slightly higher than in the above procedures; therefore, we considered it appropriate to provide some form of prophylactic treatment. However, as changes in WBC and CRP level are minimal, there is the possibility that such treatment can be omitted.

We consider the bipolar system (B-knife), which is mainly used in the colon ESD procedure, to be safe^[21]. Although the monopolar system is available as a backup, the IT-knife with an insulated tip that enhances safety is being used^[22,23]. In other institutions, there are those that

mainly use a dual-knife (Olympus Co.) with the monopolar system. Differences between such devices can create differences in the rate of complications and the method of post-ESD management.

The colon ESD performed at our institution has the indications mentioned above and is discussed in the context of the CP. The purpose of this study was to investigate the appropriateness and effectiveness of the 5 d hospitalization period, including 1 d for bowel preparation, as the CP to prepare patients for ESD and to determine any sign of post-procedure complications. However, the attending physician mainly judged the prolongation of the hospitalization period. Although there is no particularly clear standard, the attending physician usually orders the prolongation under any of the following circumstances: (1) when complications, such as perforation and bleeding, are observed; (2) when an ablation on the intrinsic muscle layer at the time of ESD is judged as invasive; and (3) when there may be problems with blood sampling or physical findings the following day. It became clear that in such a case, the time to restart ingestion of water and food was commonly prolonged. At our institution, the incidence of post-ESD bleeding following gastric ESD is approximately 5% and the CP for gastric ESD is 7 d (patients discharged on day 5 after ESD). With the introduction of the CP for colon ESD, the incidence of post-ESD bleeding was lower than gastric ESD bleeding; thus, the period of hospitalization was set at 5 d and safety could be maintained for many patients. The lesions, method and bowel preparation in colon ESD differ according to the institution; therefore, the risks of complications during and after ESD are likely to differ. Hereafter, to stratify the risks in the CP, addition of the status after resection (complete suturing) and the site of the lesion (rectum or colon) as parameters should increase safety.

Limitations

There is no doubt that a 5 d hospitalization period may not be possible in many countries for financial reasons. A randomized control trial would be the best method to evaluate the necessity of post-procedure hospital admission. However, we would like to share our findings from this retrospective observational study which confirm the safety of discharging ESD patients without any complications 1 d after the procedure.

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COMMENTS

Background

Conventional laparotomy is the standard treatment for early colon cancer.

Subsequently, endoscopic mucosal resection (EMR) was developed for small polyps. Analysis of surgically resected specimens revealed that in cases of early colon cancer with a depth of invasion of < 1000 μm into the submucosal layer (SM 1), no lymphatic invasion, no vascular involvement, or without a poorly differentiated adenocarcinoma component, curative resection can be obtained by endoscopic treatment.

Research frontiers

Endoscopic submucosal dissection (ESD) is an advanced technique, compared with EMR, by which higher *en-bloc* resection and lower rates of tumor recurrence are achieved when treating large tumors > 20 mm in diameter.

Innovations and breakthroughs

This is a retrospective observational study which included 189 patients consecutively treated by ESD at the National Cancer Center Hospital from May 2007 to March 2009. The following data were collected for both groups: mean hospitalization period, tumor site, median tumor size, post-ESD rectal bleeding requiring urgent endoscopy, perforation during or after ESD, abdominal pain, fever above 38 $^{\circ}\text{C}$, and blood test results positive for inflammatory markers before and after ESD. Each parameter was compared after data collection.

Applications

The lesions, method and bowel preparation in colon ESD differ according to the institution; therefore, the risks of complications during and after ESD are likely to differ. Hereafter, to stratify the risks in the clinical pathway, addition of the status after resection (complete suturing) and the site of the lesion (rectum or colon) as parameters should increase safety.

Peer review

The paper covers an important topic related to the ESD procedure: the length of the hospital stay and the quality of the monitoring of the patient after the procedure. The clinical problem is well exposed, the picture is impressive and the paper opens a new area of discussion on colonic ESD.

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Analysis of hepcidin expression: *In situ* hybridization and quantitative polymerase chain reaction from paraffin sections

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Abstract

AIM: To establish methods for quantitative polymerase chain reaction (PCR) for hepcidin using RNAs isolated from paraffin-embedded sections and *in situ* hybridization of hepatocellular carcinoma (HCC).

METHODS: Total RNA from paraffin-embedded sections was isolated from 68 paraffin-embedded samples of HCC. Samples came from 54 male and 14 female patients with a mean age of 66.8 ± 7.8 years. Quantitative PCR was performed. Immunohistochemistry and *in situ* hybridization for hepcidin were also performed.

RESULTS: Quantitative PCR for hepcidin using RNAs isolated from paraffin-embedded sections of HCC was

performed successfully. The expression level of hepcidin mRNA in cancer tissues was significantly higher than that in non-cancer tissues. A method of *in situ* hybridization for hepcidin was established successfully, and this demonstrated that hepcidin mRNA was expressed in non-cancerous tissue but absent in cancerous tissue.

CONCLUSION: We have established novel methods for quantitative PCR for hepcidin using RNAs isolated from paraffin-embedded sections and *in situ* hybridization of HCC.

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Key words: Hepcidin; Expression; *In situ* hybridization; Immunohistochemistry; Real-time polymerase chain reaction

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INTRODUCTION

Hepcidin is a key regulator of iron metabolism, binding to the iron-exporter ferroportin and triggering internal-

ization and degradation^[1]. Because both iron deficiency and overload are toxic for the human body, iron concentration is tightly regulated, and hepcidin plays a major role in this process.

Hepcidin was discovered initially as an anti-microbial peptide^[2,3], and a recent study has indicated that hepcidin functions as an acute inflammatory molecule^[4]. Furthermore, the roles of hepcidin in other diseases, including cancer, have been extensively studied^[5,6]. We have previously reported that the expression of hepcidin mRNA is suppressed in hepatocellular carcinoma (HCC)^[7]. In the present study, we isolated RNAs from fresh surgical specimens. There have been no previous reports describing the isolation of RNA from paraffin-embedded sections and quantitative reverse-transcriptional polymerase chain reaction (RT-PCR). It would be valuable if quantitative RT-PCR could be performed using RNAs isolated from paraffin-embedded sections. However, it is well known that hepcidin is a difficult molecule to analyze in this way. Here we report a simple way to isolate hepcidin RNA for RT-PCR from paraffin-embedded sections, and also in situ hybridization for hepcidin.

MATERIALS AND METHODS

Patients

A total of 68 paraffin-embedded samples of HCC were used in this study. The sections were taken from areas that were clearly in the border zone between cancerous and non-cancerous tissues. The mean age of the patients was 66.8 ± 7.8 years, and there were 54 males and 14 females. Liver cirrhosis was observed in 42 patients and chronic hepatitis was diagnosed in 21; only 5 patients lacked chronic hepatitis or liver cirrhosis. Tumor stage was determined based on the classification of the Liver Cancer Study Group of Japan.

Isolation of RNAs from paraffin-embedded sections

Isolation of total RNA from paraffin-embedded sections was performed using an Agencourt Formapure Kit (Beckman-Coulter, Beverly, MA). Briefly, tissue sections were moistened with 20 μ L of lysis buffer, and cancerous and non-cancerous parts were separately cut off with a scalpel and transferred to a 96-well plate (IWAKI, Tokyo, Japan). The removed samples were heated at 72 °C for 60 min, then 20 μ L proteinase K was added, followed by incubation at 55 °C for 60 min. After 2 min of cooling, the lysates were mixed with 150 μ L of Bind I buffer and 320 μ L of Bind II buffer, and incubated at 55 °C for 5 min. The plate containing the digests was placed on a magnetic sheet for 5 min, and then the supernatants were removed. The RNA and bead complexes were washed twice, and the complexes were resuspended with 70% ethanol. The plate was then placed back on the magnetic sheet and the supernatant was again removed. Then, the plate was removed from the magnetic sheet and 100 μ L of DNase solution was added. After 15 min of incubation at 37 °C, 550 μ L of Wash Buffer was added and the plate was left at room

temperature for 5 min. It was then placed again on the magnetic sheet for 10 min, and the supernatant was again removed. The plate was then removed from the magnetic sheet and 750 μ L of 70% ethanol was added, followed by replacement on the magnetic sheet. After 5 min, the supernatant was removed, the plate was taken from the magnetic sheet, and the complex was resuspended in 500 μ L of 90% isopropanol. The plate was then incubated at 70 °C for 3 min, followed by removal from the magnetic sheet and aspiration of the supernatant. The complex was resuspended in 750 μ L of 70% ethanol and the plate was placed back on the magnetic sheet for 5 min, followed by removal of the supernatant. The complex was then air-dried for 10 min and resuspended in 80 μ L nuclease-free water. The plate was then incubated at 65 °C for 30 s, placed back on the magnetic sheet for 1 min, and the supernatant containing RNA was obtained.

Real-time PCR

Reverse transcription reactions were performed using a Rever Tra Ace α -First Strand cDNA Synthesis Kit (Toyobo, Osaka, Japan). Briefly, 1 μ g of total RNA, oligo dT-primer, and dNTPs were incubated at 65 °C for 5 min, then 10 μ L of a cDNA synthesis mixture was added and the mixture was incubated at 50 °C for 50 min. The reaction was terminated by adding 1 μ L of RNaseH and incubating the mixture at 37 °C for 20 min.

Real-time PCR was performed with an ABI Prism 7700 sequence detector (Applied Biosystems, Warrington, United Kingdom). The PCR reaction was carried out in a final volume of 2 μ L cDNA, 12.5 μ L 2 \times SYBR Green (Applied Biosystems), 0.5 μ L of 25 nmol/L sense and antisense primers, and H₂O up to 25 μ L. The PCR conditions consisted of 40 cycles at 95 °C for 30 s and 60 °C for 30 s. The sequences of the primers were as follows: GAPDH: sense-primer 5'-CCACCCAGAAGACTGTGGAT-3', anti-sense 5'-TTCAGCTCAGGGATGACCTT-3'; β -actin: sense-primer 5'-GTCGTACCCTGGCATTGTG-3', anti-sense 5'-CCATCTCTTGCTCGAAGTCC-3'; hepcidin 1: sense-primer 5'-CACAACAGACGGGACAACCTT-3', anti-sense 5'-CGCAGCAGAAAATGCAGATG-3'; hepcidin 2: sense-primer 5'-GACCAGTGGCTCTGTTTTCC-3', anti-sense 5'-CACATCCCACACTTTGATCG-3'. The primer sets for GAPDH, β -actin, and hepcidin 2 were designed using Primer 3 software (ver. 4.0), and that for hepcidin 1 was based on the previous report^[8].

The level of expression was calculated using the formula: Relative expression (t) = (Copy number of target molecule/Copy number of GAPDH)^[8]. Samples were assayed in triplicate. Means and standard deviations were calculated from the data obtained. The *t* value was calculated from the mean of three different assays.

Immunohistochemistry

The tissues were fixed with Tissue Fixative (Genostaff), and then embedded in paraffin, and sectioned at 6 μ m. Tissue sections were deparaffinized with xylene and

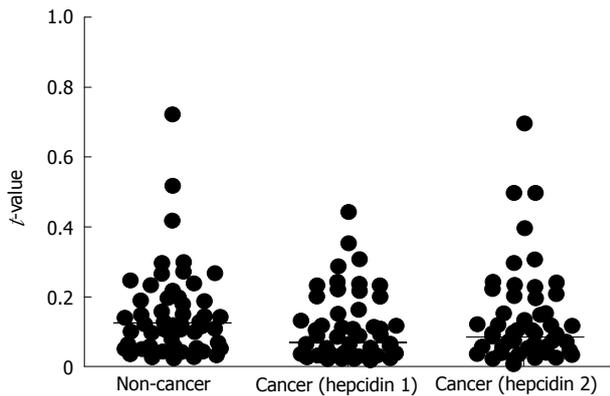


Figure 1 Expression of hepcidin mRNA in hepatocellular carcinoma. Expression of hepcidin mRNA, extracted from paraffin-embedded sections, was suppressed in cancer tissue. The median *t*-values for non-cancer, and cancer tissues with hepcidin 1 and hepcidin 2 primers, were 0.124 (0.021-3.433), 0.067 (0.009-2.063), and 0.0825 (0.007-2.1), respectively. The *t*-values for cancer tissues were significantly higher than that for non-cancer tissues ($P = 0.0057$).

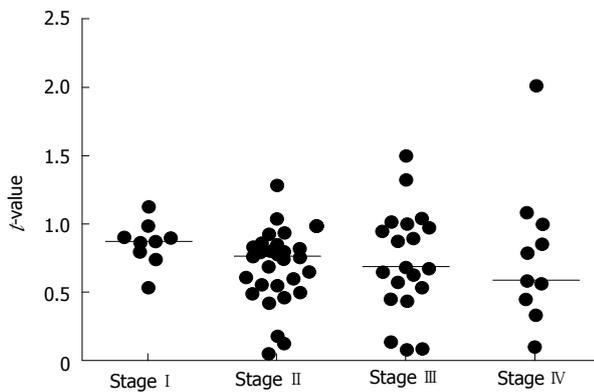


Figure 2 Ratio of hepcidin expression in non-cancer relative to cancer tissues, according to tumor stage. The median *t*-values for stages I, II, III and IV were 0.874 (0.537-1.127), 0.754 (0.052-1.286), 0.678 (0.069-1.494), and 0.579 (0.090-2.013), respectively ($P = 0.3381$, one-way analysis of variance).

rehydrated through an ethanol series and Tris-buffered saline (TBS). Antigen retrieval was performed by microwave treatment for 10 min at 500 W in 1 mmol/L ethylenediaminetetraacetic acid (EDTA) buffer, pH 9.0. Endogenous peroxidase was blocked with 3% H₂O₂ in methanol for 15 min, followed by incubation with Protein Block (DAKO). The sections were incubated with anti-hepcidin-25 antibody (HEPC13-S, Alpha Diagnostic International, San Antonio, TX) at 4 °C overnight. After washing with TBS, the sections were treated with a Biotin Blocking System (DAKO) and biotin-conjugated goat anti-rabbit Ig (DAKO) diluted 1:600, for 30 min at room temperature, followed by addition of peroxidase-conjugated streptavidin (Nichirei) for 5 min. Peroxidase activity was visualized using diaminobenzidine. The sections were counterstained with Mayer's hematoxylin (Muto), dehydrated, and then mounted with Malinol (Muto). Some sections were also stained with hematoxylin-eosin.

In situ hybridization

The tissue sections were dewaxed with xylene and rehy-

drated through an ethanol series and phosphate buffered saline (PBS). The sections were then fixed with 4% paraformaldehyde in PBS for 15 min and washed with PBS. The sections were treated with 15 µg/mL proteinase K in PBS for 30 min at 37 °C, washed with PBS, refixed with 4% paraformaldehyde in PBS, washed again with PBS, and placed in 0.2 mol/L HCl for 10 min, followed by acetylation by incubation in 0.1 mol/L triethanolamine-HCl, pH 8.0, and 0.25% acetic anhydride for 10 min. After a wash with PBS, the sections were dehydrated through an ethanol series. Hybridization was performed with digoxigenin-labeled RNA probes at a concentration of 100 ng/mL in Probe Diluent (Genostaff Co., Ltd., Tokyo, Japan) at 60 °C for 16 h. After hybridization, the sections were washed in 5 × HybriWash (Genostaff Co., Ltd.), equal to 5 × SSC, at 60 °C for 20 min and then in 50% formamide, 2 × HybriWash at 60 °C for 20 min, followed by RNase treatment in 50 µg/mL RNaseA in 10 mmol/L Tris-HCl, pH 8.0, 1 mol/L NaCl and 1 mmol/L EDTA. Then the sections were washed twice with 2 × HybriWash at 60 °C for 20 min, twice with 0.2 × HybriWash at 60 °C for 20 min and once with TBS containing 0.1% Tween 20 (TBST). After treatment with 0.5% blocking reagent (Roche) in TBST for 30 min, the sections were incubated with anti-DIG alkaline phosphatase (AP) conjugate (Roche) diluted 1:1000 with TBST for 2 h. The sections were washed twice with TBST and then incubated in 100 mmol/L NaCl, 50 mmol/L MgCl₂, 0.1% Tween 20, and 100 mmol/L Tris-HCl, pH 9.5. Coloring reactions were performed with BM purple AP substrate (Roche) overnight and then washed with PBS. The sections were counterstained with Kemechtrot stain solution (Muto), dehydrated, and then mounted with Malinol (Muto). The DNA fragment used for the probes was a 246-bp fragment corresponding to nucleotide positions 72-317 of human hepcidin (GenBank accession number NM_021175).

RESULTS

Real-time PCR

Using the method described in Materials and Methods, quantitative RT-PCR was performed for all samples. The median *t*-values for non-cancer and cancer tissues with the hepcidin 1 and hepcidin 2 primers were 0.124 (0.021-3.433), 0.067 (0.009-2.063), and 0.0825 (0.007-2.1), respectively. The *t*-values for cancer tissues were significantly higher than those for non-cancer tissues ($P = 0.0057$) (Figure 1). The assays were repeated using primers for β-actin, and the results were the same as those shown in Figure 1. Figure 2 shows the ratio of *t*-values for non-cancer to cancer tissues according to tumor stage. The median ratio of *t*-values for stages I, II, III and IV were 0.874 (0.537-1.127), 0.754 (0.052-1.286), 0.678 (0.069-1.494), and 0.579 (0.090-2.013), respectively ($P = 0.3381$; one-way analysis of variance).

Figure 3 shows a representative result of *in situ* hybridization for hepcidin. Positive staining for hepcidin was observed in non-cancerous tissues by immunohisto-

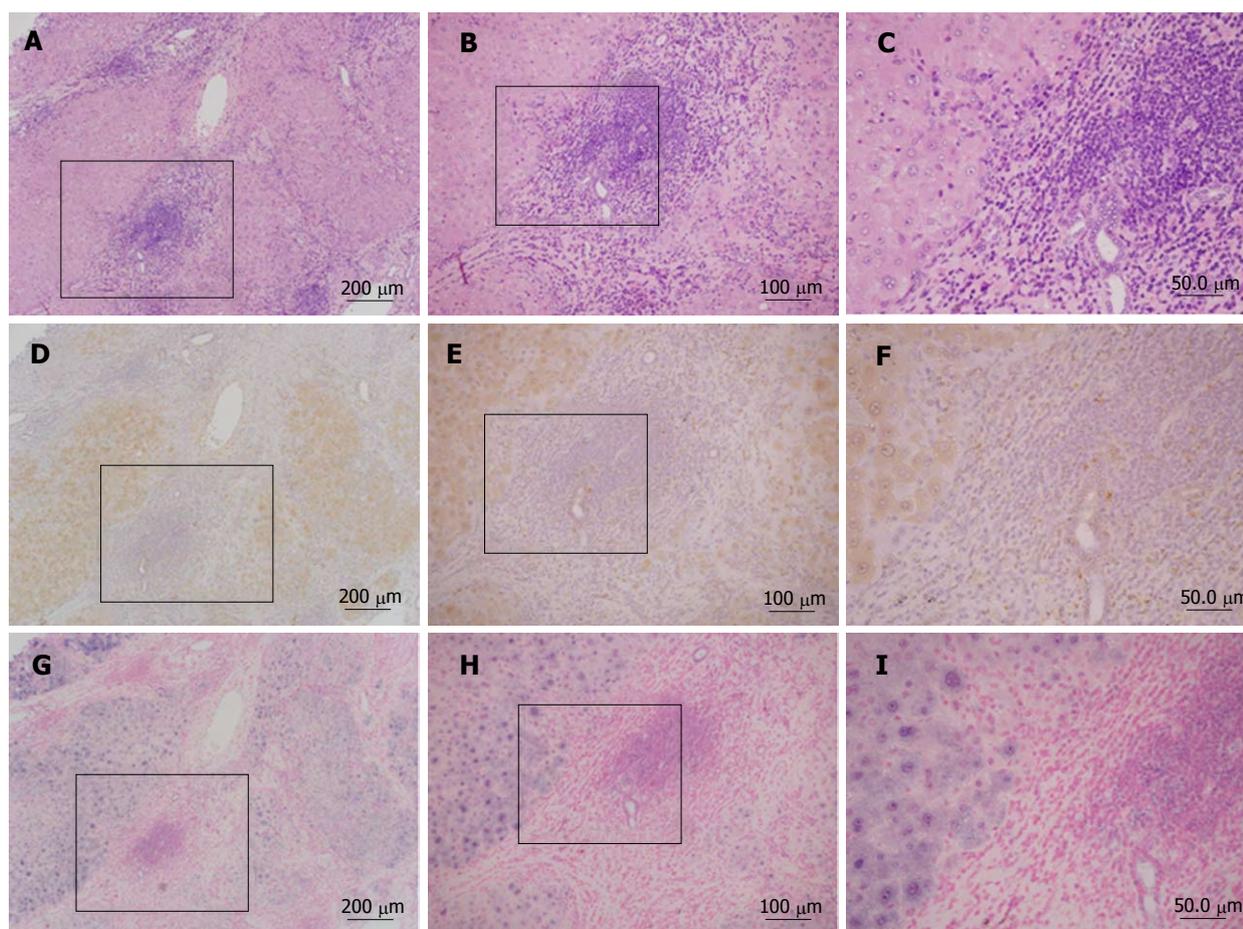


Figure 3 *In situ* hybridization for hepcidin. A surgical specimen was stained with hematoxylin-eosin (A: $\times 100$, B: $\times 200$, C: $\times 400$), and also subjected to immunohistochemistry (D: $\times 100$, E: $\times 200$, F: $\times 400$) and *in situ* hybridization (G: $\times 100$, H: $\times 200$, I: $\times 400$). Hematoxylin-eosin staining shows cancer tissues surrounded by non-cancerous hepatic lobules. Immunohistochemistry shows positive staining for hepcidin in the non-cancerous hepatic lobules (indicated as yellow in the figures). *In situ* hybridization for hepcidin shows clear expression of hepcidin mRNA in non-cancerous tissues (blue in the figures). Squares represent magnified areas.

chemistry (Figure 3D-F). Accordingly, by *in situ* hybridization, hepcidin mRNA was expressed in non-cancerous tissue but was absent in cancerous tissue (Figure 3G-I).

DISCUSSION

As we have reported previously, hepcidin gene expression was decreased in HCC^[7]. In the previous study, we extracted RNAs from freshly resected surgical specimens. This time, however, we aimed to investigate hepcidin gene expression using RNAs obtained from paraffin-embedded sections. However, for reasons that are unclear, this was not an easy process. We finally succeeded in isolating hepcidin RNA using the method described here. The expression of hepcidin mRNA was lower in cancerous than in non-cancerous tissues in most of the samples, and although not statistically significant, the ratios of the level of hepcidin mRNA in non-cancer to cancer tissues tended to be lower in poorly differentiated than in well differentiated HCCs.

Visualizing the expression of hepcidin is very important when analyzing which types of cells express or

do not express the molecule. For this purpose, *in situ* hybridization is an ideal approach, but so far no studies have succeeded with *in situ* hybridization for hepcidin. As shown in Figure 3, hepcidin mRNA was clearly detected in non-cancerous tissue, and not in cancerous tissue. Thus, the present *in situ* hybridization technique should contribute to investigations of the role of hepcidin in HCC.

COMMENTS

Background

Hepcidin is a key regulator of iron metabolism, binding to the iron-exporter ferroportin and triggering internalization and degradation. Hepcidin functions as an acute inflammatory molecule. The authors have previously reported that the expression of hepcidin mRNA is suppressed in hepatocellular carcinoma (HCC). In the present study, the authors isolated RNAs from fresh surgical specimens. There have been no previous reports describing the isolation of RNA from paraffin-embedded sections and quantitative reverse-transcriptional polymerase reaction (RT-PCR). It would be valuable if quantitative RT-PCR could be performed using RNAs isolated from paraffin-embedded sections. However, it is well known that hepcidin is a difficult molecule to analyze in this way. In the present study, the authors devised a simple way to isolate hepcidin RNA for RT-PCR from paraffin-embedded sections, and also performed *in situ* hybridization for hepcidin.

Research frontiers

There have been no previous reports of isolation of RNA from paraffin-embedded sections and quantitative RT-PCR. It would be valuable if quantitative RT-PCR could be performed using RNAs isolated from paraffin-embedded sections.

Innovations and breakthroughs

The authors established novel methods for quantitative PCR for hepcidin using RNAs isolated from paraffin-embedded sections and *in situ* hybridization of HCC.

Applications

The methods described in this paper will help to clarify the role of hepcidin in various disorders.

Peer review

This technical report relates to the analysis of hepcidin expression in the liver tissues of paraffin sections from patients with hepatocellular carcinoma using *in situ* hybridization and quantitative polymerase chain reaction. It is a nice technical note and well written, and should be relevant to other investigators in general.

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Double layered self-expanding metal stents for malignant esophageal obstruction, especially across the gastroesophageal junction

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Abstract

AIM: To evaluate the clinical outcomes of double-layered self-expanding metal stents (SEMS) for treatment of malignant esophageal obstruction according to whether SEMS crosses the gastroesophageal junction (GEJ).

METHODS: Forty eight patients who underwent the SEMS insertion for malignant esophageal obstruction were enrolled. Patients were classified as GEJ group

(SEMS across GEJ, 18 patients) and non-GEJ group (SEMS above GEJ, 30 patients) according to SEMS position. Double layered (outer uncovered and inner covered stent) esophageal stents were placed.

RESULTS: The SEMS insertion and the clinical improvement were achieved in all patients in both groups. Stent malfunction occurred in seven patients in the GEJ group and nine patients in the non-GEJ group. Tumor overgrowth occurred in five and eight patients, respectively, food impaction occurred in one patient in each group, and stent migration occurred in one and no patient, respectively. There were no significant differences between the two groups. Reflux esophagitis occurred more frequently in the GEJ group (eight vs five patients, $P = 0.036$) and was controlled by proton pump inhibitor. Aspiration pneumonia occurred in zero and five patients, respectively, and tracheoesophageal fistula occurred in zero and two patients, respectively.

CONCLUSION: Double-layered SEMS are a feasible and effective treatment when placed across the GEJ for malignant esophageal obstruction. Double-layered SEMS provide acceptable complications, especially migration, although reflux esophagitis is more common in the GEJ group.

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Key words: Metal stent; Gastroesophageal junction; Malignant esophageal obstruction

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INTRODUCTION

Despite recent developments in diagnosis and treatment, more than 50% of malignancies involving the esophagus are inoperable and the 5-year survival rates are < 10% at present^[1,2]. In advanced or metastatic patients, therapy is usually palliative in nature, with the major aims being relief of dysphagia, maintenance of nutrition, or closure of the tracheoesophageal fistula. Self-expanding metal stents (SEMS) are widely used for the relief of patients with dysphagia, providing rapid, safe, and effective symptomatic relief in most patients^[3-6]. However, placing a stent across the gastroesophageal junction (GEJ) face several limitations due to anatomic features leading to a higher migration rate and gastroesophageal reflux^[7-10].

Covered stents are now the most commonly used type in esophageal obstruction to prevent tumor ingrowth. But, covered stents are more likely to migrate than uncovered stents, especially in the region of the GEJ. Therefore, uncovered stents should be considered to prevent migration in those cases. However, considering the risk of stent obstruction owing to tumor in-growth and stent migration in GEJ tumors, a newly designed stent with advantages of uncovered (low migration) and covered (low tumor in-growth) stents should be considered^[11-13].

Although several studies have reported the efficacy of SEMS in malignant esophageal obstruction, there are only limited data about stenting across the GEJ^[14-16]. Because placement of SEMS across the GEJ has a tendency of higher migration, we evaluated the clinical outcomes of double-layered SEMS for treatment of malignant esophageal obstruction according to whether SEMS crossed the GEJ.

MATERIALS AND METHODS

Patients

Between February 2005 and June 2010, we enrolled a consecutive series of 48 patients with malignant esophageal obstruction who underwent SEMS placement. All patients had pathologically proven esophageal or gastric cardiac carcinoma. None were candidates for curative surgical treatment because of advanced or metastatic disease or poor functional status. Informed consent for stent placement was obtained from each patient before

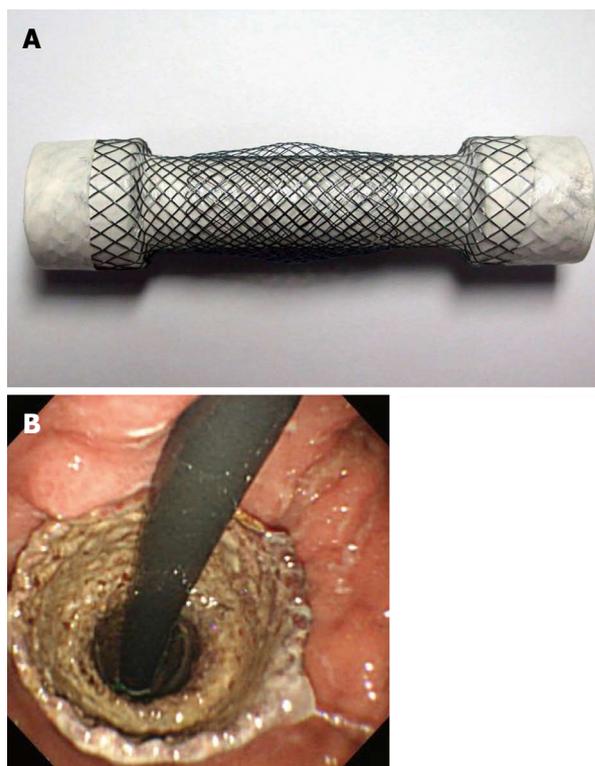


Figure 1 Niti-S double layered stent and endoscopic finding. A: Niti-S double layered esophageal stent, consisting of an inner polyurethane membrane layer and an outer uncovered nitinol wire; B: Endoscopic view of an expanding Niti-S stent, seen from the stomach.

SEMS placement.

SEMS construction and placement

Double-layered stent (Niti-S double layered esophageal stent; Taewoong, Seoul, South Korea) was used throughout the study. The double-layered stent was designed to have the advantages of both uncovered (low migration rate) and covered (low in-growth rate) stents to resolve some of the disadvantages of preexisting metal stents. The stents consist of an inner polyurethane membrane covered stent to prevent tumor ingrowth and an outer uncovered stent to reduce migration. The outer uncovered stent overlapped in the central portion of the covered stent (Figure 1). Because there is space between the covered and uncovered stent in the central portion, this space permits tissue and tumor growth throughout. Thus, the double-layered stent becomes anchored to the esophageal wall.

Patients were consciously sedated with intravenous midazolam during the procedure. Under fluoroscopic and endoscopic visualization, the length of the stricture was measured. A stent 2-4 cm longer than the length of the stricture was chosen to allow for a 1-2 cm extension above and below the proximal and distal tumor margin, respectively. After the guidewire was inserted through the stricture, the delivery system of the stent was gently introduced. After the stent placement, the positioning of the stent was assessed both radiographically and endoscopically.

	Overall (<i>n</i> = 48)	GEJ obstruction (<i>n</i> = 18)	Non-GEJ obstruction (<i>n</i> = 30)	<i>P</i> value
Age (yr, mean ± SD)	68.4 ± 10.5	65.8 ± 10.3	69 ± 10.5	0.749
Male	39 (81.3)	12 (25.0)	27 (56.2)	0.045
Histological diagnosis				
Squamous cell carcinoma	32 (66.6)	2	30	0.001
Adenocarcinoma	16 (33.4)	16	0	0.001
Type of treatment before SEMs placement				
Supportive care	29 (60.4)	10	19	0.594
Chemotherapy	11 (22.9)	7	4	0.041
Radiation therapy	7 (14.6)	1	6	0.170
Chemoradiation therapy	1 (2.1)	0	1	0.434

GEJ obstruction, distal esophageal and gastroesophageal junction obstruction; Non-GEJ obstruction, middle and upper esophageal obstruction. GEJ: Gastroesophageal junction; SEMs: Self-expanding metal stents.

Assessment of clinical outcomes and complications

The technical success of stent placement was defined as adequate positioning and deployment of the stent with complete bridging of the stricture. For the assessment of clinical improvement, we used a dysphagia score: score 0, ability to eat a normal diet; score 1, ability to eat some solid food; score 2, ability to eat some semisolids only; score 3, ability to swallow liquids only; score 4, complete inability to swallow^[17]. This was measured before and 1–2 weeks after the stent placement. We evaluated stent malfunction due to tumor overgrowth, stent migration, or food impaction and other complications including reflux esophagitis, aspiration pneumonia, or fistula.

Statistical analysis

Differences between categorical variables were examined statistically using the χ^2 test. Dysphagia scores before and after the stent placements were analyzed by the paired-*t* test. The incidence of complications was compared between two groups using the χ^2 test. All statistical analyses were performed with SPSS 12.0 (SPSS, Chicago, IL). A *P* value < 0.05 was considered statistically significant.

RESULTS

The clinical characteristics of the patients are shown in Table 1. Forty eight patients (39 men, mean age 68.4 years, range 45–86 years) with esophageal obstruction underwent SEMs placement. The histological types were squamous cell carcinoma in 32 patients (66.6%) and adenocarcinoma in 16 (33.4%). The location of obstruction was in the GEJ in 18 patients (37.5%) and non-GEJ in 30 patients (62.5%). In our study, GEJ obstruction meant that the location of obstruction was in the far distal esophagus and GEJ. Non-GEJ obstruction meant that the location of obstruction were in the upper, middle, and proximal distal esophagus. Before stent placement, 39.6% (19/48) of patients received anticancer treatments

Outcomes	Overall (<i>n</i> = 48)	GEJ obstruction (<i>n</i> = 18)	Non-GEJ obstruction (<i>n</i> = 30)
Technical success	48 (100)	18 (100)	30 (100)
Clinical success	48 (100)	18 (100)	30 (100)
Mean dysphagia score preprocedure (mean ± SD)	3.20 ± 0.68	3.05 ± 0.63	3.30 ± 0.70
Mean dysphagia score postprocedure (mean ± SD)	1.77 ± 0.77	1.77 ± 0.73	1.76 ± 0.81
Change dysphagia score (paired- <i>t</i> test) (<i>P</i> value)	< 0.001	< 0.001	< 0.001

GEJ obstruction, distal esophageal and gastroesophageal junction obstruction; Non-GEJ obstruction, middle and upper esophageal obstruction. GEJ: Gastroesophageal junction.

such as chemotherapy (*n* = 11, 22.9%), radiation therapy (*n* = 7, 14.6%) or chemoradiation therapy (*n* = 1, 2.1%). The remaining (*n* = 29, 60.4%) patients received supportive care. Patients were classified as GEJ (18 patients including distal esophageal cancer and GEJ cancer patients) and non-GEJ groups (30 patients) according to SEMs position. In GEJ group, SEMs placed across GEJ and distal flange portion of SEMs did not anchor to the esophageal wall but was free in the cardia portion. Baseline characteristics are similar except for histologic types.

The placement of esophageal stents was technically successful in all patients. Clinical success rates for malignant esophageal obstruction were 100%. The mean dysphagia score improved from 3.20 ± 0.68 to 1.77 ± 0.77 (*P* < 0.001) by stent placement. The mean dysphagia score improved from 3.05 ± 0.63 to 1.77 ± 0.73 (*P* < 0.001) and 3.30 ± 0.70 to 1.76 ± 0.81 (*P* < 0.001) in GEJ obstruction and in non-GEJ obstruction, respectively (Table 2). Most patients complained of mild foreign body sensation or pain immediately after the stent placement and were getting better and free of these symptoms after several days. There was no procedure-related mortality within 24 h of the intervention.

Stent malfunctions developed in 16 (33.3%) patients [seven (38.9%) for GEJ group and nine (30.0%) for non-GEJ group], the causes of which consisted of tumor overgrowth (13 patients, 27%), stent migration (one patient, 2.1%) and food impaction (two patients, 4.2%). Tumor overgrowth, stent migration, and food impaction occurred in five (27.8%) and eight (26.7%) patients, zero (5.6%) and zero patient, and one (5.6%) and one (3.3%) patient in the GEJ and non-GEJ obstruction, respectively (Table 3). There was no significant difference between GEJ and non-GEJ groups.

Twenty patients (41.6%) showed complications such as reflux esophagitis (13 patients, 27.1%), aspiration pneumonia (five patients, 10.4%), and tracheoesophageal fistula (two patients, 4.2%). Only reflux esophagitis occurred more frequently in GEJ group [eight (44.4%) *vs* five (16.7%), *P* = 0.036]. Aspiration pneumonia and tracheoesophageal fistula occurred in zero and five (16.7%) and zero and two (6.7%) patients in the GEJ and non-GEJ

Table 3 Complications and causes of stent malfunctions *n* (%)

	Overall (<i>n</i> = 48)	GEJ obstruction (<i>n</i> = 18)	Non-GEJ obstruction (<i>n</i> = 30)	<i>P</i> value
Causes of stent malfunctions				
Overgrowth	13 (27.1)	5 (27.8)	8 (26.7)	0.933
Migration	1 (2.1)	1 (5.6)	0 (0.0)	0.192
Food impaction	2 (4.2)	1 (5.6)	1 (3.3)	0.709
Other complications				
Reflux esophagitis	13 (27.1)	8 (44.4)	5 (16.7)	0.036
Aspiration pneumonia	5 (10.4)	0 (0.0)	5 (16.7)	0.067
Fistula	2 (4.2)	0 (0.0)	2 (6.7)	0.263

GEJ obstruction, distal esophageal and gastroesophageal junction obstruction; Non-GEJ obstruction, middle and upper esophageal obstruction. GEJ: Gastroesophageal junction.

group, respectively. These complications were managed successfully with administration of proton pump inhibitors, antibiotics, or another covered stent, respectively.

DISCUSSION

Given the increased incidence of carcinoma involving the distal esophagus and gastric cardia, SEMs are increasingly being deployed across the gastroesophageal junction. Although endoscopic placement of SEMs has become an easy and safe palliative treatment for malignant esophageal obstructions, placing a stent across the GEJ still has several problems associated with anatomic location, such as stent migration and reflux esophagitis^[10]. However, little data has been published focusing on esophageal stenting across the GEJ.

In the present study, SEMs were shown to be a safe and effective treatment for malignant GEJ obstruction, providing a technical and clinical success rate of 100% and no procedure-related major complications. In comparison to another study^[18], our technical and clinical success rate was similar.

Stent placement in GEJ is especially vulnerable to migration, because the distal portion of the stent projects freely into the gastric fundus without fixation to the esophageal or gastric wall^[9]. In other studies, migration rates for covered stents have varied considerable, from 7% to as high as 50%^[18-22]. Especially, migration rates for covered stent placement in GEJ was reported as 20%^[18]. But, another study of migration rates for double layered stent placement in GEJ was reported as 4.7%^[11]. In our study, stent migration developed in one (5.6%) patient in the GEJ group. It has been recognized that stent design may play a role in reducing migration. The double layered stent was designed to reduce stent migration (Figure 1). This device combines two specific characteristics. First, it flares to 24-28 mm at both ends; this size was chosen to minimize the risk of stent-related esophageal complications. Second, it has a double-layered configuration, consisting of an inner silicone membrane layer and an outer uncovered nitinol wire to allow the mesh of the stent to embed itself in esophageal wall. So, our low migration

seems to be associated with outer uncovered stent portion. A central uncovered stent makes space between the uncovered and covered stent in the central portion of double layered SEMs. Adjacent tumor or normal tissue invades through mesh and anchor SEMs firmly to the esophageal wall. Furthermore, inner covered stent resists to tumor in-growth. So, double layered SEMs placement in GEJ is effective for prevention of stent migration with advantage of covered stent.

Overall 27% of patients experienced a symptomatic reflux esophagitis, which is similar to incidence of other studies (27%)^[10,19]. Placement of the stent across the GEJ eliminates the sphincter function of esophagus and permits free reflux of gastric contents into esophagus. Patients experience reflux from an increase in intraabdominal pressure and also passive reflux when gravity is eliminated^[23]. Thus, stenting across the GEJ seems to lead gastroesophageal reflux and even aspiration pneumonia. Weston *et al*^[24] reported gastroesophageal reflux disease, including cases of aspiration and death, in 27% of patients who had SEMs placed across the GEJ. Valbuena also reported cases of heartburn and coughing spells in 27% of patients in whom a standard open stent was used for palliative treatment of GEJ cancer^[25]. It is widely known that reflux esophagitis is more common westerners than asians. But, in our study, reflux esophagitis (44.4%) was more frequent developed by SEMs insertion in GEJ obstruction, compared to other studies (27%). Because our study was retrospective in nature, reflux esophagitis might be overestimated and multiple post-procedure symptoms could be considered as reflux esophagitis symptoms. For example, not only heartburn and regurgitation, but also chest pain and epigastric pain could be regarded as reflux esophagitis symptoms. Asians complain of atypical reflux esophagitis symptoms more frequently than westerners^[26] and 30% of atypical reflux esophagitis symptoms are related chest pain and epigastric pain^[27].

As reported on another study^[28], proton pump inhibitors were effective for controlling gastroesophageal reflux disease symptoms in most cases. Although several anti-reflux stents have been developed to prevent reflux, the results are conflicting^[29-31]. These stents also have demerits of higher rates of migration and gas bloating after meals^[32]. As our and Sabharwal *et al*^[33] results, conventional SEMs placement with a proton pump inhibitor seems to be useful alternative to manage reflux in patients with stenting across GEJ.

Our study has several limitations. First, since our study was a retrospective study, bias in evaluation of reflux esophagitis might be involved. Second, we did not take into consideration of anticancer treatment including chemotherapy and/or radiation therapy before SEMs placement. It is possible that these may cause different clinical outcomes in stent patency according to the oncologic outcomes of anticancer treatments. Consequently, such differences might have affected results of our study.

In conclusion, double-layered SEMs are feasible and

effective treatment when placing SEMS across GEJ for malignant esophageal obstruction. Double-layered SEMS provide acceptable complications, especially migration, although reflux esophagitis is more common in GEJ group.

COMMENTS

Background

There is not yet few data focusing esophageal stenting across gastroesophageal junction (GEJ). Because placement of self-expanding metal stents (SEMS) across the GEJ has a tendency of higher migration, the authors evaluated the clinical outcomes of double-layered SEMS for treatment of malignant esophageal obstruction according to whether SEMS across gastroesophageal junction.

Research frontiers

Placing a stent across the GEJ have several limitations due to anatomic features leading to a higher migration rate and gastroesophageal reflux. Double-layered SEMS provide acceptable complications, especially migration, although reflux esophagitis is more common in GEJ group.

Innovations and breakthroughs

The double-layered stent has the advantages of both uncovered (low migration rate) and covered (low in-growth rate) stents. The authors demonstrated the effectiveness of double-layered SEMS for treatment of malignant esophageal obstruction.

Applications

Double-layered SEMS are feasible and effective treatment when placing SEMS across GEJ for malignant esophageal obstruction.

Terminology

Double layered SEMS means that the stent consists of an outer uncovered and an inner covered stent. Dysphagia score is defined as follows: score 0, ability to eat a normal diet; 1, ability to eat some solid food; 2, ability to eat some semisolids only; 3, ability to swallow liquids only; and 4, complete inability to swallow.

Peer review

The paper is well structured and the results are presented and discussed in a good way.

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Vitamin D deficiency: Correlation to interleukin-17, interleukin-23 and P^{III}NP in hepatitis C virus genotype 4

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Abstract

AIM: To assess vitamin D (Vit D) abnormalities in hepatitis C infected patients and their relationship with interleukin (IL)-17, IL-23 and N-terminal propeptide of type III pro-collagen (P^{III}NP) as immune response mediators.

METHODS: The study was conducted on 50 Egyptian patients (36 male, 14 female) with hepatitis C virus (HCV) infection, who visited the Hepatology Outpatient Clinic in the Endemic Disease Hospital at Cairo University. Patients were compared with 25 age- and sex-matched healthy individuals. Inclusion criteria were based on a history of liver disease with HCV genotype 4 (HCV-4) infection (as new patients or under follow-up). Based on ultrasonography, patients were classified into four subgroups; 14 with bright hepatomegaly; 11 with perihepatic fibrosis; 11 with hepatic cirrhosis; and 14 with cirrhosis and hepatocellular carcinoma (HCC).

Total Vit D (i.e., 25-OH-Vit D) and active Vit D [i.e., 1,25-(OH)₂-Vit D] assays were carried out using commercial kits. IL-17, IL-23 and P^{III}NP levels were assayed using enzyme linked immunosorbent assay kits, while HCV virus was measured by quantitative and qualitative polymerase chain reaction.

RESULTS: Levels of Vit D and its active form were significantly lower in advanced liver disease (hepatic cirrhosis and/or carcinoma) patients, compared to those with bright hepatomegaly and perihepatic fibrosis. IL-17, IL-23 and P^{III}NP levels were markedly increased in HCV patients and correlated with the progression of hepatic damage. The decrease in Vit D and active Vit D was concomitant with an increase in viral load, as well as levels of IL-17, IL-23 and P^{III}NP among all subgroups of HCV-infected patients, compared to normal healthy controls. A significant negative correlation was evident between active Vit D and each of IL-17, IL-23 and P^{III}NP ($r = -0.679, -0.801$ and -0.920 at $P < 0.001$, respectively). HCV-infected men and women showed no differences with respect to Vit D levels. The viral load was negatively correlated with Vit D and active Vit D ($r = -0.084$ and -0.846 at $P < 0.001$, respectively), and positively correlated with IL-17, IL-23 and P^{III}NP ($r = 0.951, 0.922$ and 0.94 at $P < 0.001$, respectively). Whether the deficiency in Vit D was related to HCV-induced chronic liver disease or was a predisposing factor for a higher viral load remains to be elucidated.

CONCLUSION: The negative correlations between Vit D and IL-17, IL-23 and P^{III}NP highlight their involvement in the immune response in patients with HCV-4-related liver diseases in Egypt.

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Key words: Vitamin D; Interleukin-17; Interleukin-23; N-terminal propeptide of type III pro-collagen; Hepatitis genotype 4

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INTRODUCTION

Hepatitis C virus genotype 4 (HCV-4) is the most common variant of hepatitis C virus (HCV) in the Middle East and Africa, particularly Egypt. This region has the highest prevalence of HCV worldwide, with > 90% of infections due to HCV-4, which is considered a major cause of chronic hepatitis, liver cirrhosis, hepatocellular carcinoma (HCC), and liver transplantation in the country^[1]. HCV-4 has recently spread beyond its strongholds in Africa and the Middle East to several western countries, particularly in Europe, due to variations in population structure, immigration and routes of transmission. However, the features of this genotype and management strategies for patients infected with this genotype are not well developed^[2].

HCV is remarkably efficient at establishing persistent infections. This suggests that HCV has evolved one or more strategies to evade the host immune response, among which are effects upon T-lymphocyte responses, including interferon (IFN)- γ production; documented by the severely suppressed T-lymphocyte responses in patients with chronic HCV infections^[3].

Vitamin D (Vit D) is a critical regulator of immunity, playing a role in both innate and cell-mediated immune responses^[4]. Vit D suppresses production of T-helper (Th)-1 lymphocyte type cytokines, such as IFN- γ and interleukin (IL)-2, and consequently leads to an enhanced production of Th-2 cytokines, such as IL-4 and IL-5, thereby promoting humoral immune responses. Vit D also endorses innate immunity by directly inducing gene expression of antimicrobial peptides, such as cathelicidin and β -defensin 2, in various human cell types^[5-8]. Vit D deficiency has been shown to associate several immune-mediated diseases, as well as to increase susceptibility to both infections and cancer. Specifically, a 25(OH)-Vit D concentration < 50 nmol/L (i.e., 20 ng/mL) is accepted as a marker of deficiency, whereas a concentration of 51-74 nmol/L (21-29 ng/mL) indicates insufficiency^[8,9].

Chronic hepatic cirrhotic patients with HCV genotype 1 have low serum 25(OH)-Vit D levels, and a low Vit D status is linked to severe fibrosis and a low sustained virological response (SVR) during IFN- α -based

therapy^[10,11]. Moreover, there is interesting preliminary data that indicate that 1,25-(OH)₂ Vit D suppresses Th-17 driven cytokine responses and the differentiation and maturation of B lymphocytes, while it induces formation/activation of T-regulatory lymphocytes, stimulates IL-4 production (Th-2), and enhances natural-killer-T-cell functions^[12,13].

It has also been shown that treatment with Vit D receptor (VDR) agonists inhibits T-lymphocyte production of IL-17, which is a potent mediator of delayed-type reactions. It achieves this effect (in a manner similar to IFN γ) by elevating chemokine production in various tissues that, in turn, leads to recruitment of monocytes and neutrophils to the site of inflammation. Furthermore, IL-17 acts synergistically with tumor necrosis factor (TNF)- α and IL-1^[14] to stimulate immune functions, and its production is sustained by IL-23, an IL-12 family member, the latter of which is strongly inhibited by VDR agonists^[15]. IL-23, in conjunction with IL-6 and transforming growth factor (TGF)- β , also stimulate the differentiation of Th-17 cells with the subsequent production of IL-17^[16].

The aim of the work was to assess Vit D status in HCV-4-infected patients and its relationship to levels of IL-17 and IL-23, as well as the N-terminal propeptide of type III pro-collagen (PIII^{NP}), the direct serologic marker of collagen turnover, as immuno-inflammatory mediators.

MATERIALS AND METHODS

Subjects

Prior to initiation, this study received approval by the Ethical Committee of the Faculty of Medicine, Cairo University. The study recruited 50 patients with HCV-related chronic liver disease (minimum duration of 7 years; group I) who visited the Hepatology Outpatient Clinic in the Endemic Disease Hospital at Cairo University. Inclusion criteria were based on a history of liver disease with HCV-4 infection (as new patients or under follow-up). Patients with hepatitis B virus (HBV) or co-infection with HBV and human immunodeficiency virus were excluded. All included patients underwent tests for liver function and abdominal ultrasonography, and were tested for the presence of HCV antibodies. When fulfilled, the investigated patients included 36 men and 14 women, ranging in age from 30 to 55 years (mean age = 42.5 years). Twenty-five age- and sex-matched healthy individuals were then recruited as a control group (group II). The controls had normal liver functions and abdominal ultrasonography and negligible HCV antibody levels. Informed consent was obtained from the patients and controls regarding all the procedures. All patients were subjected to a thorough history taking.

Based on ultrasonography results, patients were classified into four subgroups: 14 with bright hepatomegaly; 11 with perihepatic fibrosis; 11 with hepatic cirrhosis; and 14 with cirrhosis and HCC. After subclassification, venous blood samples (5 mL) were obtained (after overnight fast-

Table 1 Levels of vitamin D, its active form, interleukin-17, interleukin-23, N-terminal propeptide of type III pro-collagen and viral load in the four subgroups of hepatitis C virus-infected patients

Item	Group (I a) Bright hepatomegaly (n = 14)	Group (I b) Perihepatic fibrosis (n = 11)	Group (I c) Liver cirrhosis (n = 11)	Group (I d) HCC (n = 14)	Group II Normal (n = 25)
Vit D (ng/mL)	19.80 ± 3.33 ^h	19.40 ± 3.52 ^h	10.90 ± 3.74 ^{b,d,h}	9.70 ± 3.88 ^{b,d,h}	39.70 ± 10.80
Active VitD (ng/mL)	20.60 ± 3.50 ^h	21.00 ± 3.44 ^h	13.00 ± 2.10 ^{b,d,h}	11.70 ± 2.52 ^{b,d,h}	41.90 ± 7.90
IL-17 (ng/mL)	7.60 ± 2.66 ^h	5.10 ± 2.44 ^h	115.90 ± 38.70 ^{b,d,h}	150.30 ± 46.80 ^{b,d,f,h}	1.20 ± 0.40
IL-23 (ng/mL)	76.80 ± 14.51 ^h	51.20 ± 14.60 ^h	259.30 ± 49.4 ^{b,d,h}	225.90 ± 42.10 ^{b,d,h}	6.70 ± 2.17
Viral load (IU/mL)	66.30 ± 23.55 ^h	42.40 ± 9.66 ^h	165.10 ± 31.40 ^{b,d,h}	231.10 ± 44.60 ^{b,d,f,h}	0 ± 0
P ^{III} NP (μg/L)	91.03 ± 18.99 ^h	83.88 ± 28.77 ^h	209.09 ± 31.3 ^{b,d,h}	244.80 ± 34.10 ^{b,d,f,h}	22.61 ± 0.54

Values shown are means ± SD of data in four subgroups of hepatitis C virus-infected patients and normal controls. b, d, f, h means within lines with no common superscripts differ significantly. As compared with ^bgroup (I a), ^dgroup (I b), ^fgroup (I c), ^hcontrol (group II) (one-way analysis of variance followed by Tukey-Kramer test), $P < 0.01$. HCC: Hepatocellular carcinoma; IL: Interleukin.

ing) from all patients/controls. Samples were allowed to clot and sera were then separated by centrifugation (3500 rpm, 20 min, 25 °C) and then stored at -20 °C until used for analysis of the various parameters outlined below.

Assessment of Vit D, active Vit D, IL-17, IL-23 and P^{III}NP levels

Total Vit D (i.e., 25-OH-Vit D) assay was carried out using a commercial solid phase radioimmunoassay kit (Medgenix Diagnostics SA Zoning Industrial, Fleurus, Belgium) according to the method of Mawer^[17]. The active Vit D (i.e., 1,25-[OH]₂-Vit D) assay was carried out according to Hollis^[18] using a commercial kit from Incstar Corporation (Stillwater, MN, United States). IL-17, IL-23 and P^{III}NP levels were assayed using enzyme linked immunosorbent assay kits obtained from Biosource Europe SA (Nivelles, Belgium). The sensitivity of the 25-OH-Vit D, 1,25-(OH)₂-Vit D, IL-17, IL-23 and P^{III}NP kits were 2.4 ng/mL, 5.5 pg/mL, 10 pg/mL, 5 pg/mL, and 10 pg/mL, respectively.

Assessment of HCV levels

Quantitative reverse transcription polymerase chain reaction (RT-PCR) for HCV was done using TaqMan technology according to the method of Scott and Gretch^[19], and only HCV-4-infected patients were included in the study. Typically, an RT-PCR has a limit of quantification (LOQ) of 25 IU/mL and a limit of detection (LOD) of 10-15 IU/mL; in the assays used here for HCV-RNA testing, the LOQ was 24 IU/mL and the LOD 12 IU/mL.

For genotyping, HCV type-specific primers designed by Okamoto *et al.*^[20] were utilized. Assessments of genotype burdens required three steps: (1) RNA virus was extracted from patient samples using a Tripure Method (Roche, Mannheim, Germany); (2) isolated RNA was converted to cDNA using random hexamers and Moloney Murine Leukemia Virus Reverse Transcriptase enzymes from Promega (Madison, WI, United States); and (3) the product cDNA was amplified using an allele-specific PCR method. The PCR program was set for 1 cycle at 96 °C for 6 min, then for 40 cycles at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, and a final extension cycle of 72 °C for 10 min. For each patient,

two vials containing primer specific for virus types 1a/1b and for 2a and 3a were used. A positive control for each genotype (supplied by the kit manufacturer) was also run in parallel with each set of samples. In addition, HCV viral load was also determined using an Artus Real Art PCR Kit (Qiagen, Valencia, CA, United States). For both assays, a LightCycler[®] 480 real-time PCR System (Roche) was used. The slope of each reaction was between 3.2 and 3.4, and the error < 0.002. All results of the quantitative HCV analyses were expressed as IU/mL.

Statistical analysis

All data are expressed as mean ± SD. All analyses utilized SPSS for Windows version 15.0 (SPSS, Chicago, IL, United States). Analysis of variance was employed for comparisons of means of the different parameters, with Tukey-Kramer test as the post hoc test. $P < 0.05$ was accepted as statistically significant. Correlation analyses were done using Pearson's correlation.

RESULTS

Table 1 demonstrates significantly decreasing levels of Vit D and active Vit D [1,25-(OH)₂-Vit D], along with the progressive hepatic state induced by chronic HCV infection, ranging from bright hepatomegaly and perihepatic fibrosis to hepatic cirrhosis and further HCC. Levels of Vit D and active 1,25-(OH)₂-Vit D were significantly lower in advanced liver disease (i.e., hepatic cirrhosis and/or carcinoma), compared to bright hepatomegaly and perihepatic fibrosis. A similar pattern was seen for the levels of IL-17, IL-23 and P^{III}NP and the viral load, for which the levels were significantly elevated in hepatic cirrhosis and/or carcinoma, compared to bright hepatomegaly and perihepatic fibrosis. Only the IL-17, P^{III}NP and the viral load of patients with HCC were significantly higher than in cirrhotic patients. The decrease in Vit D and active Vit D was concomitant with increases in viral load, as well as levels of IL-17, IL-23 and P^{III}NP among all subgroups of HCV-infected patients, compared to normal healthy controls.

Vit D insufficiency (21-29 ng/mL) was detected in 14 (28%) HCV patients and three (12%) controls, while Vit

Table 2 Vitamin D, its active form, interleukin-17, interleukin-23, N-terminal propeptide of type III pro-collagen and viral load in male and female subgroups of hepatitis C virus-infected patients

Item	Male group (n = 36)	Female group (n = 14)	P value
Vit D (ng/mL)	10.30 ± 2.12	10.00 ± 2.60	1.00
Active Vit D (ng/mL)	12.10 ± 2.80	12.40 ± 2.60	0.96
Viral load (IU/mL)	198.00 ± 38.10	199.00 ± 31.10	0.93
IL-17 (ng/mL)	133.80 ± 32.50	134.90 ± 32.60	0.90
IL-23 (ng/mL)	241.00 ± 28.10	243.00 ± 26.50	0.92
P ^{III} NP (μg/L)	226.57 ± 346.54	227.94 ± 36.45	0.90

Values shown are means ± SD of data for both male and female hepatitis C virus-infected subjects. Values shown include group 1c (liver cirrhosis) and 1d (hepatocellular carcinoma) subjects. Significant difference is determined at $P < 0.05$. IL: Interleukin; Vit: Vitamin.

D deficiency, defined as serum level < 20 ng/mL, was present in 36 (72%) of the HCV-infected patients and none of the controls. Lastly, Vit D deficiency was seen in all 25 cirrhotic patients and 10 (40%) of the non-cirrhotic HCV-infected patients.

The parameters studied in HCV-infected patients when classified into two subgroups according to sex are shown in Table 2. HCV-infected men and women showed no differences with respect to Vit D levels. There were significant correlations between different parameters in HCV-infected patients and controls. There was a significant negative correlation between Vit D and IL-17, IL-23 and P^{III}NP. Viral load was negatively correlated with Vit D and active Vit D levels ($r = -0.084$ and -0.846 at $P < 0.001$, respectively), and positively correlated with IL-17, IL-23 and P^{III}NP ($r = 0.951$, 0.922 and 0.94 at $P < 0.001$). The significant negative correlations between active Vit D and IL-17 ($r = -0.679$), IL-23 ($r = -0.801$), and viral load ($r = -0.84$), are illustrated in Figure 1.

DISCUSSION

The liver plays a central role in Vit D metabolism, and its inadequacy is common in non-cholestatic chronic liver diseases and correlates with disease severity^[21]. The current study showed a significant reduction of Vit D and its active metabolite in HCV-4-infected patients compared to healthy controls. HCV-infected patients were classified according to sonar finding into four groups with progressive hepatic states; bright hepatomegaly and perihepatic fibrosis to hepatic cirrhosis and further HCC. The reduction of the levels of Vit D and its active form was more prevalent and severe in cirrhotic patients (versus non-cirrhotic patients), and much lower in patients with HCC; these differences were each highly significant. This is consistent with previous studies in patients with HCV genotype 1, which showed that Vit D deficiency is universal (92%) among patients with chronic liver disease, and at least one-third of the patients have severe Vit D deficiency^[21-23].

A significant negative correlation was reported between viral load and Vit D and active Vit D. Vit D is an impor-

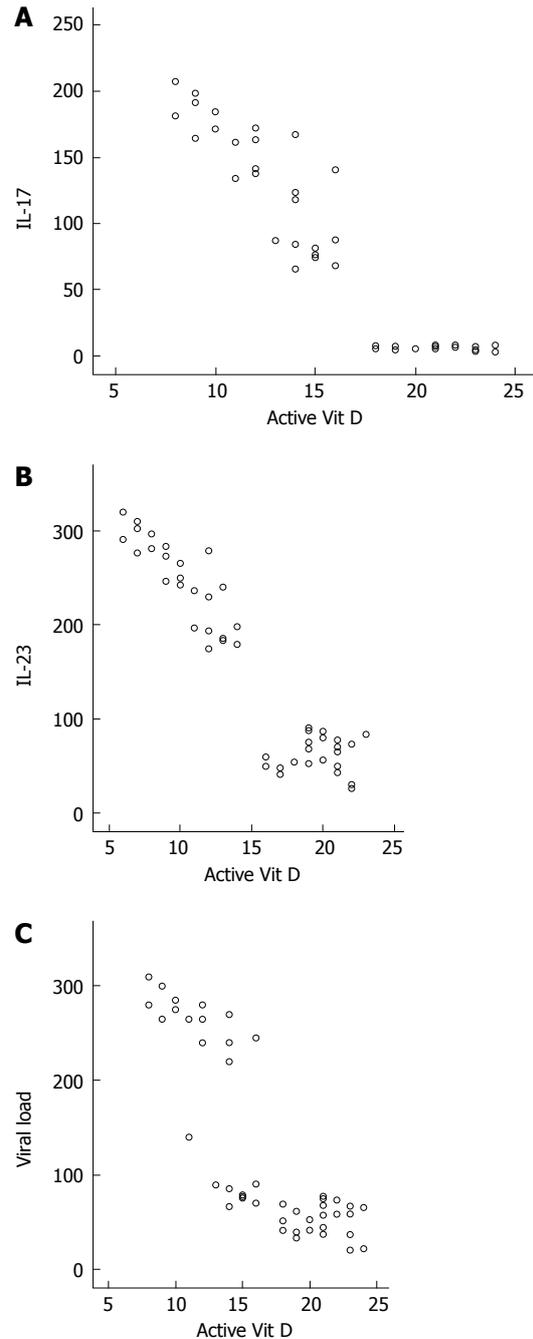


Figure 1 Correlation between active vitamin D levels and interleukin-17, interleukin-23 and viral load. A: Correlation between active Vit D levels and interleukin (IL)-17, revealing a negative correlation ($r = -0.679$); B: Correlation between active Vit D levels and IL-23, revealing a negative correlation ($r = -0.801$); C: Correlation between active Vit D levels and viral load, revealing a negative correlation ($r = -0.846$). Vit: Vitamin.

tant immune modulator and preliminary data have indicated associations between Vit D deficiency and failure to achieve SVR rates in HCV patients^[24]. It has been reported that the reduced (25-OH Vit D) levels and CYP27-1260 promoter polymorphism lead to reduced [1,25-(OH)₂ Vit D] levels, and are associated with failure to achieve an SVR in patients infected with HCV genotypes 1, 2 or 3^[14,25]. The patients in the present study with HCV-4 need further follow-up to confirm the effect of Vit D deficiency upon

their responses to treatment.

Furthermore, the present study showed that IL-17 and IL-23 were markedly increased in HCV-infected patients in comparison to controls. The difference in viral load among these groups may explain, in part, the differences noted in levels of inflammatory cytokines in the patients in the current study. Regulation of Th1 and Th17 responses in HCV-infected individuals has previously been studied^[25], and it has been reported that TGF- β and IL-6 promote differentiation of naïve murine CD4⁺ T lymphocytes into IL-17-secreting Th17 lymphocytes. In addition, it has been reported that other innate cytokines, including IL-1, IL-23, TNF- α and IL-21, in different combinations or with TGF- β , are also involved in the differentiation, amplification, or stabilization of the Th17 phenotype^[26]. A significant negative correlation between Vit D and both IL-17 and IL-23 was a prominent finding in the current investigation. Previous studies in mice have shown that Vit D is a strong inhibitor of Th17 polarization and Th17 cytokine expression by splenic CD4⁺ T lymphocytes. Furthermore, Th17 differentiation from naïve T lymphocytes is affected by Vit D. These data imply a regulatory effect on Th17 cells by Vit D, through the reduction of retinoic acid-related orphan receptor (ROR) γ t expression^[22]. The effect of Vit D on the behavior of Th17 cells has been investigated in different diseases and Vit D suppresses the expression of IL-17 and IL-23^[27-32], as documented in the current study.

We reported a positive correlation between IL-17 and IL-23 and viral load; a finding that supports our hypothesis regarding a link between Vit D and both IL-17 and IL-23 in immunoregulation of HCV-4-related chronic liver disease, and may explain how Vit D deficiency plays a role in increasing liver fibrosis. Our results also revealed no significant differences between HCV-infected men and women with respect to Vit D status. In contrast, Arteh *et al.*^[33] have reported that African American women with chronic liver disease are at higher risk of Vit D deficiency.

During the process of liver fibrosis, type III procollagen is converted to type III collagen by cleavage of its amino terminal and carboxy terminal propeptides. Serum levels of PIII^{NP}, which are direct serologic markers of collagen turnover in liver fibrosis, are elevated in both acute and chronic liver diseases; and further reflect the histologic stage of hepatic fibrosis in various chronic liver diseases^[34]. There was a significant increase (in comparison to controls) in the serum levels of PIII^{NP} in patients with all grades of hepatic disease; results that are in agreement with Walsh *et al.*^[34]. However, Panasiuk *et al.*^[35] have reported a decrease in PIII^{NP} levels in cirrhotic patients in comparison to controls, and have not shown any inflammatory process in the cirrhosis, hence more studies are needed to resolve this point of controversy. The results of the current study also revealed a significant negative correlation between Vit D and PIII^{NP} levels, supporting a role for decreased Vit D in inflammation and fibrosis; a relationship that has not previously been

investigated in patients with hepatic disease. Interestingly, Zehnder *et al.*^[36] have reported that reduction of the Vit D hormonal system in kidney diseases is associated with increased renal inflammation and fibrosis. These investigators have also reported a significant negative correlation between Vit D and PIII^{NP} levels. Logistic regression analysis with urinary PIII^{NP}, as a binary outcome, has shown that a 10-U increase in serum 1,25(OH)₂-D or 25-OH-Vit D resulted in lower renal inflammation^[37].

In conclusion, Vit D deficiency is prevalent in HCV-4-infected patients and the viral load is negatively correlated with Vit D status. In view of the role of Vit D in maintaining optimal immune function, Vit D status may be assessed and supplements may be considered to achieve an SVR during IFN- α -based therapy. The negative correlation between Vit D and IL-17, IL-23 and PIII^{NP} levels appears to highlight, at least in part, how these cytokines are involved with Vit D in immune responses in HCV-4-related liver disease, and could explain how Vit D deficiency plays a role in liver fibrosis.

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COMMENTS

Background

Hepatitis C virus (HCV) infects primarily the hepatocytes, leads to the development of fibrosis or cirrhosis of the liver, and is a significant risk factor for the development of hepatocellular carcinoma (HCC). The cell-mediated immune response plays a central role in hepatocellular necrosis and in the immunopathogenic mechanisms involved in viral clearance and persistence in liver disease of viral etiology, such as HCV-related chronic liver disease. In this context, cytokines modulate the immune system and exert direct antiviral activity by cytopathic and non-cytopathic mechanisms. T-cell immunoregulatory cytokines influence the persistence of HCV chronic infection and the extent of liver damage.

Research frontiers

Vitamin (Vit D) abnormalities are well documented in patients with chronic liver disease, but the association of the degree of Vit D abnormality with the progressive hepatic necroinflammatory state has not been thoroughly investigated. Furthermore, the proinflammatory cytokine profile in patients infected with HCV genotype 4 (HCV-4) needs further study. The research hotspot is how to determine/assess the extent of Vit D abnormality in HCV-4-infected patients and determine its relationship with proinflammatory markers, namely, interleukin (IL)-17, IL-23, and the N-terminal propeptide of type III pro-collagen (PIII^{NP}) as immune response mediators.

Innovations and breakthroughs

A plethora of studies has investigated the association of Vit D abnormalities with individual liver diseases, including hepatitis B, alcoholic hepatitis, autoimmune hepatitis, and HCC. Nevertheless, the role of Vit D abnormalities in the progression of HCV to cirrhosis and then to HCC remains to be elucidated. To this end, this study sought to investigate the potential association between levels of Vit D and IL-17, IL-23 and exacerbation of hepatic damage in chronic HCV patients. The results showed that levels of Vit D and its active form were significantly lower in patients with advanced liver disease (hepatic cirrhosis and/or carcinoma), compared to those with bright hepatomegaly and perihepatic fibrosis. IL-17, IL-23 and PIII^{NP} levels were markedly increased in HCV patients and correlated with progression of hepatic damage. The decrease in Vit D and its active form was concomitant with increases in viral load, as well as levels of IL-17, IL-23 and PIII^{NP} among all subgroups of HCV-infected patients, com-

pared to normal healthy controls. The negative correlation between Vit D and IL-17, IL-23 and PIII^{NP} may highlight, at least in part, how these cytokines are involved with Vit D in the immune response to HCV-4-related liver disease, and may explain how Vit D deficiency plays a role in the progression of liver fibrosis. In view of the role of Vit D in maintenance of immune function, its status may be assessed and supplements should be considered to achieve a sustained virological response during therapy.

Applications

The actual role of Vit D in the context of hepatic inflammatory process is still not fully elucidated. Given the major significance of the inflammatory response in mediating HCV clearance, as well as the anti-inflammatory actions displayed by Vit D *in vitro*, Vit D could have a positive influence on HCV infection. Further studies are needed to explain which stages of HCV infection require higher levels of Vit D and the mechanism of Vit D supplementation for these patients.

Terminology

Hepatitis C is a chronic liver infection that can be complicated by liver failure and liver cancer. In the liver, cytokines coordinate physiological and pathological processes such as liver growth and regeneration, inflammatory processes including viral liver disease, liver fibrosis and cirrhosis. T-cell immunoregulatory cytokines may play a key role in influencing the persistence of HCV infection and the extent of liver damage. IL-6 and transforming growth factor β , as the differentiation factors for Th17 cells, both cytokines together induce massive amounts of IL-17 from naïve T cells. Vit D has an important role in the treatment of different bacterial and viral infections; this vitamin is synthesized in the skin by absorption of ultraviolet light from the sun. The mechanism of action of this vitamin is unknown, but it may improve the activities of immune cells that are important in the eradication of HCV.

Peer review

This article reveals the importance of Vit D and/or its active form in HCV-4 infection, which is the most common form of hepatitis C in Egypt. The study also showed that this deficiency progresses with disease deterioration. This may indicate that Vit D supplements could be efficient in early stages of the disease.

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PI3K expression and PIK3CA mutations are related to colorectal cancer metastases

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Abstract

AIM: To assess the significance of phosphatidylinositol 3-kinase (PI3K) in colorectal cancer (CRC) and toxicity of LY294002 in CRC cells with different metastatic abilities.

METHODS: Sixty formalin-fixed and paraffin-embedded CRC tumor specimens were investigated. Adjacent normal colonic mucosa specimens from 10 of these cases were selected as controls. PI3K protein was detected by immunohistochemistry and PIK3CA mutations were investigated by gene sequencing analysis. A flow-cytometry-based apoptosis detection kit was used to determine PI3K inhibitor-induced apoptosis in CRC cell lines SW480 and SW620. Expression of phosphorylated protein kinase B in CRC cell lines was detected by Western blotting.

RESULTS: There was a significant difference in the proportion of primary lesions (30%, 18/60) vs metastatic lesions (46.7%, 28/60) that were positive for PI3K ($P < 0.05$). Mutations were detected in exon 9 (13.3%) and

exon 20 (8.3%). Out of 60 cases, seven mutations were identified: two hotspot mutations, C.1633G>A resulting in E545A, and C.3140A>G resulting in H1047R; two novel missense mutations C.1624G>A and C.3079G>A; and three synonymous mutations (C.1641G>A, C.1581C>T and C.3027T>A). Exposure of SW480 cells to PI3K inhibitor for 48 h resulted in a significant increase of apoptotic cells in a dose-dependent manner [3.2% apoptotic cells in 0 $\mu\text{mol/L}$, 4.3% in 5 $\mu\text{mol/L}$, 6.3% in 10 $\mu\text{mol/L}$ ($P < 0.05$), and 6.7% in 20 $\mu\text{mol/L}$ ($P < 0.05$)]. Moreover, PI3K inhibitor induced a similar significant increase of apoptotic cells in the SW620 cell line for 48 h [3.3% apoptotic cells in 0 $\mu\text{mol/L}$, 13.3% in 5 $\mu\text{mol/L}$ ($P < 0.01$), 19.2% in 10 $\mu\text{mol/L}$ ($P < 0.01$), and 21.3% in 20 $\mu\text{mol/L}$ ($P < 0.01$)].

CONCLUSION: High PI3K expression is associated with CRC metastasis. PI3K inhibitor induced apoptosis in CRC cells and displayed strong cytotoxicity for highly metastatic cells. PI3K inhibition may be an effective treatment for CRC.

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Key words: Colorectal cancer; PI3K; PIK3CA; Metastasis

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INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer-related death in the United States. The majority

of these deaths are due to metastatic disease^[1]. There is at least a 25-fold variation in occurrence of CRC worldwide, although rates are generally increasing. In high-risk areas, such as Europe, the rates are gradually increasing. The incidence tends to be low in Africa and Asia and intermediate in southern parts of South America^[2]. However, in China, the incidence of CRC is increasing rapidly^[3]. The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway is activated in multiple cancers and it is an important regulator of cell growth, metabolism, proliferation, survival, motility, and invasion^[4-7]. Highlighting the importance of the PI3K signaling pathway in human cancers, genetic alterations have been reported in various tumors including amplification of AKT1 and PIK3CA^[8], somatic mutations of PIK3CA and AKT^[9,10], and deletion of PTEN^[11]. Somatic mutations in the catalytic subunit of PIK3CA have been found in a significant fraction of breast carcinomas, in which PIK3CA exon 20 mutation is associated with poor prognosis^[12]. However, the different significance of PIK3CA mutations in primary CRC *vs* metastatic lesions has not been explored. Mutations in PIK3CA have been reported to occur in 13.6%-31.6% of CRC, making it one of the most frequently mutated genes^[13,14]. A majority of mutations have been identified in the helical (exon 9) and kinase (exon 20) domains of PIK3CA. Expression of cancer-derived PIK3CA mutants in cultured human-derived CRC cells increases kinase activity, invasion, and resistance to apoptosis, suggesting that mutant PIK3CA plays a role in the initiating steps of CRC tumorigenesis^[15,16]. In addition, the higher frequency of PIK3CA mutations in other advanced stage invasive tumors suggests a putative role in tumor progression^[4,17].

Alongside genetic, molecular biological, and biochemical studies, chemical inhibitors have been valuable as tools in PI3K research^[18-21]. They are helpful in understanding the role of PI3K enzymes in signal transduction and downstream physiological and pathological processes, as well as to assist validation of PI3Ks preclinically as therapeutic targets^[18]. The earliest and still widely utilized inhibitors are wortmannin and LY294002. We utilized the PI3K inhibitor LY294002 in this study.

Based on PIK3CA data in other malignancies, we hypothesized that mutations resulting in the activation of the PIK3CA pathway may play a role in the progression of CRC. To test this hypothesis, we analyzed expression of PI3K protein in both primary and metastatic lesions; the mutational status of the two hotspot regions of PIK3CA (exons 9 and 20) in CRC; and the effect of PI3K inhibitor on cell apoptosis and AKT phosphorylation using two CRC cell lines.

MATERIALS AND METHODS

Samples and cell lines

Formalin-fixed and paraffin-embedded tumor specimens were obtained from surgical specimens from Shanghai Xuhui District Center Hospital. Specimens were from

sporadic patients with colorectal adenocarcinoma who had not received preoperative radiotherapy, chemotherapy, or immunotherapy. All tumors were reviewed and confirmed by two senior pathologists using the World Health Organization classification of tumors of the digestive system^[22]. Among the 60 patients with CRC, 33 were male and 27 were female. Their mean age was 52 years, ranging from 24 years to 78 years. There were 58 cases in stage III and two in stage IV according to the tumor, nodes, metastasis system. Lymph node metastasis was seen in 58 cases and liver metastasis in two. Twenty-six cases occurred in the colon and 34 in the rectum. Adjacent normal colonic mucosa specimens from 10 of these cases were selected as controls. Human colon cell lines SW480 and SW620 (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences); annexin V-fluorescein iso-thiocyanate (FITC) Apoptosis Detection kit (BioVision, Palo Alto, CA, United States); and the detergent-compatible protein assay kit and ECL Plus Western blotting Detection System (Bio-Rad Laboratories, Hercules, CA, United States) were also used.

Immunohistochemistry

Paraffin-embedded blocks of CRC primary and metastatic tissue specimens were cut into 5 μ m thick sections. One section from each block was stained with hematoxylin and eosin as a control. Known positive biopsies were used as positive controls, while specimens treated with phosphate buffered saline (PBS) instead of primary antibody were used as negative controls. All further steps were performed in strict accordance with the Envision two-step method instructions. PI3K (p110 α) rabbit anti-human monoclonal antibody (Cell Signaling Technology, Beverly, MA, United States) was diluted 1:50. Envision Kit and DAB color kit (Zhongshan Biotechnology Company, Beijing, China) were used. PI3K presence was expressed in the cytoplasm as brown-yellow or brown granules. We observed the distribution of PI3K protein and scored the specimens^[23]. The score was 0 if no positive tumor cells were found; 1 if positive tumor cells were < 10%; and 2 if positive tumor cells were > 10%. Tissues with scores of 0 or 1 were considered negative; those with scores of 2 were considered positive.

Polymerase chain reaction and sequencing

Genomic DNA was extracted from all samples using phenol-chloroform and ethanol precipitation. The DNA concentration of each sample was measured using NanoVue ultraviolet spectrophotometer. Exons 9 and 20 of PIK3CA were amplified using previously described polymerase chain reaction (PCR) primers^[24]. The exon 9 forward and reverse primers were respectively 5'-GATTG-GTTCTTTCCTGTCTCTG-3' and 5'-CCACAAATATCAA TTTACAACCATTG-3', and yielded a 487-bp expected product. The exon 20 forward and reverse primers were respectively 5'-TGGGGTAAAGGGAAT-CAAAAG-3' and 5'-CCTATGCAATCGGTCTT TGC-3', and yielded a 525-bp expected product. The PCR

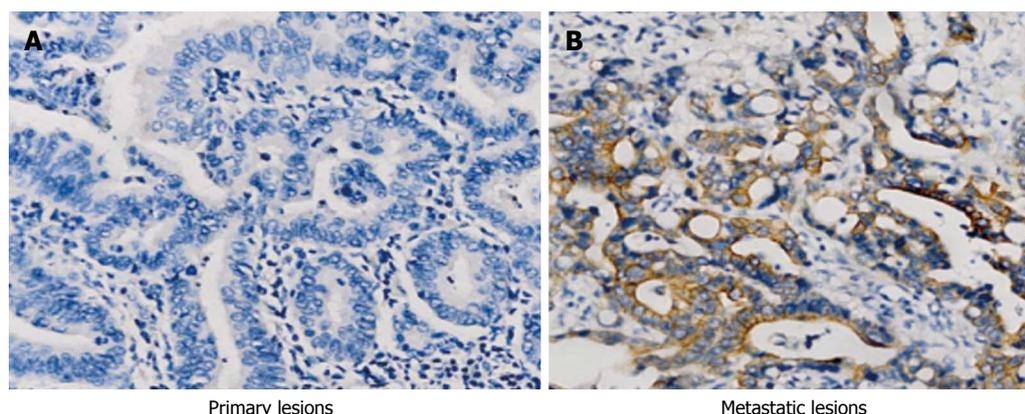


Figure 1 Expression of phosphatidylinositol 3-kinase in colorectal cancer by immunohistochemistry staining (enVision $\times 400$).

mixture contained 150 ng DNA, 5 pmol/L each primer, 2.5 nmol/L each dNTP, and 1.25 U Taq DNA polymerase in 20 μ L buffer with 0.04 μ mol/L Mg^{2+} . After an initial denaturation step at 94 $^{\circ}C$ for 5 min, 35 PCR cycles were performed, which included 45 s for denaturation at 94 $^{\circ}C$, 30 s for annealing at 60 $^{\circ}C$, and 45 s for extension at 68 $^{\circ}C$. PCR products were analyzed by 2% agarose gel electrophoresis. After observing clear and accurately sized bands, amplification products were then purified and directly sequenced on an ABI Prism 3730 sequence detection system (Applied Biosystems, Foster City, CA, United States).

Cells apoptosis analysis

SW480 and SW620 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin. All cell lines were incubated in 5% CO_2 at 37 $^{\circ}C$. PI3K-inhibitor-induced apoptosis in SW480 and SW620 cells was determined by flow cytometry using the annexin V-FITC Apoptosis Detection kit following the manufacturer's instructions. Briefly, 3×10^5 cells were treated with PI3K inhibitor (0 μ mol/L, 5 μ mol/L, 10 μ mol/L, and 20 μ mol/L) for 24 h. The cells were then harvested, washed in PBS, and incubated with annexin V and propidium iodide for staining in binding buffer at room temperature for 10 min in the dark. The stained cells were analyzed using the fluorescence-activated cell sorting (FACS) Aria instrument (BD Biosciences, CA, United States).

Western blotting

Whole cell lysates were generated with cell lysis solution for Western blotting. After centrifugation, the supernatant fraction was collected for Western blotting. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. After blocking with 5% nonfat milk in blocking buffer [20 mmol/L tris-buffered saline (pH 7.5) containing 0.1% Tween 20], the membrane was incubated with the primary antibody at 4 $^{\circ}C$ overnight and then incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. The immunoreac-

tive bands were visualized using the ECL Plus Western Blotting Detection System. The level of β -actin for each sample was used as a loading control. PI3K (p110 α) rabbit anti-human monoclonal antibody was purchased from Cell Signaling Technology. β -actin (1-19) and the secondary antibodies (HRP-linked anti-mouse IgG) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States).

Statistical analysis

The statistical analysis software package SPSS 11.5 was used to conduct the Fisher's exact test and χ^2 test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Expression of PI3K protein in CRC

PI3K protein was localized in the cytoplasm of CRC tumor cells; brown granules were detected within the cytoplasm. The expression of PI3K in primary and metastatic lesions in the same patient is shown in Figure 1A and B. The positive PI3K rates were significantly lower in the primary tumors than in the associated metastases: 30% (18/60) *vs* 46.7% (28/60) ($P < 0.05$). Moreover, the positive rate of 10% (1/10) in normal mucosa was significantly lower than in cancer tissue ($P < 0.05$).

Mutational analysis of PIK3CA exon 9 and exon 20 in CRC

PCR products of PIK3CA exon 9 and exon 20 were analyzed by 2% agarose gel electrophoresis (Figure 2). After observing clear and accurately sized bands, amplification products were then purified and directly sequenced on an ABI Prism 3730 sequence detection system. Mutational analysis of PIK3CA was done in 60 primary CRC and 10 normal tissues (Figure 3). Mutation rates of PIK3CA exon 9 and exon 20 were 13.33% (8/60) and 8.33% (5/60) in CRC, respectively. A total of eight mutations were identified (Table 1). All the mutations were not detected in corresponding normal tissues, therefore, these mutations were confirmed as somatic mutations. Of these 13 mutations, eight were clustered in exon 9 and the remain-

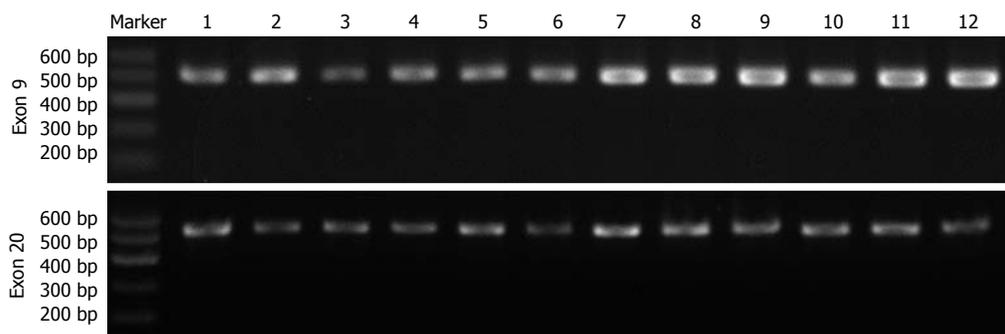


Figure 2 Representative examples (1-12) of 2% agarose gel electrophoresis results of PIK3CA (exon 9) and PIK3CA (exon 20) polymerase chain reaction products.

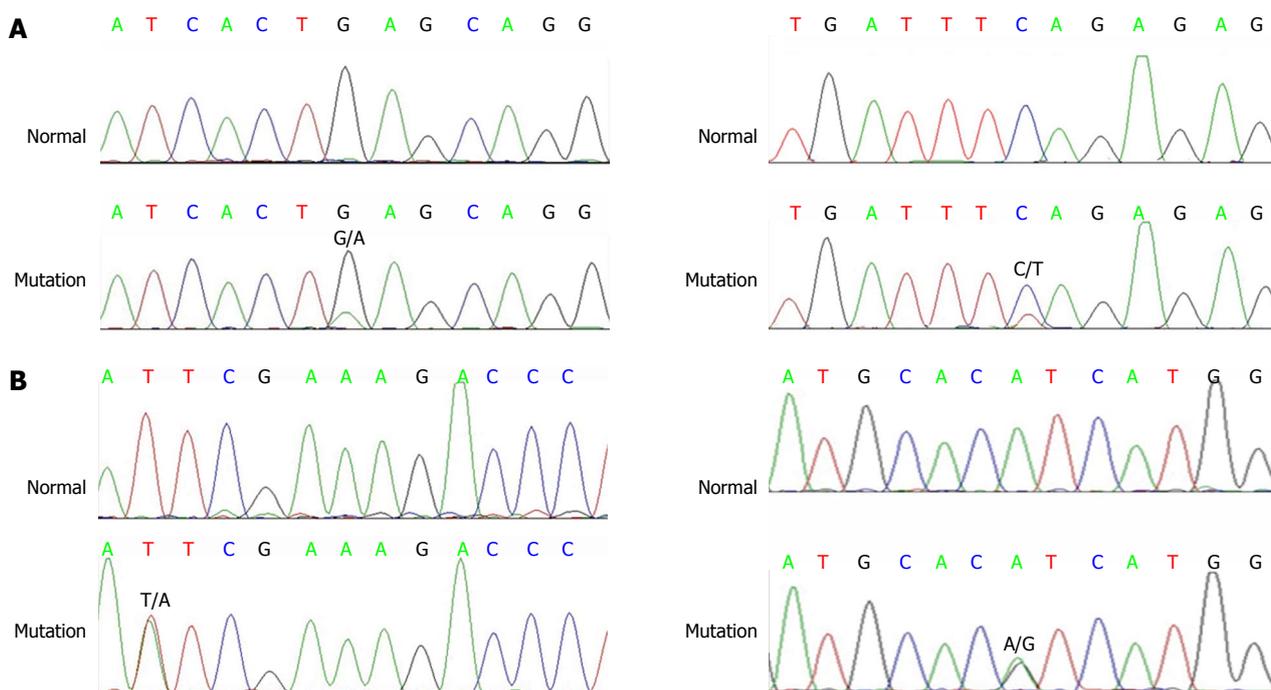


Figure 3 Sequencing traces are shown for exon 9 (A) and exon 20 (B) with cancer mutant sequence (bottom) and normal wild-type sequence (top).

Exon	Nucleotide	Codon	Domain	Cases
9	c.1624G>A	p.E541K	Helical	3
9	c.1633G>A	p.E545K	Helical	3
9	c.1581C>T	p.D527D	Helical	1
9	c.1641G>A	p.E547E	Helical	1
20	c.3027T>A	p.G1009G	Kinase	3
20	c.3079G>A	p.A1027H	Kinase	1
20	c.3140A>G	p.H1047R	Kinase	1

ing five in exon 20.

Analysis of PI3K-inhibitor-induced apoptosis in human CRC cells with different metastatic activities

To determine whether the PI3K inhibitor (LY294002)-induced growth inhibition in human CRC cells was associated with metastasis of CRC, SW480 and SW620 cells were treated with PI3K inhibitor. The numbers of apop-

totic cells were assessed using the annexin V-FITC Apoptosis Detection kit by the FACS Aria instrument. Exposure of SW480 cells to PI3K inhibitor for 48 h resulted in a significant increase of apoptotic cells in a dose-dependent manner [3.2% apoptotic cells in 0 μmol/L, 4.3% in 5 μmol/L, 6.3% in 10 μmol/L (*P* < 0.05), and 6.7% in 20 μmol/L (*P* < 0.05)]. Inhibition was also obtained when the SW620 cell lines were exposed to the same PI3K inhibitor for 48 h [3.3% apoptotic cells in 0 μmol/L, 13.3% in 5 μmol/L (*P* < 0.01), 19.2% in 10 μmol/L (*P* < 0.01), and 21.3% in 20 μmol/L (*P* < 0.01)] (Figure 4). PI3K inhibitor, at a concentration of 20 μmol/L, induced apoptosis to a greater extent in SW620 cells, which have higher metastatic potential than SW480 cells.

Effect of PI3K inhibition on AKT phosphorylation in CRC cells with different metastatic activities

To determine the potential effect of PI3K inhibition on AKT phosphorylation, phosphorylated AKT (pAKT)

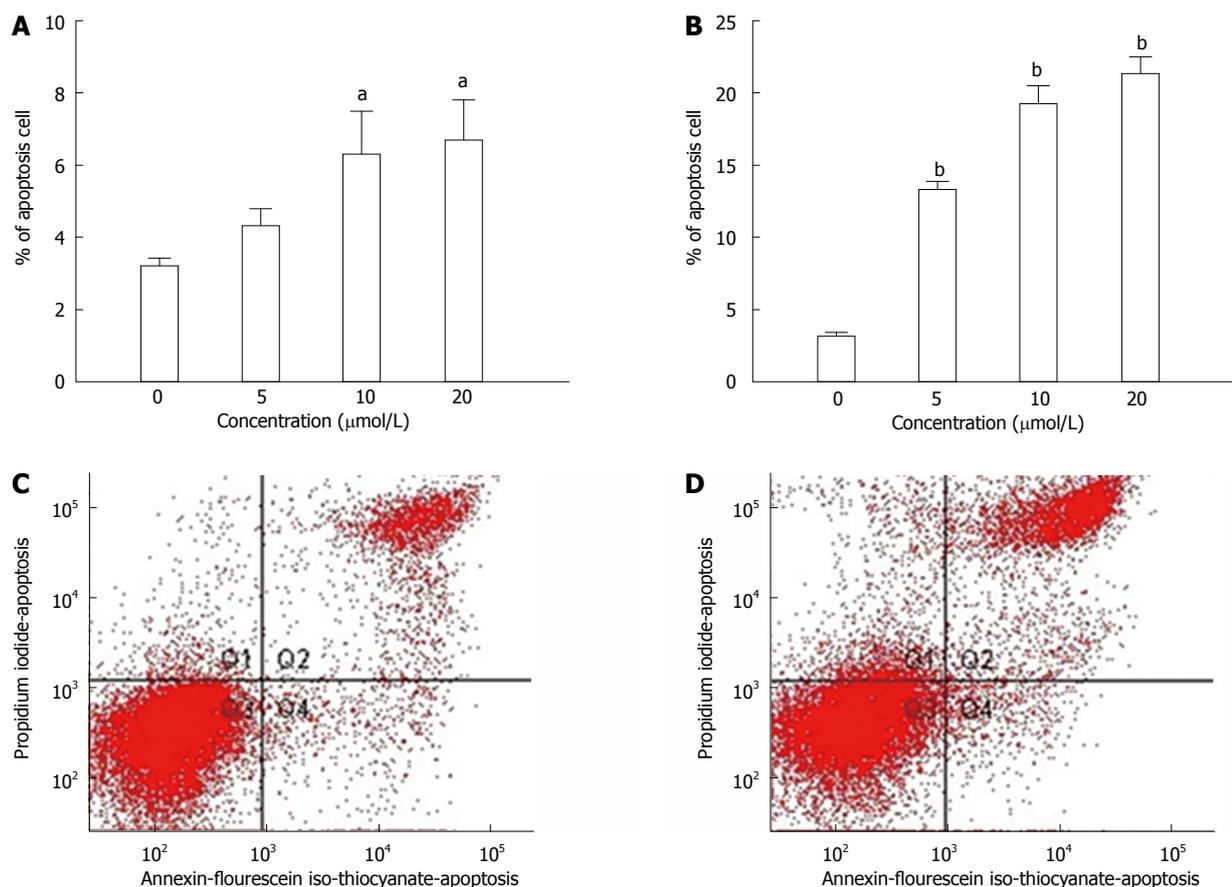


Figure 4 LY294002 induced apoptosis in human colorectal cancer cells with different metastatic activities. Induction of apoptosis in human colorectal cancer cells SW480 (A) and SW620 (B) by LY294002. Cells were exposed to various concentrations of LY294002 for 48 h followed by examination with annexin V-flourescein iso-thiocyanate Apoptosis Detection kit. SW620 cells were exposed to 5 μmol/L (C) and 20 μmol/L (D) LY294002 and analyzed by flow cytometry. All assays were done in triplicate [^a*P* < 0.05, ^b*P* < 0.01 vs control group (0 μmol/L)].

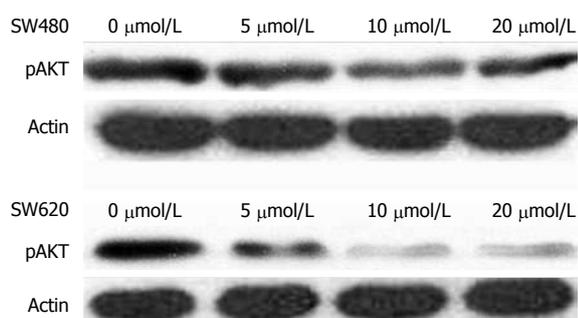


Figure 5 Effect of phosphatidylinositol 3-kinase inhibitors on protein kinase B phosphorylation in colon cancer cells.

was detected using different concentrations of PI3K inhibitors by Western blotting in two colon cancer cell lines with different metastatic activities. As shown in Figure 5, pAKT was detected in cells with 0 μmol/L inhibitor concentration, indicating the constitutive activation of AKT in two colon cancer cell lines. In SW620 and SW480 cells, the level of pAKT gradually decreased as inhibitor concentration gradually increased from 5 μmol/L to 10 μmol/L and 20 μmol/L. In SW620 and SW480 cells, pAKT at high inhibitory concentration was lower than that at low inhibitory concentration. In addition, pAKT

was marginally lower in SW620 than SW480 cells at the same inhibitory concentration. These findings confirmed that this PI3K inhibitor was strongly cytotoxic for highly metastatic CRC cells.

DISCUSSION

It is well known that tumors are often genetically influenced and somatic mutations of oncogenes and tumor suppressor genes are the initiators of the carcinogenic process. The PI3K/AKT signaling pathway has previously been implicated in tumorigenesis; evidence over the past several years suggests a vital role for the PI3K catalytic subunit PIK3CA in human cancers^[25,26]. In this study, we found that the expression of PI3K protein differed between primary and metastatic lesions in CRC. The positive rate of PI3K was higher in metastatic than in primary tumors, indicating that PI3K might be involved in CRC development, progression and metastases. The exact molecular mechanism deserves further study.

To probe the mechanism of PI3K high expression, we investigated the frequency of PIK3CA mutations in CRC and normal tissue adjacent to cancer. Due to the limited number of metastatic samples, PIK3CA mutations in metastases could not be evaluated. Instead, two

previously characterized mutational hotspots, exon 9 and exon 20, were analyzed. PIK3CA mutations were identified in 21.67% of the tumor tissues. No mutations were detected in corresponding normal tissues, therefore, these mutations were confirmed as somatic mutations. We also found that the PI3K mutation rate was less than the expression rate of PI3K protein, indicating that PI3K mutations were not the sole cause of the high expression of PI3K protein in CRC samples.

Of these 13 mutations, eight were clustered in exon 9 and five in exon 20. In the two previously reported hotspots, we identified the point mutations c.1633G>A and c.3140A>G, which resulted in the amino acid substitutions E545A and H1047R, respectively^[27,28]. Previous studies have also shown that mutations are more common in exon 9 in CRC^[14]. We also explored associations between any PIK3CA mutation and other variables, as well as interactions between histological groups, and found no significant correlation with mutation frequency. This does not agree with previous studies^[29], which might be related to the small number of cases in the present study. However, some similar reports have been found in breast cancer research. Dupont Jensen *et al.*^[30] have reported that PIK3CA mutations may be discordant between primary and corresponding metastatic disease in breast cancer, and PIK3CA mutation was detected in 45% of the primary tumors; and there was a net gain in mutation in metastatic disease, to 53%. Nonetheless, there were instances where metastases were wild type in patients with PIK3CA mutant primary tumors. Thus, we believe that PIK3CA is altered between primary and metastatic disease, and it is necessary to assess the PIK3CA status in the metastatic lesions to select patients that would benefit from PIK3CA inhibitor treatment.

To remedy the problem that PIK3CA mutations in metastases were not evaluated due to the limited number of metastatic samples, we further investigated the effect of PI3K inhibitor on human CRC cells with different metastatic activities. We found that PI3K inhibitor (LY294002) induced growth inhibition in human CRC cells, which was associated with the induction of apoptosis. The number of apoptotic cells in SW620 cell line was obviously increased compared with that in SW480 after LY294002 (20 $\mu\text{mol/L}$) treatment. Other research has confirmed that LY294002 has a strong sensitizing effect similar to boswellic-acid-induced apoptosis in colon cancer cells, and inhibits bone-morphogenetic-protein-2-induced epithelial-mesenchymal transition and invasion^[31,32]. Our results indicate that PI3K inhibitor induced different levels of apoptosis in cell lines with various metastatic capabilities, which suggests that PI3K is involved in the process of CRC metastasis. PIK3CA mutation and subsequent activation of the AKT pathway play an important role in colorectal carcinogenesis^[29]. Therefore, we also investigated the influence of LY294002 on AKT activation in our study. We found that LY294002 (5 $\mu\text{mol/L}$, 10 $\mu\text{mol/L}$ or 20 $\mu\text{mol/L}$) inhibited the phosphorylation of AKT significantly in two colon cancer cell lines, SW620 and SW480. PI3K inhibitor may have

potential for the treatment of metastatic CRC. It is worth investigating further the relationship among the death receptors Fas, tumor necrosis factor-related apoptosis-inducing ligand receptor (TRAILR)1, TRAILR2 and PI3K. These experiments are planned in our future work.

In summary, our results suggest that activation of the PI3K pathway plays an important role in colorectal tumor metastasis, and PI3K mutation is likely to be involved in promoting tumor invasion. However, PI3K mutation rate was not in accordance with the expression rate of PI3K protein, indicating that PI3K mutations were not the unique cause leading to the high expression of PI3K protein in CRC samples. PI3K inhibitors might become a new therapeutic method for CRC metastasis. However, the association between exon 9 and exon 20 mutations and the risk of CRC progression needs further investigation of a larger number of samples. In addition, the upstream and downstream genes of the PI3K/AKT pathway including insulin-like growth factor 1 receptor and ligand, phosphatidylinositol-3,4,5-triphosphate (PIP3), phosphatase and tensin homolog deleted on chromosome 10 (PTEN), mammalian target of rapamycin complex 2 and pyruvate dehydrogenase kinase warrant further study.

ACKNOWLEDGMENTS

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COMMENTS

Background

Colorectal cancer (CRC) is the second leading cause of cancer-related death in the United States. The majority of these deaths are due to metastatic disease. The phosphatidylinositol 3-kinase (PI3K)/protein kinase B signaling pathway is an important regulator of cell growth, metabolism, proliferation, survival, motility, and invasion. This pathway is activated in multiple cancers. It is of important to identify expression of PI3K protein in both primary and metastatic lesions to understand better the molecular mechanism of CRC, and evaluate PI3K inhibitors as a new treatment method for metastatic CRC.

Research frontiers

The expression of PI3K in primary CRC and metastatic tissues remains unclear. The differential effects of PI3K inhibition in CRC cell lines with various metastatic capabilities have been less well reported.

Innovations and breakthroughs

This is an important study that validates the hypothesis that activation of the PI3K pathway might play a role in CRC metastasis, and PI3K inhibitors should be evaluated as new candidates for the treatment of metastatic CRC.

Applications

These results suggest that the PI3K/AKT signaling pathway plays an important role in colorectal tumor metastasis. However, the PI3K mutation rate was not in accordance with the expression rate of PI3K protein, indicating that PI3K mutations were not the unique cause of the high expression of PI3K protein in CRC. PI3K inhibitor was strongly cytotoxic for cells with high metastatic ability, making PI3K targeting a new therapeutic candidate for the treatment of CRC metastasis. Further bioinformatics and functional studies using a large number of patient specimens will further determine the significance of these genes in CRC pathogenesis.

Terminology

Flow cytometry (FCM) is a technique used to analyze cells rapidly and individually, and it allows for the quantitative analysis of distributions of a property or properties in a population. FCM has been a fundamental tool for biological dis-

covery in recent years. It offers many advantages over conventional measurements for the analysis of biological cells, and it is a generally accepted tool to analyze apoptotic cells.

Peer review

The intention of the authors is to focus on the PI3K/AKT pathway. This pathway is indeed very interesting concerning oncogenesis.

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Evaluation of a novel hybrid bioartificial liver based on a multi-layer flat-plate bioreactor

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Abstract

AIM: To evaluate the efficacy and safety of a hybrid bioartificial liver (HBAL) system in the treatment of acute liver failure.

METHODS: Canine models with acute liver failure were introduced with intravenous administration of D-galactosamine. The animals were divided into: the HBAL treatment group ($n = 8$), in which the canines received a 3-h treatment of HBAL; the bioartificial liver (BAL) treatment group ($n = 8$), in which the canines received a 3-h treatment of BAL; the non-bioartificial liver (NBAL) treatment group ($n = 8$), in which the canines received a 3-h treatment of NBAL; the control group ($n = 8$), in which the canines received no additional treat-

ment. Biochemical parameters and survival time were determined. Levels of xenoantibodies, RNA of porcine endogenous retrovirus (PERV) and reverse transcriptase (RT) activity in the plasma were detected.

RESULTS: Biochemical parameters were significantly decreased in all treatment groups. The TBIL level in the HBAL group was lower than that in other groups ($2.19 \pm 0.55 \mu\text{mol/L}$ vs $24.2 \pm 6.45 \mu\text{mol/L}$, $12.47 \pm 3.62 \mu\text{mol/L}$, $3.77 \pm 1.83 \mu\text{mol/L}$, $P < 0.05$). The prothrombin time (PT) in the BAL and HBAL groups was significantly shorter than the NBAL and control groups (18.47 ± 4.41 s, 15.5 ± 1.56 s vs 28.67 ± 5.71 s, 21.71 ± 3.4 s, $P < 0.05$), and the PT in the HBAL group was shortest of all the groups. The albumin in the BAL and HBAL groups significantly increased and a significantly higher level was observed in the HBAL group compared with the BAL group (27.7 ± 1.7 g/L vs 25.24 ± 1.93 g/L). In the HBAL group, the ammonia levels significantly decreased from 54.37 ± 6.86 to 37.75 ± 6.09 after treatment ($P < 0.05$); there were significant difference in ammonia levels between other the groups ($P < 0.05$). The levels of antibodies were similar before and after treatment. The PERV RNA and the RT activity in the canine plasma were all negative.

CONCLUSION: The HBAL showed great efficiency and safety in the treatment of acute liver failure.

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Key words: Hybrid bioartificial liver; Acute liver failure; Flat plate bioreactor; Co-culture; Nanofiber scaffold

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INTRODUCTION

The mortality associated with acute liver failure or acute-on-chronic liver failure remains dismally high^[1]. Orthotopic liver transplantation is a unique and effective treatment for acute liver failure^[2]. However, many patients still die while waiting for liver transplantation due to a persistent scarcity of donors. Therefore, many investigators have attempted to develop diversified extracorporeal liver assist devices as a bridge for patients until recovery or liver transplantation^[3-5]. Extracorporeal liver assist devices can be classified into artificial systems and bioartificial systems. An effective liver assist device should include three primary functions: detoxification, biosynthesis and regulation. The main functions of artificial systems include the removal of toxins (e.g., aromatic amino acids and ammonia), but does not include the synthesis of products that take place in the liver. Bioartificial systems could be the ideal replacement therapy in the long run. However, bioartificial liver systems have not reached their full efficiency yet, since enhancing the viability and functions of hepatocytes in the bioreactor remains a difficult problem, and the function of detoxification is inadequate^[6-8]. For these reasons, we consider the application of artificial systems in combination with bioartificial liver systems presents a potential method for aiding patients waiting for liver transplantation.

In this study, we first developed a newly bioartificial liver system based on multi-layer flat-plate bioreactor with co-cultured pig hepatocytes and bone marrow mesenchymal stem cells, so as to mimic the microenvironment *in vivo*. We then combined this newly bioartificial liver system with an anionic resin adsorption column to form a novel hybrid bioartificial liver (HBAL) system. The efficacy and safety of this novel HBAL system was evaluated *via* treatment of canines with acute liver failure.

MATERIALS AND METHODS

Animals and reagents

Outbred white pigs with a weight of 15-20 kg, as well as dogs with a weight of 10-15 kg, received humane care. All animal procedures were performed according to institutional and national guidelines and approved by the Animal Care Ethics Committee of Nanjing University and Nanjing Drum Tower Hospital. RPMI 1640 were purchased from GIBCO (United States). Lactobionic acid and chitosan (low molecular weight, Brookfield viscosity 20 000 cps, 85% deacetylation) were purchased from Sigma-Aldrich (Saint Louis, United States). N-Hydroxysuccinimide was purchased from Thermo-Pierce (Rockford,

United States). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N, N, N', N'-tetramethylethylenediamine were obtained from TCI (Tokyo, Japan). Polyethylenoxid (M_w ≈ 10⁶) was supplied by Guoren Chemical Co. (Beijing, China). All other reagents were of analytical reagent grade.

Cell isolation and culture

Porcine mesenchymal stem cells were isolated by bone marrow aspirates from the iliac crest of pigs, as described previously, with slight modification^[9]. Briefly, mononuclear cells were collected by gradient centrifugation over a Ficoll Histopaque layer (20 min, 400 g, density 1.077 g/mL) and seeded at a density of 1 × 10⁶ cells/cm² in growth medium containing low-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 IU/mL) and streptomycin (100 µg/mL). The non-adherent cells were removed after the first 24 h and changed every 3 d to 4 d thereafter. The primary pig hepatocytes were then harvested by a two-step *in situ* collagenase perfusion technique^[10]. The viability of the isolated primary hepatocytes, determined by trypan blue exclusion, was more than 95%.

Non-bioartificial liver system

Whole blood was removed at a rate of 30 mL/min from the jugular vein of the canine and separated to plasma by a plasma separator (Bellco, Italy) at a rate of 30 mL/min. The separated plasma was pumped into an anionic resin adsorption column (Aier, China) where the toxic substances were absorbed, and then reconstituted with red blood cells and returned to the canine *via* the venous cannula (Figure 1A).

Bioartificial liver system

Bioreactor configuration: The multi-layer bioreactor consisted of housing, a hollow column stent, and stacked flat plates, all of which were made of polycarbonate. The fully assembled bioreactor contained a stack of 65-layer round flat plates, on which galactosylated chitosan nanofiber scaffolds were electrospun for hepatocyte immobilization and aggregation. The diameter and thickness of each plate were 10.4 cm and 1 mmol, respectively. There was a hole with a diameter of 1 cm in the center of each plate, which was used to fix them onto the stent. The channel height between every two neighboring plates was maintained at 0.5 mmol with the spacers attached to the bottom of each plate. The stent was open on the top and closed at the bottom, with four vertical-side holes on the lateral wall, which were broken up into eyelets between every two plates. The last component was housing, which was a cylindrical container with an outlet on the bottom. When the stacked plates were fixed onto the stent, they were put into the housing, and the lid with an O-ring was screwed onto the housing in order to provide a water tight seal. The culture medium entered the bioreactor from the top opening of the stent, then flowed onto the surface of each flat plate through the eyelets, and

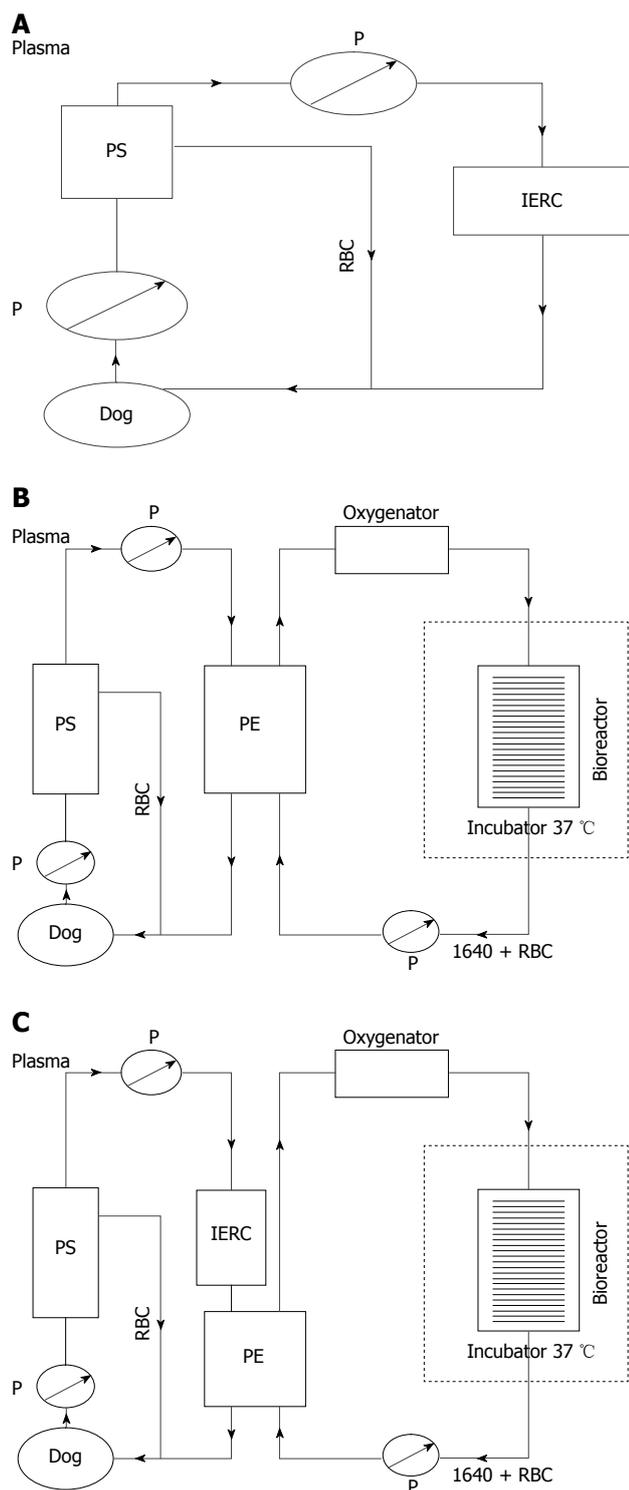


Figure 1 Schematic assembly of three artificial liver systems. A: Schematic assembly of non-bioartificial liver; B: Schematic assembly of bioartificial liver; C: Schematic assembly of hybrid bioartificial liver. P: Pump; RBC: Red blood cells; PS: Plasma separator; PE: Plasma component exchange column; IERC: Anionic resin adsorption column.

finally flowed out from the outlet of housing. The height of this bioreactor was about 10 cm, and the effective volume was 480 mL.

The multi-layer flat-plate bioartificial liver consists of a cell circuit and a blood circuit. Freshly isolated porcine

hepatocytes (approximately 10^{10}) were pre-mixed in 480 mL RPMI 1640 culture medium containing 10% BSA, 0.5 mg/mL insulin, 10 mmol NaHCO_3 , 50 mg/mL penicillin and streptomycin, and 100 mg/mL neomycin. The medium was then filled into the bioreactor by a peristaltic pump (JHBP-2000B, Guangzhou, China). The whole bioreactor was incubated for 1 h at 37 °C and 5% CO_2 until the cells were adhered onto the surface of plates. The culture medium was pumped through an oxygenator (Affinity, United States) via Fresenius silicone tubing (Fresenius, Germany) by a peristaltic pump before flowing into the bioreactor, and then circulated into a blood component exchange column (Asahi Kasei Corporation, Japan) at 25 mL/min, which constitute the cell circuit. As for the blood circuit, whole blood was removed at a rate of 30 mL/min from the jugular vein of the canine and separated to plasma by a plasma separator (Bellco, Italy) at a rate of 15 mL/min. The separated plasma was pumped into the blood component exchange column where the plasma component and culture medium component exchanged, and then reconstituted with red blood cells and returned to the canine via the venous cannula (Figure 1B).

HBAL system

We combined the bioartificial liver system with an anionic resin absorption column to form a novel HBAL system. This HBAL system differs from the bioartificial liver system in that the separated plasma was pumped into an anionic resin adsorption column where the toxic substances were absorbed, and then passed through the blood component exchange column (Figure 1C).

Canine models of acute liver failure and treatment groups

The canines were injected intravenously with D-galactosamine to induce acute liver failure and then randomly divided into 4 groups: the HBAL system treatment group ($n = 8$), in which the canines received a 3 h treatment of HBAL system (as described above) 24 h after the administration of the drug; the bioartificial liver system treatment group ($n = 8$), in which the canines received a 3 h treatment of bioartificial liver system; the non-bioartificial liver system (artificial support system) treatment group ($n = 8$), in which the canines received a 3 h treatment of artificial support system; the control group ($n = 8$), in which the canines received no additional treatment.

Blood biochemistry and survival rate

Blood samples were measured with an automatic analyzer (Hitachi 7600, Tokyo, Japan) for alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), total bilirubin (TBIL), albumin (ALB), ammonia, and prothrombin time (PT). Survival was observed for 7 d.

Safety evaluation

Levels of xenoantibodies (IgG, IgM) were detected by

Table 1 Comparison of biochemical index between different groups in different time points

Groups	Time points	ALT	AST	LDH	TBIL	ALB	Ammonia	PT
Control (<i>n</i> = 8)								
	Before modeling	64 ± 22.67	44.87 ± 11.6	210.14 ± 48.32	1.67 ± 0.57	30 ± 5	46.5 ± 11.98	8.4 ± 1.7
	After modeling	485.28 ± 140.2	651.35 ± 125.9	694.14 ± 232.8	28.45 ± 9.8	26.6 ± 5	62.25 ± 21.56	16.4 ± 5.2
	1 d after treatment	2438.64 ± 720.3	3201.9 ± 903	2433 ± 838.47	49 ± 15.77	24.8 ± 6.5	98.67 ± 21.7	28.67 ± 5.71
	3 d after treatment	860 ± 237.96	230.37 ± 92.37	867.25 ± 86.57	56.1 ± 5.3	22.3 ± 3.6	66.5 ± 8.54	24.83 ± 2.55
	5 d after treatment	144.6 ± 55.4	172.9 ± 36.57	639 ± 117.88	24.2 ± 6.45	21.4 ± 3.8	39.3 ± 9.5	10.5 ± 4.1
Bioartificial liver system (<i>n</i> = 8)								
	Before modeling	51.78 ± 13.1	58.77 ± 13.4	285.5 ± 83.18	1.1 ± 0.4	29.42 ± 2.48	50.28 ± 14.28	8.2 ± 1.43
	After modeling	439.7 ± 180.98	811.7 ± 209.85	438.5 ± 138.2	18.12 ± 4.85	24.46 ± 1.97	55.5 ± 15.4	13.5 ± 4.1
	1 d after treatment	1511.54 ± 183.4	1472.46 ± 365	463 ± 76	25.92 ± 6.2	24.76 ± 4.46	46.4 ± 18.1	18.47 ± 4.41
	3 d after treatment	331.8 ± 31	51.2 ± 15.1	278.8 ± 67.2	28.84 ± 6.23	25.44 ± 3.52	38.2 ± 10.2	15.2 ± 7.6
	5 d after treatment	86.2 ± 24.5	46.3 ± 10.54	311.5 ± 84.35	12.47 ± 3.62	25.24 ± 1.93	34 ± 9.46	8.8 ± 2.7
Non-bioartificial liver system (<i>n</i> = 8)								
	Before modeling	43.11 ± 19.44	41.77 ± 14.32	216.87 ± 46.2	2.44 ± 1	29.35 ± 3.08	50.25 ± 17.07	7.67 ± 0.315
	After modeling	447.5 ± 99.44	660.15 ± 152.13	536 ± 110	24.65 ± 2.57	24.36 ± 5.56	60.25 ± 15.12	14.6 ± 2.17
	1 d after treatment	1164.5 ± 332.5	1531.31 ± 294.96	485.5 ± 119.41	28.07 ± 9	23.61 ± 3.77	46 ± 13.6	21.71 ± 3.4
	3 d after treatment	286.2 ± 99.8	190.62 ± 30.9	379.8 ± 67.36	7.21 ± 3.6	23.94 ± 2.13	42.6 ± 8.17	19.48 ± 3.19
	5 d after treatment	89.15 ± 5.32	85.9 ± 11.98	530.25 ± 136.55	3.77 ± 1.83	23.92 ± 0.82	39.5 ± 7.59	7.95 ± 0.64
Hybrid bioartificial liver system (<i>n</i> = 8)								
	Before modeling	56.5 ± 12.62	45.9 ± 10.56	233.25 ± 39	1.71 ± 0.28	29.4 ± 2.6	51.5 ± 8.94	7.62 ± 0.47
	After modeling	459.05 ± 80.57	681.95 ± 101	520.75 ± 101.22	18.8 ± 3.49	25.8 ± 3.2	54.37 ± 6.86	13.67 ± 2.66
	1 d after treatment	655.45 ± 97	1010.67 ± 118.95	301.12 ± 69.26	20.35 ± 2.68	28 ± 1.7	37.75 ± 6.09	15.5 ± 1.56
	3 d after treatment	117.97 ± 30.61	44.52 ± 9.7	222.5 ± 53.19	4.44 ± 1.02	28.3 ± 1.4	173.7 ± 8	8.36 ± 0.99
	5 d after treatment	55 ± 10.73	39.81 ± 8.72	334.25 ± 76.99	2.19 ± 0.55	27.7 ± 1.7	17.75 ± 3.77	8.3 ± 1.22

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LDH: Lactate dehydrogenase; TBIL: Total bilirubin; ALB: Albumin; PT: Prothrombin time.

enzyme linked immunosorbent assay (ELISA) kit (Alpha Diagnostic International Inc., United States). To identify the transmission of porcine endogenous retroviruses (PERVs), the PERV RNA and reverse transcriptase (RT) activity in the plasma was detected with reverse transcription-polymerase chain reaction (RT-PCR) and RT activity assay kits (Cavidi-Tech, Uppsala, Sweden), respectively.

Statistical analysis

All values were expressed as the mean ± SD. The two-tailed unpaired Student's *t*-test or one-way analysis of variance was used to evaluate the statistical significance of differences, which were set with a *P* value < 0.05. The survival rates were analyzed using the Kaplan-Maier method and compared using a log-rank test.

RESULTS

Common conditions

After treatment, hepatic encephalopathy was significantly improved in all treatment groups; most of the canines could stand, eat and drink by themselves. However, the canines in the control group became gradually worse, progressing into a coma and eventually death. Body temperature was stable in all treatment groups (38 ± 0.5 °C in non-bioartificial liver (NBAL) group; 37 ± 1 °C in bioartificial liver (BAL) group; 38 ± 0.8 °C in HBAL group); however, the canines in the control group became lower than other groups.

Liver function parameters

Several laboratory parameters have been determined,

such as ALT, AST, LDH, TBIL, ALB, PT and ammonia. As Table 1 and Figure 2 show, there was no significant difference between any of the groups before treatment. In all treatment groups, ALT, AST, LDH, TNIL and ammonia were shown to be statistically decreased compared to the control group. Moreover, the TBIL level in the HBAL group was lower than that in the other groups (2.19 ± 0.55 μmol/L *vs* 24.2 ± 6.45 μmol/L, 12.47 ± 3.62 μmol/L, 3.77 ± 1.83 μmol/L, *P* < 0.05). There was no significant difference in PT and ALB between the NBAL and control groups. On the contrary, the PT in the BAL and HBAL groups was significantly shorter than in the NBAL and control groups (18.47 ± 4.41 s, 15.5 ± 1.56 s *vs* 28.67 ± 5.71 s, 21.71 ± 3.4 s, *P* < 0.05), and the PT in the HBAL group was the shortest of all the groups. The ALB in the BAL and HBAL groups significantly increased and a significantly higher level was observed in the HBAL group compared with the BAL group (27.7 ± 1.7 g/L *vs* 25.24 ± 1.93 g/L). In the HBAL group, ammonia levels significantly decreased from 54.37 ± 6.86 to 37.75 ± 6.09 after treatment (*P* < 0.05); there were significant differences in ammonia levels between the other groups (*P* < 0.05).

Animal survival

Analysis of survival curves (Figure 3) showed that the 7 day survival rate was 37.5% (3/8) in the control group, 50% (4/8) in the NBAL group, 62.5% (5/8) in the BAL group and 87.5% (7/8) in the HBAL group. Survival time was significantly prolonged by HBAL treatment (*P* = 0.028). However, the results showed there was no significant difference between the other groups.

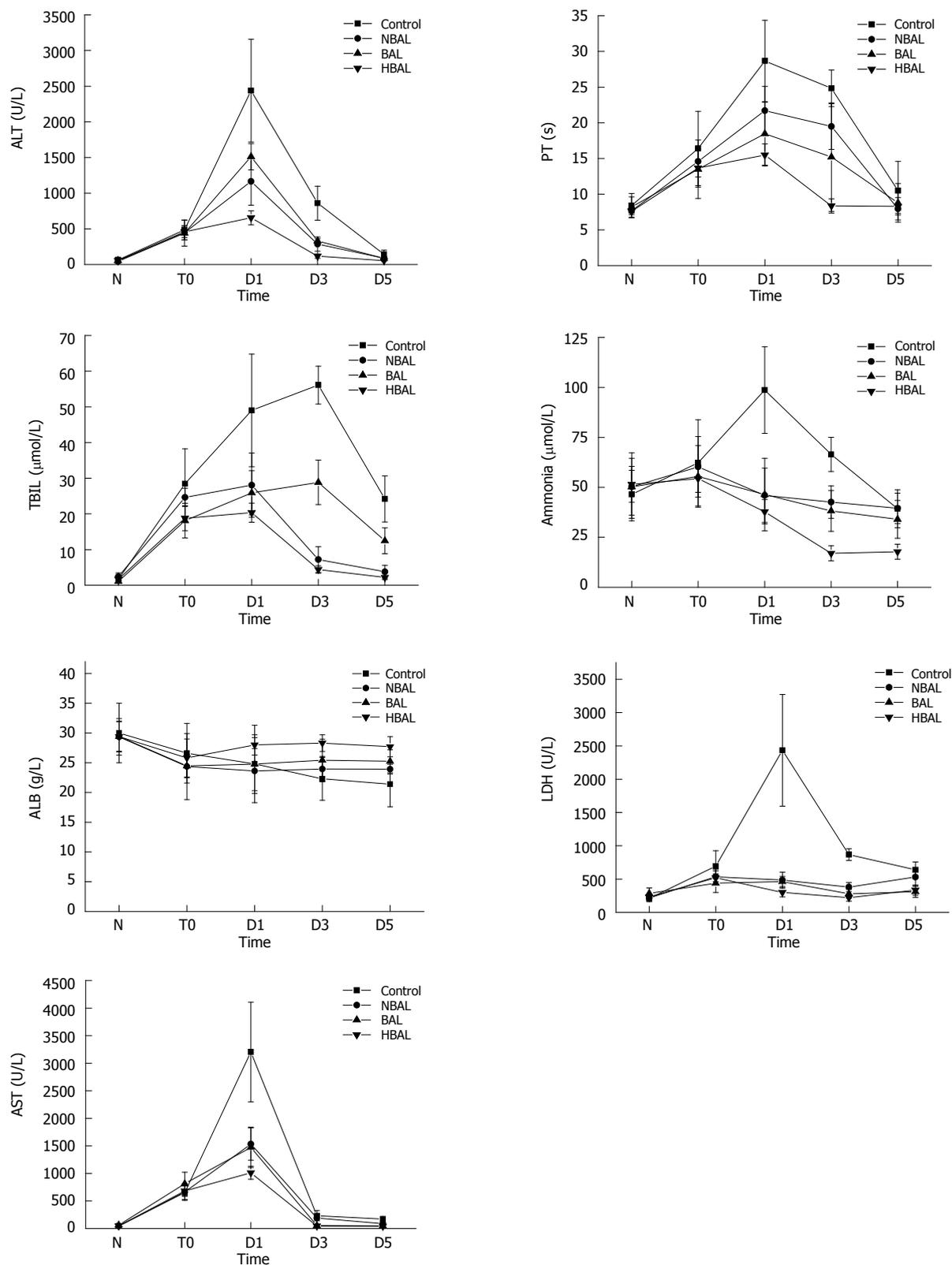


Figure 2 Comparisons of biochemical index between the treatment groups and the control group. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LDH: Lactate dehydrogenase; TBIL: Total bilirubin; ALB: Albumin; PT: Prothrombin time; HBAL: Hybrid bioartificial liver; BAL: Bioartificial liver; NBAL: Non-bioartificial liver; N: Normal; T0: Before treatment; D1: Day 1 after treatment; D3: Day 3 after treatment; D5: Day 5 after treatment.

Safety evaluation

To evaluate the humoral immune response in canine models after treatment with BAL, we examined the levels

of antibodies. The levels of antibodies were measured by ELISA in canine plasma obtained before and after BAL. As shown in Figure 4A, immediately after the first

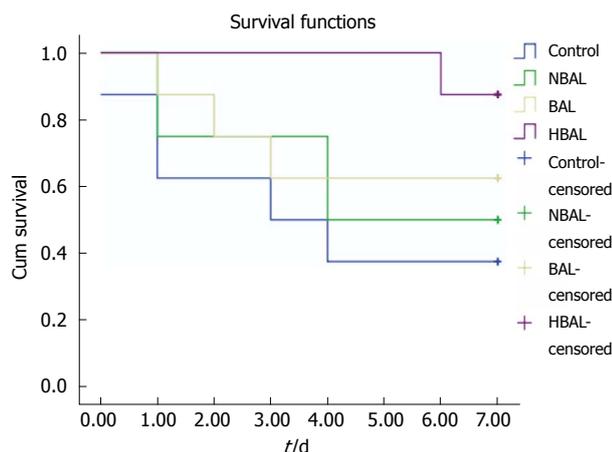


Figure 3 Kaplan-Meier analysis of survival of two groups. HBAL: Hybrid bioartificial liver; BAL: Bioartificial liver; NBAL: Non-bioartificial liver.

BAL treatment, the levels of IgG and IgM were suitable before and after treatment. All results of RT-PCR with the RNA from collected plasma were negative (Figure 4B). In addition, the RT activity in the canine plasma was negative as well.

DISCUSSION

In recent years, extracorporeal liver assist devices have been developed and widely used to bridge the gap between for patients until liver transplantation or a spontaneous recovery of liver function. There are currently two types of liver assist devices: artificial and bioartificial livers. Artificial liver systems include conventional plasmapheresis with plasma exchange, hemodialysis using large pore membranes and hemoperfusion using exchange resin columns or activated charcoal^[11-13]. These systems could remove toxic substances (e.g., ammonia, endotoxin, mercaptans and endogenous inhibitor neurotransmitters) but also some helpful cytokines, such as interleukin-6, hepatocyte growth factor. Moreover, these devices could not replace the synthetic and metabolic functions, nor could they prolong patient survival time. Bioartificial liver systems could replace the primary and most important liver functions, such as oxidative detoxification, biotransformation, excretion and synthesis. However, there are still many problems holding back the development of bioartificial livers in a clinical setting. How to maintain the functions of hepatocytes and mimic cell microenvironment *in vitro* remains a difficult problem. In addition, plasma from patients with acute liver failure have some toxic factors, which are harmful to the hepatocytes in the bioreactor. In the present study, to resolve these problems, we first developed a new bioartificial liver system based on a multi-layer flat-plate bioreactor with co-cultured pig hepatocytes and bone marrow mesenchymal stem cells.

Due to the short-term viability and rapid phenotypic de-differentiation of primary hepatocytes *in vitro*, mimicking the three-dimensional microenvironment of

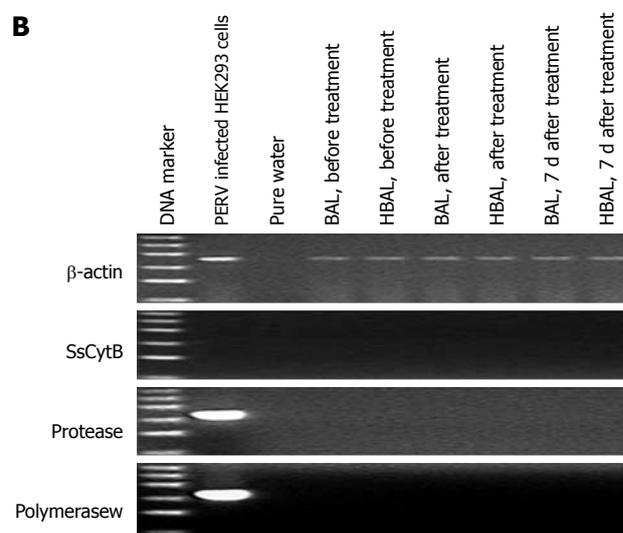
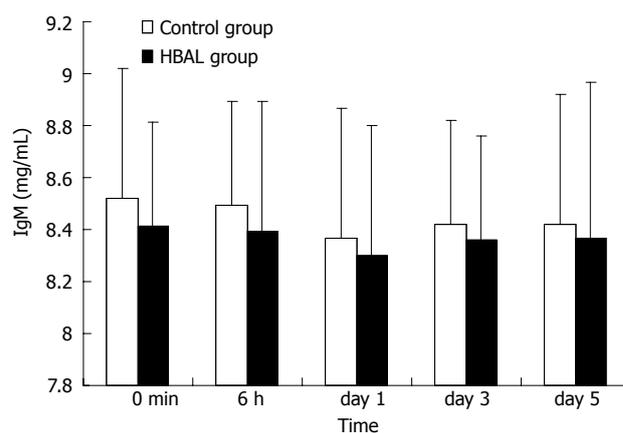
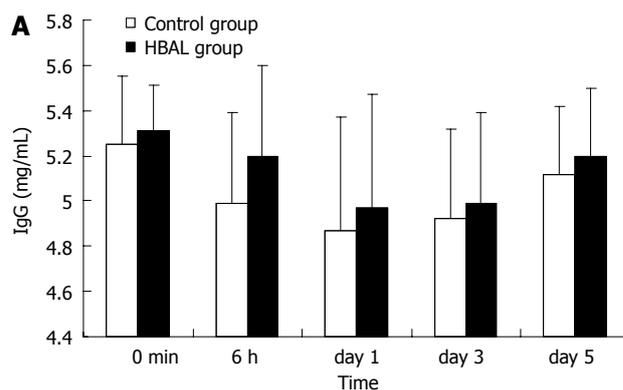


Figure 4 Safety evaluation. A: Xenoreactive antibodies levels during bioartificial liver treatments; B: Representative results of reverse transcription-polymerase chain reaction electrophoresis with the RNA extracted from the plasma. The ladder ranged from 100 bp to 600 bp. NBAL: Hybrid bioartificial liver; BAL: Bioartificial liver; PERV: Porcine endogenous retrovirus.

liver sinusoid *in vitro* should be important for reconstruction of tissue architectures and restoration of isolated hepatocyte functions. Recent studies have shown that functions of primary hepatocytes can be maintained by co-culturing with non-parenchymal cells^[14,15]. The interaction between cells and the extracellular matrix (ECM) provides direct regulatory signals to cells through adhesion. In addition, various soluble factors secreted

by mesenchymal cells have been shown to be important in maintaining hepatocyte phenotypes. In our previous study, we confirmed that the morphology and functionality of co-culturing such heterotypic cells were well-maintained and improved over hepatocytes homo-culture^[16,17]. Therefore, in this study, we adopted co-cultured porcine hepatocytes and bone marrow mesenchymal stem cells as the cell resource.

Hepatocytes *in vivo* are exposed to sinusoidal endothelial cells mediated by Disse space filled with ECM, which consists of a complex topography in the nanometer range and functional proteins that provide signals regulating cellular functions through cell surface receptors. In order to mimic the topography of ECM, various materials have been fabricated into nanometer materials and proven to affect cell migration, adhesion, proliferation and other cellular behaviors^[18]. In the past decade, nanometer scaffolds have been widely applied in various areas of tissue engineering. In our previous study, galactosylated chitosan nanofiber scaffolds could adequately mimic the topography of ECMs *in vitro* and increase the contact sites between cells and materials, thus enhancing the signal transduction between cells, promoting cell functions, and the pseudopod of hepatocytes could be formatted on the scaffolds, which further facilitated cells to adhere tightly to the nanofiber surface, and enhance cell adhesion and viability. Moreover, the galactose group grafted onto the scaffold could selectively adhere to the asialoglycoprotein receptors on the surface of hepatocytes, which could then induce the formation of hepatocyte aggregates and exhibit higher levels of liver-specific functions^[19,20]. Based on these results, we introduced galactosylated chitosan nanofiber scaffolds into the bioreactor to mimic the topography and biochemical environment of ECMs.

Bioreactors can be divided into four types: (1) hollow fibers (e.g., the HepatAssist system and the Extracorporeal Liver Assist Device, which suffer from oxygen substrate limitations^[21-23], and so a large proportion of hepatocytes may be anoxic and lose their functions); (2) beds or scaffolds (the major disadvantage of which is the direct contact between plasma and porcine liver cells, and so there is no barrier for porcine pathogens^[24,25]); (3) suspension and encapsulation chambers; and (4) flat plate and monolayer cultures. As one classical type of bioreactor, flat-plate bioreactors have many advantages^[26-28]. Firstly, the cells can adhere to the plates evenly and therefore making the microenvironment homogeneous. Secondly, the culture medium/plasma can make contact with seeded cells adequately, which allows sufficient material exchange. Thirdly, the bioreactor can be easily scaled up by adjusting the flat plates to meet the clinical requirements. Finally, the structure of multi-layer flat plates is similar to that of hepatic plates of a normal liver, which is crucial for the long-term maintenance of liver functions.

A successful liver support system is likely to depend on the viability and functions of the hepatocytes in the bioreactor. Previous research has shown that the toxic

substances of acute liver failure plasma can damage the hepatocytes^[29]. In our study, in order to reduce the damage to the hepatocytes caused by the toxic substances in acute liver failure plasma and improve the efficacy of the treatment, we combined bioartificial liver system with an anionic resin absorption column to form a novel HBAL system. An anionic resin absorption column was used before the bioreactor, which may not only protect the co-culture cells in the bioreactor from toxic effect of acute liver failure plasma, but also improve the effect of plasma detoxification.

In this study, we found that the ALT, AST, LDH, TBIL and ammonia in HBAL group appeared to be statistically decreased compared to the control group. Furthermore, the TBIL level in the HBAL group was lower than that in other treatment groups. In addition, the ALB in the BAL and HBAL groups significantly increased, and a significantly higher level was observed in the HBAL group as compared with the BAL group. The survival rate of the HBAL group was higher than in the other treatment groups and the control group. This result suggests that HBAL significantly prolonged the survival time of the canine models with acute liver failure. These data indicate that the performance of this novel HBAL system may be superior to that of the simple BAL systems.

The main recognized disadvantages of the use of porcine hepatocytes are the risk of infection of PERV and immunological rejection. Previous reports have shown that PERV successfully infected a variety of human cells *in vitro*, such as endothelial cells, fibroblasts and bone marrow stromal cells^[30-32]. Moreover, Wilson found that implanted porcine islets may lead to PERV infection in non-obese diabetic severe combined immunodeficiency mice^[33]. On the other hand, elevated titers of xenoantibodies have been found after treatment with porcine-based BAL systems, indicating that host exposure to porcine antigens occurs^[34]. In our study, the PERV RNA and RT activity in the canine plasma were all negative. No direct evidence of severe immunological complications were observed. Our results were similar to other reports^[35,36].

In conclusion, This novel HBAL system showed great efficiency and safety in the treatment of canines with acute liver failure and seemed to be applied in a clinical setting.

COMMENTS

Background

Orthotopic liver transplantation is a unique and effective treatment for acute liver failure. However, due to severe donor-liver shortage, high cost and exacerbation of disease, many patients die before they can receive the operation. Therefore, bioartificial livers have been proposed as temporary liver support for patients awaiting liver transplantation. However, bioartificial liver systems have not reached their full efficiency yet, since a method to enhance the viability and functions of hepatocytes in the bioreactor remains a difficult problem, and the function of detoxification is inadequate.

Research frontiers

Nowadays, various types of bioartificial liver are applied in clinic, and obtain

satisfactory curative effects. However, in a bioartificial liver, the problem remains as to how it would be possible to enhance the viability and functions of hepatocytes in the bioreactor.

Innovations and breakthroughs

In this study, the authors first developed a new bioartificial liver system based on a multi-layer flat-plate bioreactor with co-cultured pig hepatocytes and bone marrow mesenchymal stem cells so as to mimic the microenvironment *in vivo*. They then combined this new bioartificial liver system with an anionic resin absorption column to form a novel hybrid bioartificial liver (HBAL) system. An anionic resin absorption column was used before the bioreactor, which may not only protect the co-culture cells in the bioreactor from toxic effect of acute liver failure plasma, but also improve the effect of plasma detoxification.

Applications

The study results suggest that the HBAL showed great efficiency and safety in the treatment of acute liver failure.

Terminology

BAL: An artificial extracorporeal supportive device in which patient plasma is circulated extracorporeally through a bioreactor. The bioreactor houses metabolically active liver cells (hepatocytes) between artificial plates or capillaries. The functions of the liver are principally carried out by hepatocytes. The goal is to develop bioartificial liver devices in which hepatocytes are optimally maintained so that they carry out as many activities as possible.

Peer review

This is a good descriptive study in which authors evaluate the efficacy and safety of a HBAL system in the treatment of acute liver failure. The results are interesting and suggest that the HBAL could be used in the treatment of acute liver failure.

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Computed tomography virtual endoscopy with angiographic imaging for the treatment of type IV-A choledochal cyst

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Abstract

Type IV-A choledochal cysts (CCs) are a congenital biliary anomaly which involve dilatation of the extrahepatic and intrahepatic bile ducts. We present the case of a 30-year-old woman with type IV-A CC, on whom three-dimensional computed tomography (3D CT) and virtual endoscopy were performed. 3D CT revealed partial dilatation in the posterior branch of the intrahepatic bile duct and a relative stricture between it and the extrahepatic bile duct. Virtual endoscopy showed that this stricture was membrane-like and separated from the surrounding blood vessels. Based on these image findings, complete cyst resection, bile duct plasty for the stricture, and hepaticojejunostomy were safely performed. To the best of our knowledge, there are no reports of imaging by virtual endoscopy of the biliary tract which show the surrounding blood vessels running along the bile duct.

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Key words: Choledochal cyst; Bile duct dilatation; Computed tomography; Virtual endoscopy; Bile duct plasty

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INTRODUCTION

Choledochal cysts (CCs), namely, congenital bile duct dilatation, are high-risk factors for biliary tract cancer^[1,2]. Almost all cases of type I and type IV-A CCs are associated with pancreaticobiliary maljunction, and preventative diversion surgery is performed even without the presence of cancer^[3]. Type IV-A CCs involve dilatation of the extrahepatic and intrahepatic bile ducts, and relative stricture at the junction of both is often observed. If this stricture is left alone, several complications, including cholangitis, hepatolithiasis, and cholangiocarcinoma may occur after surgery. Therefore, bile duct plasty for stricture or bile duct resection which includes the stricture site is required^[4,5]. Moreover, if the stricture or cyst is present in the intrahepatic bile duct, hepatectomy is required to remove these lesions^[4,6]. Accordingly, for type IV-A CCs, a thorough examination of the biliary tract is necessary before surgery. Recently, three-dimensional (3D) imaging and virtual endoscopy based on computed tomography (CT) or magnetic resonance images have often been used for the evaluation of biliary tract anatomy and disease detection^[7-10]. Here, we report a case of type IV-A CC in which the condition of the bile duct stricture and blood



Figure 1 Percutaneous transhepatic bile duct drainage cholangiography demonstrating a cystic dilatation of the common bile duct and relative stricture (arrow) between the extrahepatic and intrahepatic bile ducts.

vessels running along the CC were confirmed by 3D CT imaging and virtual endoscopy, and surgery was safely performed.

CASE REPORT

A 30-year-old woman was admitted to a local hospital because of cholangitis during late pregnancy. Since marked dilatation of the common bile duct and jaundice were observed, she underwent percutaneous transhepatic bile duct drainage (PTCD). She was given a diagnosis of type IV-A CC with pancreaticobiliary maljunction and was transferred to our department for surgery. We performed cholangiography by injecting a contrast medium *via* a PTCD tube, and imaging data at 1 mm intervals obtained by multislice CT was evaluated using a 3D image analytic system (Synapse Vincent FN-7941; Fuji Film Medical Co., Ltd., Tokyo, Japan).

PTCD cholangiography and endoscopic retrograde cholangiopancreatography (ERCP) revealed marked cystic dilatation in the common bile duct (Figure 1). The end of the common bile duct was obstructed, and communication between the bile duct and the side of the papilla was not observed. Moreover, stricture was found in the transition site, which was assumed to be between the left hepatic duct and the intrahepatic bile duct. On a 3D image of the biliary tract, no dilatation in the anterior branch or the lateral branch of the intrahepatic bile duct, and no relative stricture of the extrahepatic bile duct were observed. However, the posterior branch showed partial dilatation in the transition site from the extrahepatic bile duct, and relative stricture was observed in the transition site, but with no stricture or dilatation in the peripheral bile duct (Figure 2).

A virtual endoscopic image of the biliary tract showed no stricture in the orifice of the anterior branch, but the presence of a membrane-like stricture in the orifice of the posterior branch was confirmed (Figure 3A). Furthermore, when this image was overlapped with blood vessel images obtained by 3D imaging, the distribution of a blood vessel system around the biliary tract was clearly

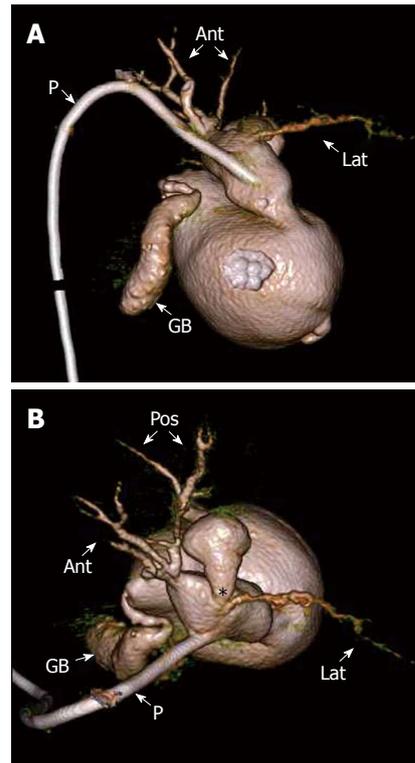


Figure 2 Three-dimensional image. A: Three-dimensional (3D) frontal image demonstrating no stricture or dilatation of the anterior and lateral branches of the bile duct; B: 3D head image showing a relative stricture (*) and partial dilatation of the posterior branch of the bile duct. P: Percutaneous transhepatic bile duct drainage tube; GB: Gallbladder; Ant: Anterior branch; Pos: Posterior branch; Lat: Lateral branch.

observed. This image showed that blood vessels did not run in the membrane-like stricture (Figure 3B).

During surgery, the gallbladder and dilated extrahepatic bile duct were removed. Next, the membrane-like stricture of the posterior branch was cut by several mm, and each cut end was sutured with 2 stitches of 5-0 absorbable sutures. This alleviated the relative stricture, and the orifice of the posterior branch expanded sufficiently (Figure 4). Finally, the bile ducts on the hepatic side and jejunum were anastomosed. In the histopathological findings of the resected specimen, chronic inflammation and hyperplasia of the gallbladder and bile duct were observed, but no malignancy was observed. One year after surgery, no complications, including cholangitis or hepatolithiasis, have been observed.

DISCUSSION

3D imaging and virtual endoscopy based on reconstructed CT images have been performed since the early 1990s, and have been applied in various fields. Initially, the images obtained were comparatively rough because they were taken from helical CT^[7], but with the introduction of multislice CT, it has become possible to obtain images almost as clear as those obtained in cholangioscopy^[8]. This imaging modality has 93% sensitivity in visualizing the biliary tract, 90% sensitivity in visualizing CCs, and 93%

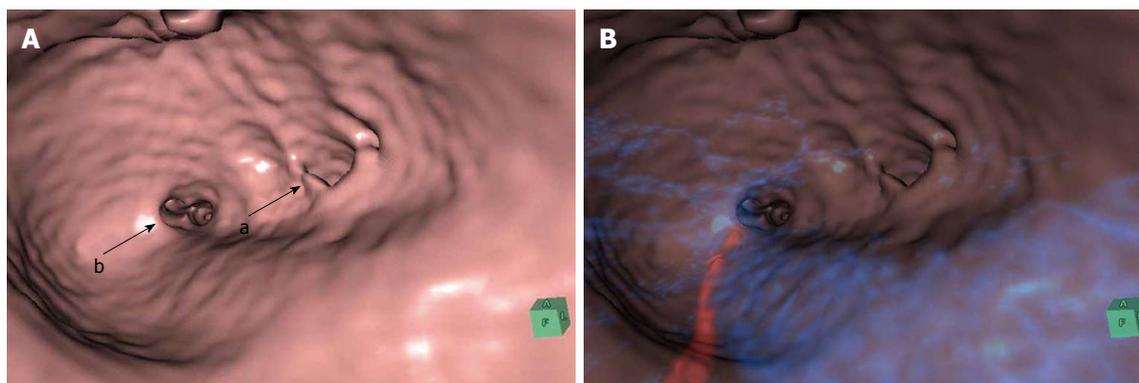


Figure 3 Virtual cholangioscopy. A: Virtual cholangioscopy demonstrating a relative stricture of the orifice in the posterior branch (a) and no stricture in the anterior branch (b); B: Composite virtual cholangioscopy demonstrating no relationship between the membrane-like stricture and the surrounding blood vessels. Red indicates the hepatic artery and blue denotes the portal vein.

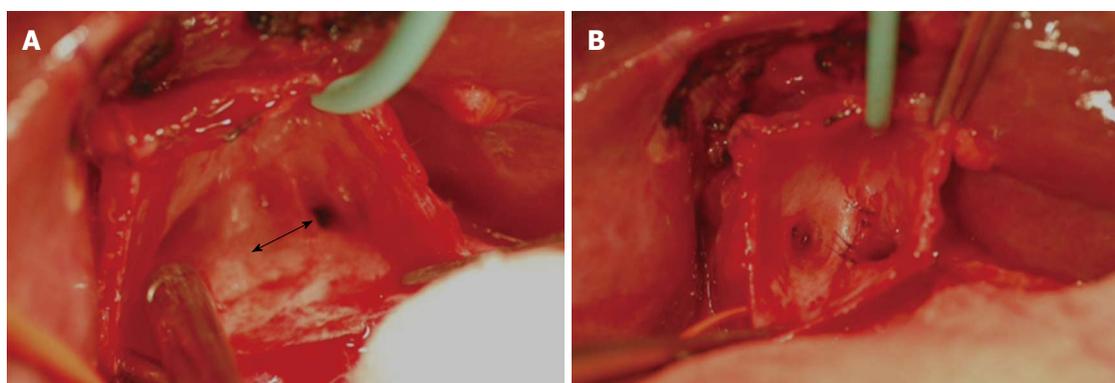


Figure 4 Operative findings. A: A membrane-like stricture, which was cut along the arrow line; B: Bile duct plasty was safely performed.

sensitivity in the diagnosis of lithiasis^[11]. Conventionally, ERCP is considered to be the best imaging modality for CCs, but its application has become less with the appearance of virtual endoscopy^[11]. One reason for this is that ERCP is invasive and poses the risk of complications including bleeding, cholangitis or pancreatitis. Moreover, if there is partial or complete obstruction of the bile duct, imaging of the peripheral bile duct may also be poor. Furthermore, since a high volume of contrast medium is needed to visualize CCs, there is a risk of missing small lesions in the biliary mucosa. In the present case, since the end of the common bile duct was completely obstructed, neither ERCP nor direct cholangioscopy could be performed. PTCD cholangiography revealed that there was a relative stricture in the transition site, which was assumed to be present between the left hepatic duct and the intrahepatic bile duct, but was clearly shown in the posterior branch of the bile duct by 3D imaging and virtual endoscopy. These results indicate that 3D imaging and virtual endoscopy can be very useful in understanding the anatomy of the biliary tract.

The basic surgical treatment for type IV-A CCs is as follows^[4,12]: (1) the gallbladder and extrahepatic bile duct portions with high carcinogenetic risk are completely removed; (2) if the transition site of the extrahepatic bile

duct and intrahepatic bile duct is narrow, bile duct plasty for stricture is performed; (3) if stricture or dilatation is observed in the intrahepatic bile duct, hepatectomy is also performed; and (4) during hepaticoenterostomy, a wide anastomotic stoma is formed to prevent cholangitis due to anastomotic stricture. In the present case, the posterior branch of the bile duct showed partial dilatation from the transition site from the extrahepatic bile duct, and a relative stricture was observed in the transition site. Moreover, according to the virtual endoscopic findings, this was a membrane-like stricture, and bile duct plasty was performed. There was no other stricture or dilatation in the intrahepatic bile duct, thus hepatectomy was not required. In the present case, by confirming blood vessels running around the bile duct using virtual endoscopy, the risk of mistakenly cutting the blood vessels by bile duct plasty could be avoided. To the best of our knowledge, there are no reports considering the overlapping of surrounding blood vessels running on the same image through observation of the bile duct by virtual endoscopy. Since the hepatic artery, and often the portal vein, may become displaced by a dilated cyst, caution is required in performing surgery of CCs. The present results clearly show that CT virtual endoscopy greatly contributes to the diagnosis and treatment of CCs.

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Direct cholangioscopy combined with double-balloon enteroscope-assisted endoscopic retrograde cholangiopancreatography

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Abstract

Double-balloon enteroscope (DBE)-assisted endoscopic retrograde cholangiopancreatography (ERCP) is an effective endoscopic approach for pancreatobiliary disorders in patients with altered gastrointestinal anatomy. Endoscopic interventions *via* DBE in these postoperative settings remain difficult because of the lack of an elevator and the use of extra-long ERCP accessories. Here, we report the usefulness of direct cholangioscopy with an ultra-slim gastroscope during DBE-assisted ERCP. Three patients with choledocholithiasis in postoperative settings (two patients after Billroth II gastrojejunostomy and one patient after Roux-en-Y gastrojejunostomy) were treated. DBE was used to gain access to the papilla under carbon dioxide insufflation, and endoscopic sphincterotomy was performed with a conventional sphincterotome. For direct cholangioscopy, the entero-

scope was exchanged for an ultra-slim gastroscope through an incision in the overtube, which was inserted directly into the bile duct. Direct cholangioscopy was used to extract retained bile duct stones in two cases and to confirm the complete clearance of stones in one case. Bile duct stones were eliminated with a 5-Fr basket catheter under direct visual control. No adverse events were noted in any of the three cases. Direct cholangioscopy with an ultra-slim gastroscope facilitates subsequent treatment within the bile duct. This procedure represents another potential option during DBE-assisted ERCP.

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Key words: Direct cholangioscopy; Double-balloon enteroscope; Endoscopic retrograde cholangiopancreatography

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INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP)-related maneuvers are difficult in patients with altered gastrointestinal (GI) anatomy due to previous abdominal surgery. ERCP success rates using conventional methods

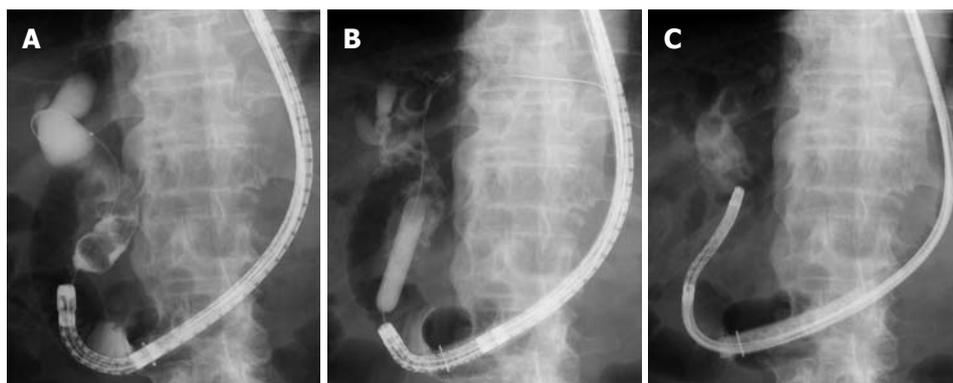


Figure 1 X-ray images. A: Cholangiography revealed a dilated bile duct and multiple stones piled up at the lower to middle part of the bile duct; B: Papillary dilation using a large balloon catheter; C: An ultra-slim gastroscope was introduced directly into the bile duct.

in patients after Billroth II gastrojejunostomy, Whipple pancreatoduodenectomy and Roux-en-Y gastrojejunostomy are reported to be as low as 52%^[1,2], 51%^[3], and 33%^[2], respectively. If the papilla or biliary anastomosis in the afferent loop cannot be reached by an ordinary duodenoscope because of excessive intestinal length and/or sharp angulation of the anastomosis, it has recently become possible to use a double-balloon enteroscope (DBE) for ERCP. There are several hurdles for this technique, including reaching the papilla or biliary anastomosis, selective duct cannulation, and accomplishment of the treatment. The enteroscope is advanced through the overtube into the afferent jejunal loop using the push-and-pull double balloon technique. Identifying the opening of the afferent loop and coping with sharp angulation of the anastomosis are the keys for the successful endoscope insertion. Once the papilla or biliary anastomosis is reached, however, endoscopic interventions *via* DBE in these postoperative settings remain the most difficult ERCP manipulations because of the lack of an elevator and the use of extra-long ERCP accessories.

Here, we report three cases with choledocholithiasis treated with DBE-assisted ERCP and demonstrate the usefulness of direct cholangioscopy with an ultra-slim gastroscope for subsequent treatment.

CASE REPORT

Case 1

An 80-year-old man was referred for the treatment of choledocholithiasis. He had undergone partial gastrectomy with Roux-en-Y gastrojejunostomy for gastric carcinoma five years before. With the DBE (EC-450BI5; Fujinon, Osaka, Japan) with a 152 cm working length and a 2.8 mm instrumental channel, the papilla could be reached *via* the Roux-en-Y anastomosis under carbon dioxide insufflation. Cannulation of the papilla and cholangiography revealed a dilated bile duct and multiple stones (maximum size 20 mm) piled up at the lower to middle part of the bile duct (Figure 1A). Endoscopic sphincterotomy (EST) was performed with a conventional sphincterotome, placing the papilla of Vater at the

6 o'clock position. After mechanical lithotripsy with a 7-Fr crusher catheter (Xemex crusher catheter; Zeon Medical, Tokyo, Japan), stone extraction was attempted with a basket catheter but frequently failed because the catheter was inserted into the dilated cystic duct. Because the same attempt was performed unsuccessfully during another session, the enteroscope was exchanged for an ultra-slim gastroscope (EG-530NW; Fujinon, outer diameter 5.9 mm), leaving the overtube with its balloon inflated. The ultra-slim endoscope was introduced through an incision in the overtube, which was made at a point 100 cm from its tip. The ultra-slim endoscope was advanced through the overtube up to the papilla and was directly inserted into the bile duct after papillary dilation using a large balloon catheter (CRE balloon dilator; Boston Scientific Japan, Tokyo, Japan) (Figure 1B and C). Cholangioscopy showed multiple stones inside the dilated bile duct and the cystic duct. The stones were all removed with a 5-Fr basket catheter (Basket catheter, diamond type; MTW Endoskopie, Wesel, Germany) under direct visual control (Figure 2). The patient had a good clinical course after the procedure, and no adverse events occurred.

Case 2

An 89-year-old man developed acute cholangitis with choledocholithiasis. He had undergone partial gastrectomy with Billroth II gastrojejunostomy for gastric carcinoma 21 years before. Because of the long afferent loop, the DBE (EC-450BI5; Fujinon, Osaka, Japan) was used to gain access to the papilla under carbon dioxide insufflation. Cannulation of the papilla and cholangiography showed two bile duct stones (maximum size 15 mm) at the middle part of the bile duct. After EST with a conventional sphincterotome, a smaller stone located distally was extracted with a basket catheter (Figure 3). Another stone could not be removed despite all possible efforts because of its impaction. The enteroscope was then exchanged for an ultra-slim gastroscope as described above. The endoscope was inserted directly into the bile duct, revealing an impacted stone inside the duct. The stone was removed successfully with a 5-Fr basket catheter under direct visual control, and the proximal bile duct was

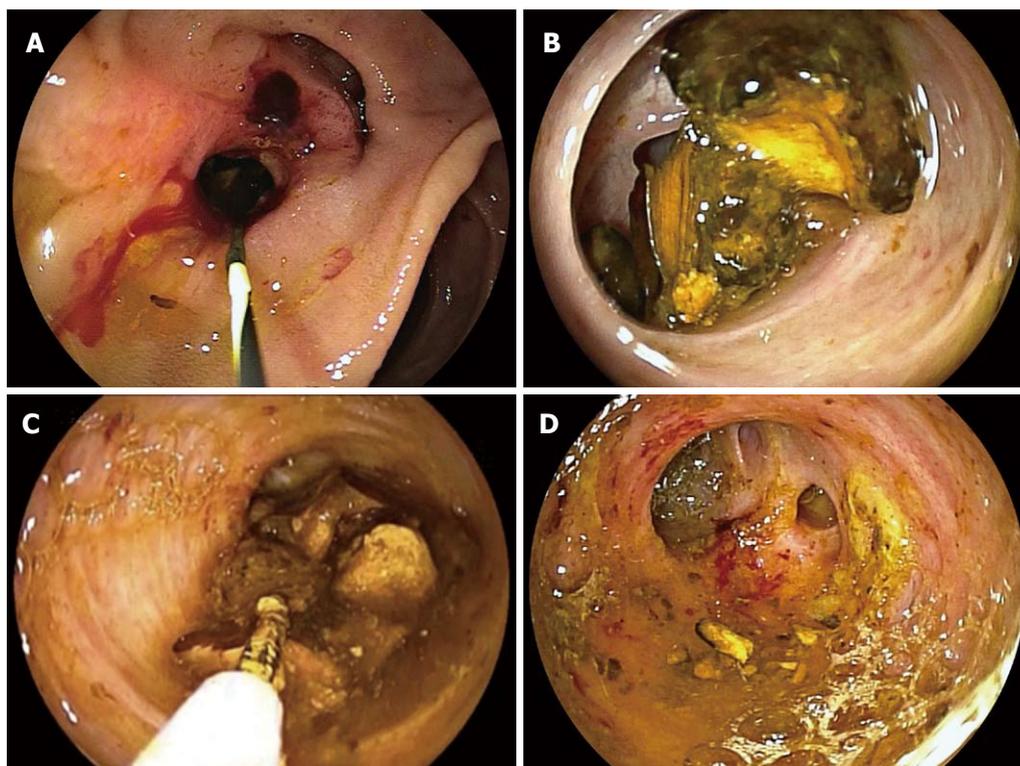


Figure 2 Endoscopic images. A: The papilla of Vater after balloon dilation; B: Cholangioscopy showed multiple stones inside the bile duct; C, D: The stones were all removed with a 5-Fr basket catheter under direct visual control.



Figure 3 Cholangiography. A smaller stone located distally was extracted with a basket catheter. Another stone was impacted inside the bile duct (arrow).

also investigated (Figure 4A-C). The patient had a good clinical course after the procedure, and no adverse events occurred.

Case 3

A 73-year-old man was referred because of cholestasis and suspected choledocholithiasis. He had undergone partial gastrectomy with Billroth II gastrojejunostomy for gastric ulcer 43 years before. The DBE (EC-450BI5; Fujinon, Osaka, Japan) was first inserted into the efferent loop and was then introduced up to the papilla *via* Braun's anastomosis under carbon dioxide insufflation. Cannulation of the papilla and cholangiography revealed several small stones (5 mm in size) inside the bile duct. After

EST with a conventional sphincterotome, the stones were extracted with a basket catheter and a retrieval balloon (Figure 5A-C). An ultra-slim gastroscope was then introduced into the bile duct as described above. Cholangioscopy confirmed complete clearance of the stones inside the bile duct (Figure 6A and B). The patient had a good clinical course after the procedure, and no adverse events occurred.

DISCUSSION

Since the first report^[4] of a successful DBE-assisted cholangiography in a pediatric case with late biliary stricture of choledochojejunostomy after living donor liver transplantation, many case series^[5-17] have reported the effectiveness of DBE-assisted ERCP for pancreatobiliary disorders in various postoperative settings. In most previous reports^[6-8,10-12,15,16], the therapeutic enteroscope, which has a 200-cm working length and a 2.8-mm instrumental channel, has been used for DBE-assisted ERCP. Recently, the single-balloon enteroscope (SBE) system (Olympus Medical Systems, Tokyo, Japan), which consists of an enteroscope without the balloon at the tip of the endoscope and an overtube equipped with a balloon, has also been used for ERCP in patients with altered postsurgical anatomy with similar technical success rates^[18-20].

The maneuverability of these enteroscopes is rather poor and only limited ERCP accessories are available for these endoscopes because of the long working length. To overcome these drawbacks, Fährndrich *et al*^[9] reported a



Figure 4 Direct cholangioscopy with an ultra-slim gastroscope. A: Cholangioscopy revealed an impacted stone inside the bile duct; B: The stone was removed with a 5-Fr basket catheter under direct visual control; C: The proximal bile duct was also investigated.

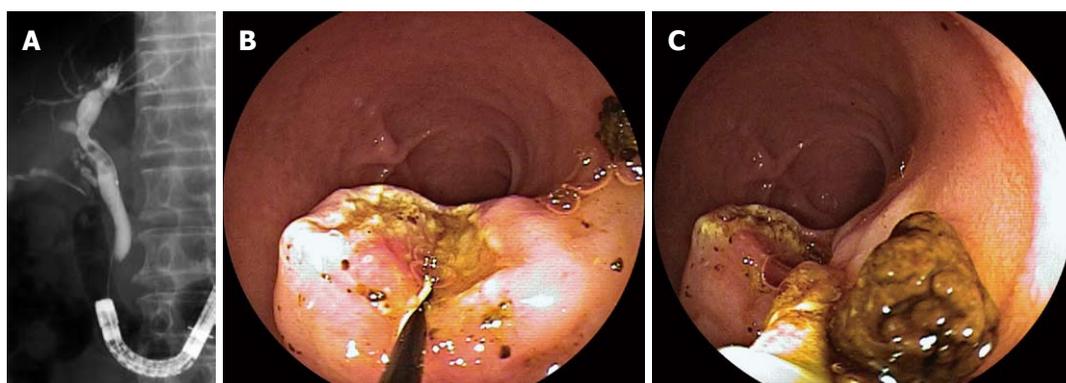


Figure 5 X-ray and endoscopic images. A: Cholangiography revealed several small stones inside the bile duct; B: The papilla of Vater after endoscopic sphincterotomy; C: The bile duct stones were extracted with a basket catheter and a retrieval balloon.

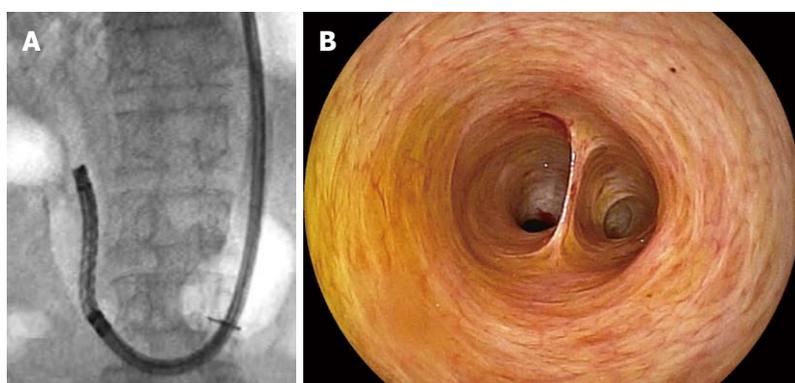


Figure 6 Direct cholangioscopy. A: An ultra-slim gastroscope was introduced directly into the bile duct after removal of bile duct stones; B: Cholangioscopy confirmed complete clearance of the duct.

facilitated method for endoscopic interventions at the bile duct during DBE-assisted ERCP that involved exchanging the enteroscope with an ordinary gastroscope after incision of the overtube once the papilla or the biliary anastomosis was reached. This technique is favorably performed in our country when the SBE or the “long” DBE is used for ERCP because long-length ERCP accessories are not commercially available in Japan. Alternatively, the DBE, which has a 152 cm working length and a 2.8 mm instrumental channel, can be used. This instrument is the “short” DBE for which conventional ERCP accessories are available. With this enteroscope, Shimatani *et al*^[13]

performed DBE-assisted ERCP and related therapeutic maneuvers in 68 patients with altered GI anatomies with an extremely high success rate.

Another disadvantage of DBE-assisted ERCP is that the enteroscope is forward-viewing. The lack of an elevator and the absence of the side-viewing perspective reduce the success rate of cannulation or sphincterotomy. Moreover, additional treatment becomes difficult in cases such as the retained bile duct stones described here, even if access to the biliary duct is obtained. Direct visualization of the bile duct with an ultra-slim gastroscope expands the options for therapy inside the duct

because the endoscope has a 2 mm instrumental channel for which 5-Fr accessories (including the biopsy forceps, the basket catheter, the laser lithotripsy probe, and the argon plasma coagulation probe) are available. The ultra-slim endoscope was introduced through incision of the overtube. The overtube was incised for three-quarters of its circumference on the side opposite of the pressure line, as described by Fährndrich *et al*^[9]. Care was taken not to damage the pressure line of the overtube to maintain the inflation of the balloon. Although this technique is only possible when the intact papilla or biliary anastomosis is reached with (at most) 100 cm of the enteroscope introduced, it is easy to advance the ultra-slim endoscope into the bile duct through the papilla treated with EST or balloon dilation because the approach is from the distal side of the afferent loop.

We did not encounter any adverse events in this case series. However, care must be taken because DBE-assisted ERCP can potentially cause serious complications, such as perforation, bleeding, or pancreatitis, during or after the scope insertion and ERCP procedures. Although no serious complications of direct cholangioscopy with an ultra-slim endoscope have been reported^[21,22], there is still a risk of air embolism; pneumobilia is often recognized after EST. Carbon dioxide insufflation during the DBE procedure may be effective in preventing this complication.

In conclusion, DBE-assisted ERCP appears to be a promising method; however, this technique still has several disadvantages. Direct cholangioscopy with an ultra-slim gastroscope compensates for drawbacks of the enteroscope and facilitates subsequent treatment within the bile duct. This procedure may represent another option during DBE-assisted ERCP.

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Multiple esophageal variceal ruptures with massive ascites due to myelofibrosis-induced portal hypertension

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Abstract

A 75-year old man had been diagnosed at 42 years of age as having polycythemia vera and had been monitored at another hospital. Progression of anemia had been recognized at about age 70 years, and the patient was thus referred to our center in 2008 where secondary myelofibrosis was diagnosed based on bone marrow biopsy findings. Hematemesis due to rupture of esophageal varices occurred in January and February of 2011. The bleeding was stopped by endoscopic variceal ligation. Furthermore, in March of the same year, hematemesis recurred and the patient was transported to our center. He was in irreversible hemorrhagic shock and died. The autopsy showed severe bone marrow fibrosis with mainly argyrophilic fibers, an observation consistent with myelofibrosis. The liver weighed 1856 g the spleen 1572 g, indicating marked hepatosplenomegaly. The liver and spleen both showed extramedullary hemopoiesis. Myelofibrosis is often complicated by portal hypertension and is occasionally associated with gastrointestinal hemorrhage due to esophageal varices. A patient diagnosed as having myelofibrosis needs to

be screened for esophageal/gastric varices. Myelofibrosis has a poor prognosis. Therefore, it is necessary to carefully decide the therapeutic strategy in consideration of the patient's concomitant conditions, treatment invasiveness and quality of life.

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Key words: Myelofibrosis; Portal hypertension; Rupture of esophageal varices

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INTRODUCTION

Myelofibrosis is a disease resulting in extensive diffuse fibrosis involving the bone marrow and is characterized by hepatosplenomegaly, leukoerythroblastosis and so on.

Portal hypertension reportedly occurs as a complication in 10%-17% of patients^[1] and may be accompanied by gastroesophageal variceal hemorrhage^[2]. Herein, we present a patient diagnosed with myelofibrosis who died 3 years later due to repeated ruptures of esophageal varices, associated with massive ascites. Autopsy findings of this case are described with a discussion of the relevant literature.

CASE REPORT

The patient was a 75-year-old man. Increases in 2 blood

Table 1 Laboratory data					
Hematology		Blood chemistry		Coagulation	
WBC	29 700/ μ L	Na	142 mEq/L	PT	0.98
BAND	35.4%	K	4.5 mEq/L	APTT	32.9 s
SEG	45%	Cl	103 mEq/L		
LYMP	2.6%	BUN	24 mg/dL		
MONO	1.4%	Cr	0.8 mg/dL	Serology	
EOSI	2.8%	AST	26 IU/L	HBsAg	(-)
BASO	4.2%	ALT	25 IU/L	HBsAb	(-)
PRO	+	ALP	599 IU/L		
MYELO	7.4%	LDH	578 IU/L		
META	0.8%	γ -GTP	65 IU/L		
BLAST	0.2%	T-Bil	1.4 mg/dL		
RBC	$641 \times 1/\mu$ L	Amy	28 IU/L		
Hb	14.2 g/dL	CK	39 IU/L		
Ht	47.3%	TP	6.3 g/dL		
MCV	73.8 fl	Alb	4.1 g/dL		
MCH	22.2 pg	CRP	1.36 mg/dL		
MCHC	30 g/dL				
Plt	$156 \times 1/\mu$ L				

WBC: White blood cell; BAND: Blood band cell; SEG: Blood segmented neutrophil; LYMP: Lymphocyte; MONO: Monocyte; EOSI: Eosinocyte; BASO: Basocyte; PRO: Promyelocyte; MYELO: Myelocyte; BLAST: Blast cell; RBC: Red blood cell; Hb: Hemoglobin; Ht: Hematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; Plt: Platelet; BUN: Blood urea nitrogen; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; LDH: Lactate dehydrogenase; γ -GTP: Gamma-glutamyl transpeptidase; T-Bil: Total bilirubin; CK: Creatine kinase; TP: Total protein; Alb: Albumin; CRP: C-reactive protein; PT: Prothrombin time; APTT: Activated partial thromboplastin time; HBsAg: Hepatitis B surface antigen; HBsAb: Hepatitis B surface antibody; META: Metamyelocyte.



Figure 1 Splenic enlargement and dilatation of the splenic vein on abdominal computed tomography.

cell lines, white and red blood cells, had been detected by medical examination at age 42 years, and polycythemia vera was diagnosed at another hospital. Blood hemoglobin (Hb) at this time was approximately 19 g/dL. Thereafter, the patient had been monitored without treatment, but blood Hb gradually decreased from 2005 onward. Thus, the patient was referred to the Department of Hematology of our center in October 2009.

The white blood cell count was 29 700/ μ L, the red blood cell count $641 \times 10^6/\mu$ L, showing an increase in 2 blood cell lines, and juvenile cells were noted (Table 1).

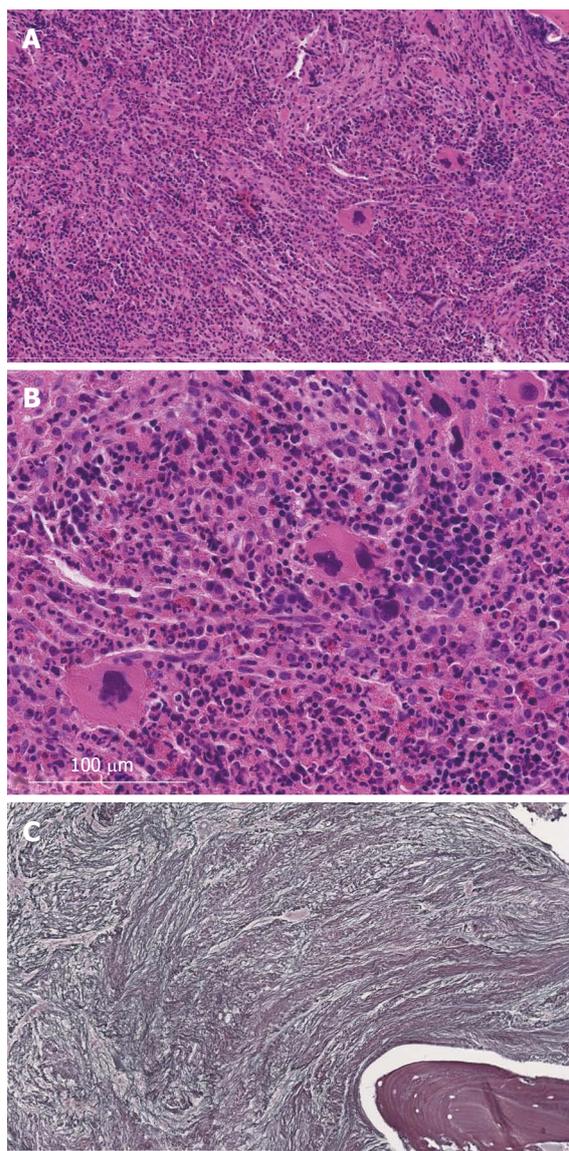


Figure 2 Microscopic findings on autopsy on bone marrow biopsy. A: Hematoxylin and eosin, $\times 20$; B: Hematoxylin and eosin, $\times 40$; C: Silver, $\times 40$. Adipocyte disappearance and markedly decreased hematopoietic cells.

Abdominal computed tomography scan revealed a large quantity of ascites in the peritoneal cavity, and marked spleen enlargement and dilatation of the splenic vein were seen (Figure 1). There were no abnormalities of the liver or other organs. No obvious blood clot formation was seen in the main blood vessels.

Bone marrow aspiration was attempted to evaluate the disease state but the tap was dry. Therefore, bone marrow biopsy was conducted from the ilium (Figure 2). Adipocytes were completely absent and hematopoietic cells markedly decreased. Thus, secondary myelofibrosis was diagnosed. There were no findings of progression to leukemia, and the patient was thus monitored by our hematology department.

The patient was urgently transported to our center due to hematemesis in January 2011. Urgent endoscopy was conducted, and esophageal varices of Lm, F2, and

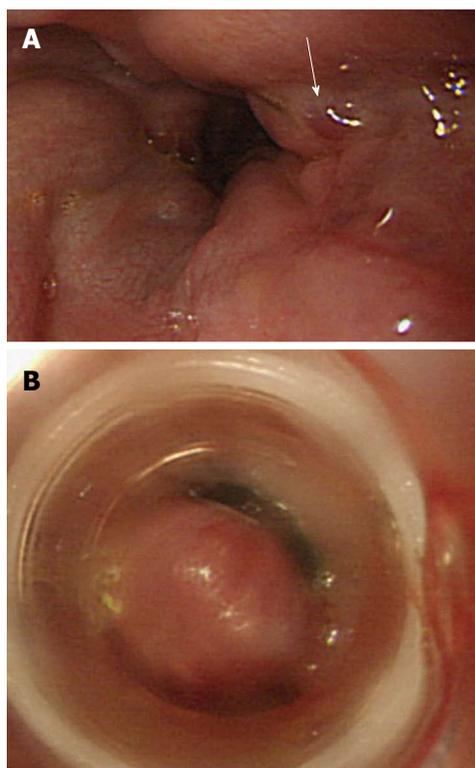


Figure 3 Endoscopic image in the lower esophagus. A: Showing a hemocystic spot by the white arrow; B: Endoscopic variceal ligation was thus conducted.

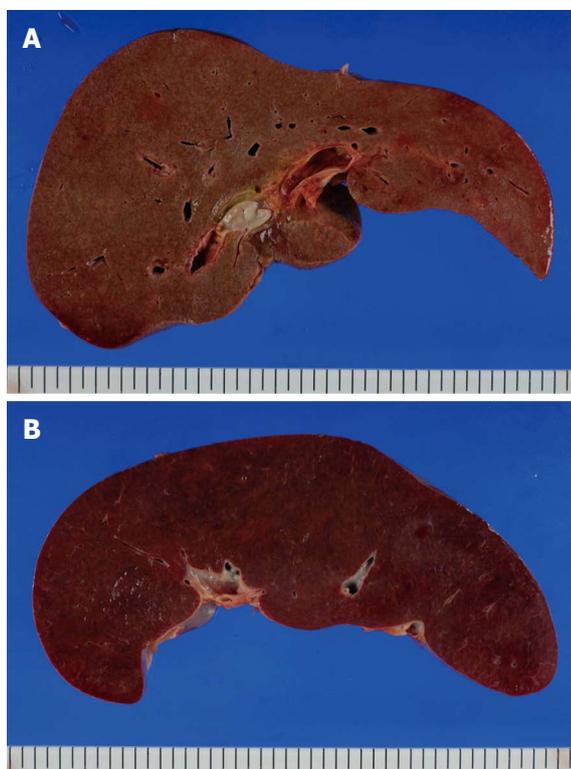


Figure 4 Macroscopic findings on autopsy. A: The liver weighed 1856 g; B: The spleen 1572 g indicating hepatosplenomegaly.

Cb and the red color sign (hemocystic spot) in the

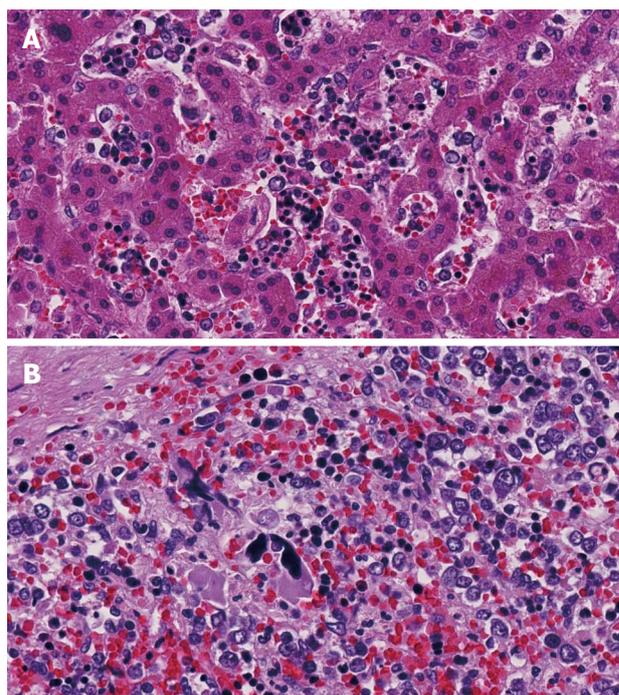


Figure 5 Microscopic findings on autopsy stained with hematoxylin and eosin. A: Liver; B: Spleen. Both the liver and the spleen showed extramedullary hemopoiesis.

lower esophagus were found (Figure 3). Therefore, endoscopic variceal ligation (EVL) was conducted. A large quantity of poorly controlled ascites was observed, and the patient's activities of daily living (ADL) were poor. Thus, no additional treatment such as endoscopic injection sclerotherapy was conducted, and the patient was discharged from the hospital. He had hematemesis in February of the same year, and bleeding was again stopped with EVL. However, no additional treatment was conducted for similar reasons. Furthermore, the patient had hematemesis again in March of the same year and was emergently transported to our center. Urgent endoscopy was conducted; however, because there was a large quantity of fresh blood in the stomach and esophagus, it was difficult to identify bleeding points. Blood pressure became unstable during the examination. Therefore, we abandoned EVL and inserted a Sengstaken-Blakemore tube. The patient was hospitalized at this time. However, the hemorrhagic shock was irreversible and he died 20 h after emergency transport. With informed consent from his family, autopsy was conducted the same day.

Autopsy findings

The enlarged liver weighed 1856 g but there were no obvious abnormalities on histopathological observation of the portal area or in hepatocytes. Extramedullary hemopoiesis was seen mainly in sinusoids. The spleen weighed 1572 g, indicating marked splenomegaly, and obscuration of white matter and the presence of bone marrow cells with splenic red pulp were the main features noted (Figures 4 and 5). In addition to these findings, small

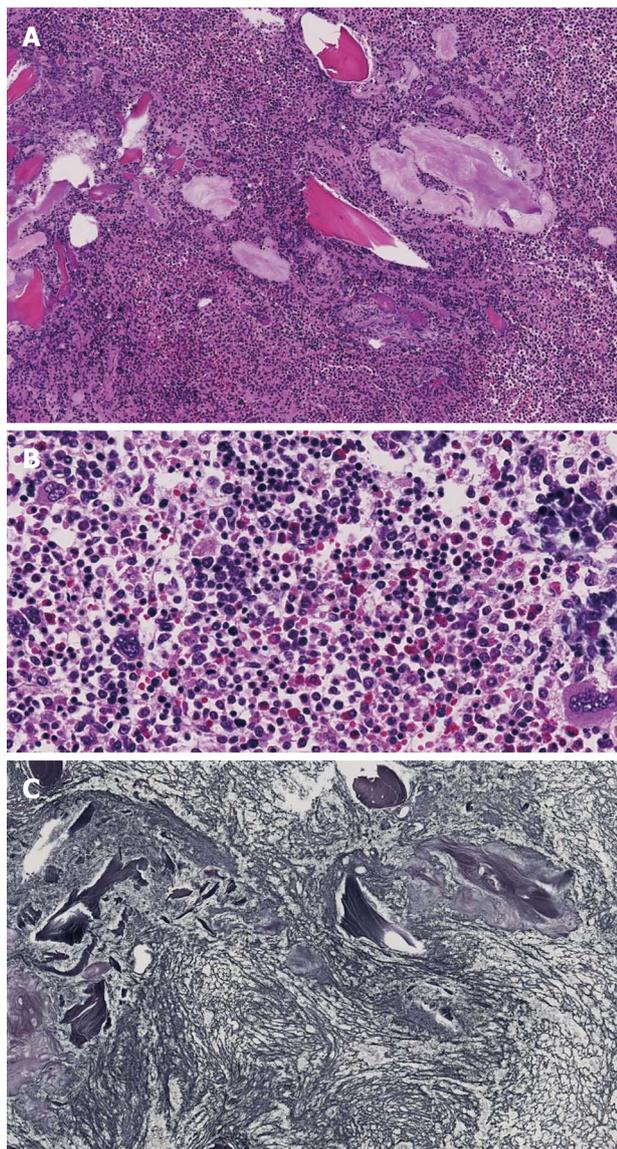


Figure 6 Microscopic findings on autopsy (bone marrow). A: Hematoxylin and eosin (HE), $\times 10$; B: HE, $\times 40$; C: Silver, $\times 10$. Adipocyte disappearance and 3-lineage differentiation were seen. Silver staining showed severe fibrosis with mainly argyrophilic fibers.

numbers of foci of extramedullary hematopoiesis were detected in the lungs, kidneys and lymph nodes around the pancreas. In bone marrow, adipocytes had completely disappeared, and 3-line cellular growth was seen. Severe fibrosis with mainly argyrophilic fibers was revealed by silver staining, and this finding was consistent with myelofibrosis (Figure 6).

DISCUSSION

Polycythemia vera is a myeloproliferative tumors resulting in absolute increases in the red blood cell count and circulating blood volume due to an acquired genetic abnormality of hematopoietic stem cells. Survival without treatment averages 18 mo, and the leading causes of death are reportedly thrombosis, progression to leukemia,

myelodysplastic syndrome or myelofibrosis, hemorrhage, and so on^[3,4].

The present case was diagnosed as having polycythemia vera, and then showed a disease shift to secondary myelofibrosis approximately 30 years later. The histology of both biopsy and autopsy osseous samples showed increased cells in the 3-lineage differentiations. Since there was no morphological abnormality in them and limited proliferation of immature cells, it was considered that malignant lymphoma and leukemia could be ruled out.

Three years thereafter, the patient repeatedly suffered rupture of esophageal varices and ultimately died of this complication. Non-cirrhotic portal hypertension has various causes. Myeloproliferative tumors are often accompanied by marked splenomegaly and can cause portal hypertension due to increased portal vein flow^[5,6]. Furthermore, Shaldon *et al*^[7] reported that pre-sinusoidal blood flow resistance increases, in association with extramedullary hemopoiesis in hepatic sinusoids. Even if the increase in blood flow is mild, impaired blood flow can be regarded as the cause of increased pressure. Other than this, portal vein thrombosis within and outside of the liver and venous thrombosis can be causes in some cases. Liver histology in the present case showed mild fibrosis in the portal area and extramedullary hemopoiesis in hepatic sinusoids but no inflammatory cell infiltrate or reconstruction of hepatic lobules.

At autopsy, extramedullary hemopoiesis was not considered to be severe enough to have resulted in impaired blood flow. In addition, no obvious portal vein thrombosis or venous thrombosis was seen, while spleen weight was markedly increased as compared with that of the liver. These findings and splenic vein enlargement were consistent with portal hypertension. Thus, portal hypertension may have been caused by the increased blood flow associated with splenomegaly in our present case.

Myelofibrosis is often associated with splenomegaly, and we advocate paying close attention to myelofibrosis that may be accompanied by portal hypertension clinically. Patients with concomitant gastroesophageal varices can reportedly be treated effectively with splenectomy, splenic embolization, and transjugular intrahepatic portal-hepatic venous shunting^[2,8].

The massive ascites in our present case was considered to have been caused by pre-sinusoidal lymphatic blockade due to extramedullary hemopoiesis; however, ascites was refractory to treatments such as the administration of diuretics. Therefore, we considered the possibility that transjugular intrahepatic portal-hepatic venous shunting would be effective.

The ADL of the present case were poor, and ascites was poorly controlled at the time of the initial hospitalization. Treatments for portal hypertension were thus considered to be too invasive and difficult; thus, they were not administered. When a patient is diagnosed as having myelofibrosis, screening endoscopy for esophageal and gastric varices should be conducted regularly, keeping in mind that portal hypertension may develop. When risk of

rupture is considered, prophylactic endoscopic therapy or endovascular treatment should be considered while the patient's general condition is good. However, the average survival period for myelofibrosis patients ranges from 3 to 7 years, and no more than 20% can expect a median survival of 10 years as this disease has a poor prognosis^[2]. Therapy should be carefully selected in full consideration of invasiveness, as it impacts quality of life and prognosis.

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Events Calendar 2012

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January 19-21, 2012 American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium San Francisco, CA 3000, United States	March 17-20, 2012 Mayo Clinic Gastroenterology and Hepatology Orlando, FL 32808, United States	April 28, 2012 Issues in Pediatric Oncology Kiev, Ukraine	September 7-9, 2012 The Viral Hepatitis Congress Frankfurt, Germany
January 19-21, 2012 2012 Gastrointestinal Cancers Symposium San Francisco, CA 94103, United States	March 26-27, 2012 26th Annual New Treatments in Chronic Liver Disease San Diego, CA 92121, United States	May 3-5, 2012 9th Congress of The Jordanian Society of Gastroenterology Amman, Jordan	September 8-9, 2012 New Advances in Inflammatory Bowel Disease La Jolla, CA 92093, United States
January 20-21, 2012 American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology Miami Beach, FL 33141, United States	March 30-April 2, 2012 Mayo Clinic Gastroenterology and Hepatology San Antonio, TX 78249, United States	May 7-10, 2012 Digestive Diseases Week Chicago, IL 60601, United States	September 8-9, 2012 Florida Gastroenterologic Society 2012 Annual Meeting Boca Raton, FL 33498, United States
February 3, 2012 The Future of Obesity Treatment London, United Kingdom	March 31-April 1, 2012 27th Annual New Treatments in Chronic Liver Disease San Diego, CA 92121, United States	May 17-21, 2012 2012 ASCRS Annual Meeting- American Society of Colon and Rectal Surgeons Hollywood, FL 1300, United States	September 15-16, 2012 Current Problems of Gastroenterology and Abdominal Surgery Kiev, Ukraine
February 16-17, 2012 4th United Kingdom Swallowing Research Group Conference London, United Kingdom	April 8-10, 2012 9th International Symposium on Functional GI Disorders Milwaukee, WI 53202, United States	May 18-19, 2012 Pancreas Club Meeting San Diego, CA 92101, United States	September 20-22, 2012 1st World Congress on Controversies in the Management of Viral Hepatitis Prague, Czech
February 23, 2012 Management of Barretts Oesophagus: Everything you need to know Cambridge, United Kingdom	April 13-15, 2012 Asian Oncology Summit 2012 Singapore, Singapore	May 18-23, 2012 SGNA: Society of Gastroenterology Nurses and Associates Annual Course Phoenix, AZ 85001, United States	October 19-24, 2012 American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course Las Vegas, NV 89085, United States
February 24-27, 2012 Canadian Digestive Diseases Week 2012 Montreal, Canada	April 15-17, 2012 European Multidisciplinary Colorectal Cancer Congress 2012 Prague, Czech	May 19-22, 2012 2012-Digestive Disease Week San Diego, CA 92121, United States	November 3-4, 2012 Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients Dnepropetrovsk, Ukraine
March 1-3, 2012 International Conference on Nutrition and Growth 2012 Paris, France	April 18-20, 2012 The International Liver Congress 2012 Barcelona, Spain	June 2-6, 2012 American Society of Colon and Rectal Surgeons Annual Meeting San Antonio, TX 78249, United States	November 4-8, 2012 The Liver Meeting San Francisco, CA 94101, United States
March 7-10, 2012 Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting San Diego, CA 92121, United States	April 19-21, 2012 Internal Medicine 2012 New Orleans, LA 70166, United States	June 18-21, 2012 Pancreatic Cancer: Progress and Challenges Lake Tahoe, NV 89101, United States	November 9-13, 2012 American Association for the Study of Liver Diseases Boston, MA 02298, United States
	April 20-22, 2012 Diffuse Small Bowel and Liver Diseases Melbourne, Australia	July 25-26, 2012 PancreasFest 2012 Pittsburgh, PA 15260, United States	December 1-4, 2012 Advances in Inflammatory Bowel Diseases Hollywood, FL 33028, United States
	April 22-24, 2012 EUROSON 2012 EFSUMB Annual	September 1-4, 2012 OESO 11th World Conference Como, Italy	
		September 6-8, 2012 2012 Joint International	

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

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 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

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

No author given

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

Volume with supplement

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No volume or issue

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

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Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary 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. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

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

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- 15 Morse SS. Factors in the emergence of infectious dis-

eases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

Patent (list all authors)

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

Statistical data

Write as mean \pm SD or mean \pm SE.

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ABOUT COVER Digestive Disease Week, May 19-22, 2012, Lian-Sheng Ma, President and Editor-in-Chief (first left) with *World Journal of Gastroenterology* Editorial Board Members, Carlos J Pirola (first right), PhD, FAHA and Silvia Sookoian (middle), MD, PhD, both from Argentina

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Alanine and aspartate aminotransferase and glutamine-cycling pathway: Their roles in pathogenesis of metabolic syndrome

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factors such as obesity, insulin resistance (IR), high blood pressure, and dyslipidemia were associated with several metabolites, including branched-chain amino acids, other hydrophobic amino acids, tryptophan breakdown products, and nucleotide metabolites. In addition, the authors found a significant association of IR traits with glutamine, glutamate and the glutamine-to-glutamate ratio. These data provide new insight into the pathogenesis of MS-associated phenotypes and introduce a crucial role of glutamine-cycling pathway as prominently involved in the development of metabolic risk. We consider that the hypothesis about the role of abnormal glutamate metabolism in the pathogenesis of the MS is certainly challenging and suggests the critical role of the liver in the global metabolic modulation as glutamate metabolism is linked with aminotransferase reactions. We discuss here the critical role of the "liver metabolism" in the pathogenesis of the MS and IR, and postulate that before fatty liver develops, abnormal levels of liver enzymes, such as alanine and aspartate aminotransferases might reflect high levels of hepatic transamination of amino acids in the liver.

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Key words: Alanine; Aspartate; Glutamine; Glutamate; 2-oxoglutarate; Glycolysis; Pyruvate

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Abstract

Although new research technologies are constantly used to look either for genes or biomarkers in the prediction of metabolic syndrome (MS), the pathogenesis and pathophysiology of this complex disease remains a major challenge. Interestingly, Cheng *et al* recently investigated possible pathways underlying MS by high-throughput metabolite profiling in two large and well characterized community-based cohorts. The authors explored by liquid chromatography and mass spectrometry the plasma concentrations of 45 distinct metabolites and examined their relation to cardiometabolic risk, and observed that metabolic risk

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INVITED COMMENTARY ON HOT ARTICLES

The metabolic syndrome (MS), a complex disorder associated with several metabolic disturbances and mostly characterized by insulin resistance (IR) in several tissues, results from a complex interplay between genetic and environmental factors^[1]. Among the environmental factors, decreased physical activity, increased nutrient availability and over nutrition, play an important role and are also largely considered to be responsible for the modern epidemic of MS-related phenotypes, such as obesity, arterial hypertension and type 2 diabetes (T2D). Moreover, the pathogenesis of IR is strongly associated with the ability of the liver to suppress endogenous glucose production, suggesting that this organ is a key player in the pathophysiology of the MS. Some metabolic disturbances in the hepatic tissue, such as abnormal triglycerides accumulation observed in fatty liver, have been suggested as the trigger events and perhaps the causative factors of IR^[2,3]. As such, nonalcoholic fatty liver disease (NAFLD) is now considered to be an additional component of the MS strongly associated with cardiovascular disease (CVD)^[1,4-6].

Although significant efforts have been made in the last years and new research technologies are constantly used to look for either genes or biomarkers in the MS prediction, the pathogenesis and pathophysiology of this complex disease remains a major challenge.

We read with great interest the article by Cheng *et al*^[7] recently published in *Circulation*. Interestingly, Cheng *et al*^[7] investigated possible pathways underlying MS by high-throughput metabolite profiling in two large and well characterized community-based cohorts, including 1015 individuals from the Framingham Heart Study and 746 from the Malmö Diet and Cancer Study. By liquid chromatography and mass spectrometry, the authors explored the plasma concentrations of 45 distinct metabolites and examined their relation to cardiometabolic risk, and found that metabolic risk factors such as obesity, IR, high blood pressure, and dyslipidemia were associated with several metabolites, including branched-chain amino acids (BCAA), other hydrophobic amino acids, tryptophan breakdown products, and nucleotide metabolites. In addition, the authors observed a significant association of IR traits with glutamine, glutamate and the glutamine-to-glutamate ratio in individuals from both cohorts. They described for the first time that a high glutamine-to-glutamate ratio is associated with a lower risk of incident diabetes mellitus. The authors also followed up these findings by a dietary-intervention study in mice, and observed that administration of glutamine led to both increased glucose tolerance and decreased blood pressure^[7]. Hence, the authors conclude

that individuals with metabolic risk factors have higher circulating concentrations of glutamate and BCAA, and lower concentrations of glutamine, and suggest that glutamate may contribute to the development of the MS. Moreover, the authors observed that circulating levels of BCAA are not only associated with obesity and impaired glucose tolerance but also with dyslipidemia and blood pressure.

What can this metabolomic data tell us about the pathogenesis of MS?

These data open new perspectives about the pathogenesis of MS-associated phenotypes and introduce a crucial role of glutamine-cycling pathway as prominently involved in the development of metabolic risk.

Actually, the role of glutamine-cycle in the regulation of metabolic syndrome-related phenotypes was postulated many years ago, as Hermanussen *et al*^[8] showed that chronic hyperglutamatemia may intoxicate arcuate nucleus neurons, thereby disrupting the hypothalamic signaling cascade of leptin action, causing hyperphagia, obesity and hyperleptinaemia. Surprisingly, glutamate has also been associated with metabolic programming and it was postulated that the thrifty phenotype, the epidemiological association between poor fetal and infant growth and the subsequent development of the MS, may be the consequence of fetal hyperglutamatemia^[8].

In addition, previous evidences from a human study, including a metabolic profiling performed on 74 obese and 67 lean subjects, identified a cluster of obesity-associated changes in specific amino acid, acylcarnitine, and organic acid metabolites in obese compared to lean subjects that was associated with IR^[9]. Newgard *et al*^[9] tested the effect of supplementation of high fat diet with BCAA in an experimental study, and showed that this model was associated with decreased levels of circulating α -ketoglutarate and increased levels of glutamate, and speculated that the accumulation of glutamate increases the transamination of pyruvate to alanine, leading to the development of obesity-associated IR. Newgard *et al*^[9] in fact extended this reasoning to that the increase in alanine, a highly gluconeogenic amino acid, contributes to the development of glucose intolerance in obesity, as circulating alanine levels are elevated in obese subjects.

Furthermore, a recent human study exploring metabolite predictors of deteriorating glucose tolerance in two Finnish population-based studies consisting of 1873 individuals and reexamination of 618 individuals after 6.5 years in one of the cohorts showed that alterations in BCAA metabolism precede hyperglycemia^[10]. In addition, alanine, lactate, and pyruvate were predictive of post-challenge glucose^[10]. A candidate gene association study in 9369 non-diabetic or newly diagnosed T2D Finnish men that explored the association of glycemia and 43 genetic risk variants showed that hyperglycemia and a variant of glucokinase (hexokinase 4) regulator (*GCKR*) are associated with the levels of eight amino acids, including alanine, leucine, isoleucine, tyrosine,

Table 1 Overview about liver aminotransferases alanine and aspartate

<p>ALT or GPT Catalyzes the reversible transamination¹ between alanine and 2-oxoglutarate to form pyruvate and glutamate: L-alanine + 2-oxoglutarate = pyruvate + L-glutamate ALT has both degradative and biosynthetic roles in the glutamate cycling ALT participates in cellular nitrogen metabolism and also in liver gluconeogenesis starting with precursors transported from skeletal muscles ALT is present in tissues including liver, kidney, heart, and skeletal muscle. AST or GOT Catalyzes the reversible transamination between L-aspartate and 2-oxoglutarate to form oxaloacetate and glutamate: L-alanine + 2-oxoglutarate L-aspartate + 2-oxoglutarate = oxaloacetate + L-glutamate Cytosolic AST (GOT 1 catalyzes the reversible reaction of oxaloacetate and glutamate to form aspartate and 2-oxoglutarate (alpha-ketoglutarate) AST has two isoforms: cytoplasmatic and mitochondrial</p>
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¹Transaminase: A subclass of enzymes that catalyze the transfer of an amino group from a donor (generally an amino acid) to an acceptor (generally 2 keto acid) in a cyclic process using pyridoxal phosphate as a cofactor. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GPT: Glutamate pyruvate transaminase; GOT: Glutamate oxaloacetate transaminase.

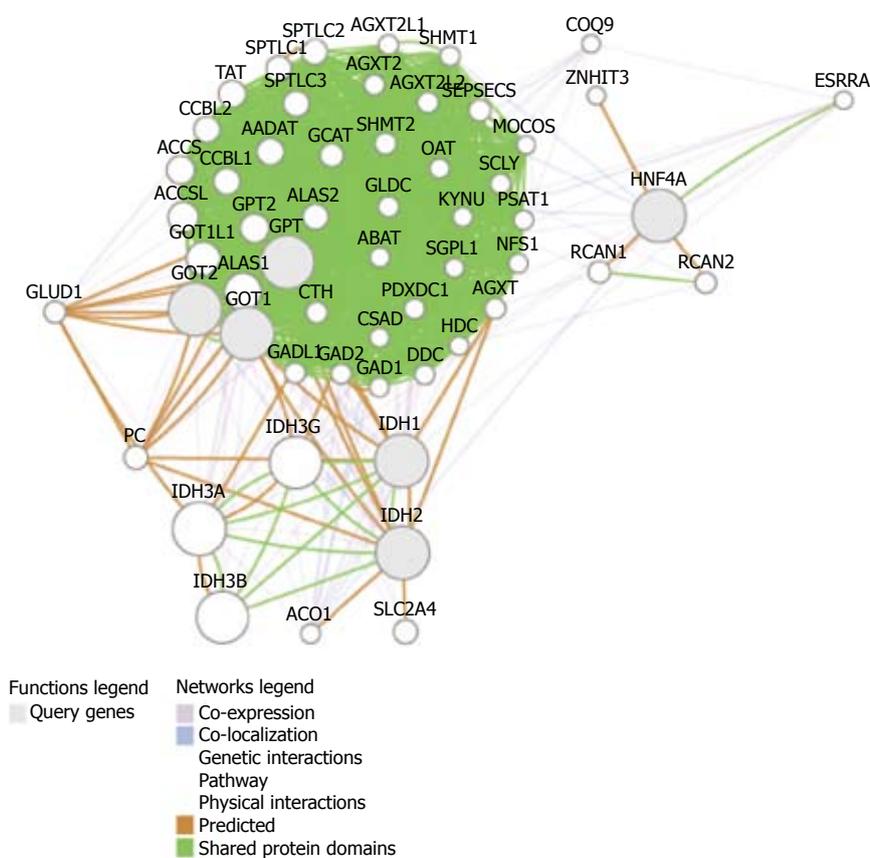


Figure 2 Integrated functional association analysis of protein and genetic interactions on alanine and aspartate. Pathways, co-expression, co-localization, and protein domain similarity were analyzed by the bioinformatics resource GenMANIA (genemania.org) for the 5 candidate genes [alanine also known as glutamate pyruvate transaminase (GPT) and GPT2], aspartate [also known as glutamate oxaloacetate transaminase (GOT)1 and GOT2, (Table 1)], isocitrate dehydrogenases 1 (IDH1), IDH2 and HNF4 (gray circles) and the predicted related genes by systems biology (open circles). List of gene symbol and gene function is shown in Table 2. Predicted functional pathways and Q values are shown in Table 3.

analyses^[14]. There was also a significant interaction between body mass index and ALT levels, and the follow-up study of these overweight and obese participants with highest ALT levels for 20 years showed a 30-fold increased risk for developing T2D^[14].

The association of ALT with the risk of development MS was also evaluated in 1097 subjects from the population-based cohort of Caucasian men and women (Hoorn Study), and ALT was significantly associated with fasting plasma glucose at follow-up^[15]. The 10-year risk of all-cause mortality, fatal and non-fatal CVD in relation to ALT was also assessed in 1439 subjects participating in the Hoorn Study, and the predictive value of ALT for coronary events, seems independent of tra-

ditional risk factors^[16].

Moreover, findings from the Western Australian Health Department data linkage system, an Australian population-based cohort study, support a strong association between ALT levels and the MS independent of insulin resistance^[17].

An overview about the epidemiological evidence of liver enzymes and cardiovascular outcomes was recently published^[18].

In spite of the epidemiological evidences mentioned above, the research community is still inconclusive about the pathobiological meaning of the elevated ALT levels and CV risk. In fact, the question of whether abnormalities in ALT levels precede the development of MS, or

Table 2 Candidate gene list (in bold) and 50 predicted genes by systems biology

Symbol	Description	Score
HNF4A	Hepatocyte nuclear factor 4, alpha [source: HGNC symbol; Acc: 5024]	66.77
IDH2	Isocitrate dehydrogenase 2 (NADP+), mitochondrial [source: HGNC symbol; Acc: 5383]	62.04
IDH1	Isocitrate dehydrogenase 1 (NADP+), soluble [source: HGNC symbol; Acc: 5382]	61.96
GPT	Glutamic-pyruvate transaminase (alanine aminotransferase) [source: HGNC symbol; Acc: 4552]	58.97
GOT1	Glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1) [source: HGNC symbol; Acc: 4432]	54.78
GOT2	Glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2) [source: HGNC symbol; Acc: 4433]	54.48
IDH3A	Isocitrate dehydrogenase 3 (NAD+) alpha [source: HGNC symbol; Acc: 5384]	2.68
IDH3G	Isocitrate dehydrogenase 3 (NAD+) gamma [source: HGNC symbol; Acc: 5386]	2.6
IDH3B	Isocitrate dehydrogenase 3 (NAD+) beta [source: HGNC symbol; Acc: 5385]	2.56
ALAS1	Aminolevulinic acid, delta-, synthase 1 [source: HGNC symbol; Acc: 396]	1.57
GOT1L1	Glutamic-oxaloacetic transaminase 1-like 1 [source: HGNC symbol; Acc: 28487]	1.35
ACCSL	1-aminocyclopropane-1-carboxylate synthase homolog (Arabidopsis)(non-functional)-like [source: HGNC symbol; Acc: 34391]	1.14
GPT2	Glutamic pyruvate transaminase (alanine aminotransferase) 2 [source: HGNC symbol; Acc: 18062]	1.03
ACCS	1-aminocyclopropane-1-carboxylate synthase homolog (Arabidopsis)(non-functional) [source: HGNC symbol; Acc: 23989]	0.98
TAT	Tyrosine aminotransferase [source: HGNC symbol; Acc: 11573]	0.93
AADAT	Amino adipate aminotransferase [source: HGNC symbol; Acc: 17929]	0.88
CCBL1	Cysteine conjugate-beta lyase, cytoplasmic [source: HGNC symbol; Acc: 1564]	0.87
CCBL2	Cysteine conjugate-beta lyase 2 [source: HGNC symbol; Acc: 33238]	0.83
SPTLC3	Serine palmitoyltransferase, long chain base subunit 3 [source: HGNC symbol; Acc: 16253]	0.82
ALAS2	Aminolevulinic acid, delta-, synthase 2 [source: HGNC symbol; Acc: 397]	0.8
SPTLC2	Serine palmitoyltransferase, long chain base subunit 2 [source: HGNC symbol; Acc: 11278]	0.79
SPTLC1	Serine palmitoyltransferase, long chain base subunit 1 [source: HGNC symbol; Acc: 11277]	0.75
PC	Pyruvate carboxylase [source: HGNC symbol; Acc: 8636]	0.69
SLC2A4	Solute carrier family 2 (facilitated glucose transporter), member 4 [source: HGNC symbol; Acc: 11009]	0.69
GCAT	Glycine C-acetyltransferase [source: HGNC symbol; Acc: 4188]	0.66
RCAN1	Regulator of calcineurin 1 [source: HGNC symbol; Acc: 3040]	0.56
SEPSECS	Sep (O-phosphoserine) tRNA: Sec (selenocysteine) tRNA synthase [source: HGNC symbol; Acc: 30605]	0.56
GLUD1	Glutamate dehydrogenase 1 [source: HGNC symbol; Acc: 4335]	0.55
SHMT2	Serine hydroxymethyltransferase 2 (mitochondrial) [source: HGNC symbol; Acc: 10852]	0.54
CTH	Cystathionase (cystathionine gamma-lyase) [source: HGNC symbol; Acc: 2501]	0.52
AGXT2L2	Alanine-glyoxylate aminotransferase 2-like 2 [source: HGNC symbol; Acc: 28249]	0.49
RCAN2	Regulator of calcineurin 2 [source: HGNC symbol; Acc: 3041]	0.49
AGXT	Alanine-glyoxylate aminotransferase [source: HGNC symbol; Acc: 341]	0.48
GLDC	Glycine dehydrogenase (decarboxylating) [source: HGNC symbol; Acc: 4313]	0.48
GADL1	Glutamate decarboxylase-like 1 [source: HGNC symbol; Acc: 27949]	0.47
PDXDC1	Pyridoxal-dependent decarboxylase domain containing 1 [source: HGNC symbol; Acc: 28995]	0.45
AGXT2	Alanine-glyoxylate aminotransferase 2 [source: HGNC symbol; Acc: 14412]	0.45
GAD2	Glutamate decarboxylase 2 (pancreatic islets and brain, 65 kDa) [source: HGNC symbol; Acc: 4093]	0.43
SCLY	Selenocysteine lyase [source: HGNC symbol; Acc: 18161]	0.43
AGXT2L1	Alanine-glyoxylate aminotransferase 2-like 1 [source: HGNC symbol; Acc: 14404]	0.42
ABAT	4-aminobutyrate aminotransferase [source: HGNC symbol; Acc: 23]	0.42
DDC	Dopa decarboxylase (aromatic L-amino acid decarboxylase) [source: HGNC symbol; Acc: 2719]	0.42
KYNU	Kynureninase [source: HGNC symbol; Acc: 6469]	0.41
OAT	Ornithine aminotransferase [source: HGNC symbol; Acc: 8091]	0.4
SHMT1	Serine hydroxymethyltransferase 1 (soluble) [source: HGNC symbol; Acc: 10850]	0.4
PSAT1	Phosphoserine aminotransferase 1 [source: HGNC symbol; Acc: 19129]	0.39
GAD1	Glutamate decarboxylase 1 (brain, 67 kDa) [source: HGNC symbol; Acc: 4092]	0.38
CSAD	Cysteine sulfonic acid decarboxylase [source: HGNC symbol; Acc: 18966]	0.38
NFS1	NFS1 nitrogen fixation 1 homolog (S. cerevisiae) [source: HGNC symbol; Acc: 15910]	0.38
ACO1	Aconitase 1, soluble [source: HGNC symbol; Acc: 117]	0.37
SGPL1	Sphingosine-1-phosphate lyase 1 [source: HGNC symbol; Acc: 10817]	0.36
HDC	Histidine decarboxylase [source: HGNC symbol; Acc: 4855]	0.36
MOCOS	Molybdenum cofactor sulfuryase [source: HGNC symbol; Acc: 18234]	0.31
ZNHIT3	Zinc finger, HIT-type containing 3 [source: HGNC symbol; Acc: 12309]	0.3
COQ9	Coenzyme Q9 homolog (S. cerevisiae) [source: HGNC symbol; Acc: 25302]	0.3
ESRRA	Estrogen-related receptor alpha [source: HGNC symbol; Acc: 3471]	0.3

IDH: Isocitrate dehydrogenases; GPT: Glutamate pyruvate transaminase; GOT: Glutamate oxaloacetate transaminase.

whether the MS components themselves can lead to the increase of ALT levels is still unanswered^[14]. Hence, the biological mechanisms responsible for the association between liver enzymes and the MS-related phenotypes are still poorly understood, and much of the speculations focus on the putative liver injury associated with

fatty liver that frequently coexists with the MS.

The metabolomic data presented by Cheng *et al*^[7] not only raised new questions about the role of glutamate-glutamine cycle in the pathogenesis of the MS, but also suggested a dramatic change in the paradigm of the meaning of elevated aminotransferase levels in the context of MS-

Table 3 Gene ontology annotation of predicted biological process

Gene ontology annotation	Q value	Genes in network	Genes in genome
Transaminase activity	4.86E-31	14	16
Transferase activity, transferring nitrogenous groups	6.17E-30	14	18
Mitochondrial matrix	3.10E-15	16	220
Cellular amino acid catabolic process	7.40E-15	12	77
Amine catabolic process	1.13E-14	12	81
Dicarboxylic acid metabolic process	1.75E-14	9	24
Cellular amino acid biosynthetic process	5.90E-13	10	54
Carboxylic acid catabolic process	2.55E-12	12	131
2-oxoglutarate metabolic process	2.55E-12	7	13
Organic acid catabolic process	2.55E-12	12	131
Amine biosynthetic process	8.11E-12	10	72
Glutamate metabolic process	1.08E-10	6	10
Carboxylic acid biosynthetic process	4.53E-10	11	154
Organic acid biosynthetic process	4.53E-10	11	154
Small molecule catabolic process	4.87E-10	12	211
Small molecule biosynthetic process	2.21E-9	12	241
Cofactor metabolic process	2.24E-8	10	163
Vitamin B6 binding	9.53E-8	5	12
Glutamine family amino acid metabolic process	9.53E-8	6	27
Cellular aromatic compound metabolic process	9.53E-8	9	135
Pyridoxal phosphate binding	9.53E-8	5	12
Cofactor binding	6.48E-7	7	69
Aromatic amino acid family catabolic process	1.24E-6	5	19
Coenzyme metabolic process	1.46E-6	8	126
Aromatic compound catabolic process	3.14E-6	5	23
Aromatic amino acid family metabolic process	3.14E-6	5	23
Vitamin binding	8.74E-6	5	28
Indolalkylamine catabolic process	1.08E-5	4	11
Indole-containing compound catabolic process	1.08E-5	4	11
Tryptophan catabolic process	1.08E-5	4	11
Tryptophan metabolic process	1.48E-5	4	12
Indolalkylamine metabolic process	1.48E-5	4	12
Indole-containing compound metabolic process	1.48E-5	4	12
Cellular biogenic amine catabolic process	3.93E-5	4	15
Serine family amino acid metabolic process	5.07E-5	4	16
Acetyl-CoA catabolic process	1.23E-4	4	20
Tricarboxylic acid cycle	1.23E-4	4	20
Coenzyme catabolic process	1.23E-4	4	20
Cofactor catabolic process	3.67E-4	4	26
Aerobic respiration	4.88E-4	4	28
Cellular biogenic amine metabolic process	6.98E-4	5	71
Lyase activity	8.37E-4	5	74
Acetyl-CoA metabolic process	1.01E-3	4	34
Transferase activity, transferring acyl groups other than amino-acyl groups	1.41E-3	5	83
Gluconeogenesis	3.05E-3	4	45
Aspartate family amino acid catabolic process	3.67E-3	3	15
Generation of a signal involved in cell-cell signaling	3.67E-3	6	178
Signal release	3.67E-3	6	178
Transferase activity, transferring acyl groups	3.67E-3	5	103
Sulfur amino acid metabolic process	4.26E-3	3	16
Neurotransmitter secretion	5.20E-3	4	53
Hexose biosynthetic process	5.50E-3	4	54
Water-soluble vitamin metabolic process	6.24E-3	4	56
Monosaccharide biosynthetic process	1.04E-2	4	64
Pteridine-containing compound metabolic process	1.05E-2	3	22

Neurotransmitter transport	1.14E-2	4	66
Carbon-carbon lyase activity	1.48E-2	3	25
Aspartate family amino acid metabolic process	1.48E-2	3	25
Regulation of neurotransmitter levels	1.60E-2	4	73
Sphingolipid metabolic process	1.82E-2	4	76
Alcohol biosynthetic process	1.82E-2	4	76
Pigment biosynthetic process	1.96E-2	3	28
Membrane lipid metabolic process	2.35E-2	4	82
Sphingolipid biosynthetic process	2.35E-2	3	30
Cofactor biosynthetic process	2.53E-2	4	84
Membrane lipid biosynthetic process	2.77E-2	3	32
Cellular modified amino acid metabolic process	2.81E-2	4	87
Cellular carbohydrate biosynthetic process	4.06E-2	4	96
Pigment metabolic process	4.80E-2	3	39
Vitamin metabolic process	4.80E-2	4	101

Q value stands for the *P* value corrected for multiple testing. In addition, number of genes in the network and in the whole genome belonging to the biological process is depicted. The candidate genes are listed in Table 2, which are involved in functional enrichment analysis using the GeneMANIA tool (genemania.org).

related phenotypes. Thus, we speculate that abnormal levels of ALT and AST are associated with a deregulation of normal amino acid metabolism in the liver, including aromatic amino acid, and then special compounds such as glutamate are released into the general circulation. This hypothesis attempts to illustrate the critical role of the “liver metabolism” in the pathogenesis of the MS and IR, and postulates that before the liver becomes fatty, abnormal levels of liver enzymes might reflect high levels of hepatic transamination of amino acids in the organ.

Is there any experimental evidence for this? Stegink *et al*^[19] have demonstrated that if a large proportion of glutamate is ingested, portal glutamate increases and this elevation results in increased hepatic glutamate metabolism, leading to release of glucose into systemic circulation, a physiopathogenic event that may perpetuate hyperglycemia.

Systems biology also provides a rational evidence for the association between liver transaminases and the metabolic abnormalities observed by Cheng *et al*^[7].

We performed a functional association analysis that included protein and genetic interactions, pathways, co-expression, co-localization, and protein domain similarity using the bioinformatics resource GenMANIA^[20]. Interestingly, several genes are regarded as direct “neighbors” of liver transaminases (GPT and GOT1/2, as described in Table 1), but including isocitrate dehydrogenases 1 (IDH1) and 2 (IDH2) and the transcription factor hepatic nuclear factor 4 alpha, because they are involved in the regulation of liver transaminases and glutamine synthetase^[21] (Figure 2). Interestingly, the predicted genes (Table 2) belong to pathways that explain most of the findings of Cheng *et al*^[7], such as glutamine family amino acid metabolic process, indolalkylamine catabolic process, indole-containing compound catabolic process, tryptophan catabolic process, tryptophan metabolic process, indolalkylamine metabolic process, indole-containing compound metabolic process, cellular biogenic amine catabolic process, among others (Table 3).

Clinical perspective

To conclude, liver transaminases should not be considered as mere biomarkers of liver damage but central players in the pathophysiology of the NAFLD in particular or the MS components in general. Further research has to be done to define whether the elevation of these enzymes is an adaptive or a causative process of the disease.

In particular, because many confounding issues are implied in a pathogenetic relationship with liver, heart and kidney, it is time to look at multi-organ pathogenetic interactions, as recently revised by Bonora *et al*^[22].

Finally, mutations in *IDH1* and *IDH2* seem to be critically involved in the generation of certain types of cancers because they create “neoenzymes” that produce 2-hydroxyglutarate, which are required for tumor cell growth from α -ketoglutarate (α -KG)^[23]. α -KG is derived from glutamine through its conversion to glutamate by glutaminase. This process may explain the glutamine dependency of the cancer cell growth^[24]. Then, it is tempting to speculate that glutamate excess as observed in the MS and NAFLD is an appropriate milieu for cancer development, which may explain the high prevalence of hepatocellular carcinoma in these patients^[25], which offers, at the same time, new avenues for its treatment.

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Diagnostic and therapeutic implications of the association between ferritin level and severity of nonalcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD), defined by excessive liver fat deposition related to the metabolic syndrome, is a leading cause of progressive liver disease, for which accurate non-invasive staging systems and effective treatments are still lacking. Evidence has shown that increased ferritin levels are associated with the metabolic insulin resistance syndrome, and higher hepatic iron and fat content. Hyperferritinemia and iron stores have been associated with the severity of liver damage in NAFLD, and iron depletion reduced insulin resistance and liver enzymes. Recently, Kowdley *et al* demonstrated in a multicenter study in 628 adult patients with NAFLD from the NAFLD-clinical research network database with central re-evaluation of liver histology and iron staining that the increased serum ferritin level is an independent predictor of liver damage in patients with NAFLD, and is useful to identify NAFLD patients at risk of non-alcoholic steatohepatitis and advanced fibrosis. These data indicate that

incorporation of serum ferritin level may improve the performance of noninvasive scoring of liver damage in patients with NAFLD, and that iron depletion still represents an attractive therapeutic target to prevent the progression of liver damage in these patients.

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Key words: Fibrosis; Ferritin; Iron overload; Nonalcoholic fatty liver disease; Steatohepatitis; Steatosis

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INVITED COMMENTARY ON HOT ARTICLES

We read with interest the article by Kowdley *et al*^[1] reporting an association between serum ferritin and the severity of liver damage in patients with nonalcoholic fatty liver disease (NAFLD), and strongly recommend it to the readers.

NAFLD is defined by the presence of liver fat deposition related to systemic insulin resistance and the metabolic syndrome^[2]. In susceptible individuals, NAFLD is associated with oxidative hepatocellular damage, inflammation, and activation of fibrogenesis, i.e., non-alcoholic steatohepatitis (NASH)^[3], with potential progression towards cirrhosis and hepatocellular carcinoma (HCC)^[4], whereas simple steatosis is believed to represent a relatively benign condition^[5]. Due to the epidemic of obesity and the metabolic syndrome, NAFLD is now the most common liver disease and the leading cause of altered liver enzymes in Western countries^[6,7], and it is supposed to become the leading cause of end-stage liver disease, liver transplantation and HCC within the next 10-20 years. Considering the high prevalence of the NAFLD (20%-34% of Western population) two key clinical challenges are expected: the development of noninvasive methods for the diagnosis, staging and follow-up of NASH, and effective treatment strategies counteracting disease progression. Although the gold standard for diagnosis is still liver biopsy, it would be impossible to perform in such a large population at risk, whereas algorithms to predict steatohepatitis and liver fibrosis are still inefficient, need validation, and leave a large grey area^[5]. In addition, although promising results have been shown for anti-oxidants^[8], no therapeutic trial has yielded convincing results in the progression of liver damage^[9], and no pharmacologic therapy is yet approved for NASH.

Over the last years, there has been accumulating evidence about a strong association between hyperferritinemia and mild iron overload unrelated to hereditary hemochromatosis and manifestations of the metabolic syndrome^[10-12], including NAFLD^[13-18], as recently reviewed by our group^[19]. Increased ferritin level was detected in about 30% of unselected patients with NAFLD^[20], which has been associated with increased hepatic iron, as determined by histological and radiological assessment, and by quantitative phlebotomy^[15,20-22]. However, it is likely that inflammation, cytokines, oxidative and endoplasmic reticulum stress, and the genetic background contribute to ferritin induction by compartmentalization of iron in macrophages in a subset of NAFLD patients without histologically detectable iron stores^[19,22-26]. Does hyperferritinemia reflect a subclinical increase in hepatic iron also in this latter subgroup? Very recent data, which have been generated thanks to the availability of magnetic resonance imaging protocols for reliable estimates of tissue iron concentration, confirmed a close link between hepatic iron stores, steatosis, and metabolic diseases. Haap *et al.*^[27] reported that in healthy non-diabetic subjects, serum ferritin is strongly associated with hepatic iron stores, and hepatic iron stores are independently associated with insulin sensitivity and hepatic fat accumulation. On the other hand, Zheng *et al.*^[28] reported that in Chinese subjects, subclinical hepatic iron overload is more frequent (60%) in subjects with pre-diabetes and diabetes than in those with normal glucose tolerance, and hepatic iron concentration explains

> 40% of glycated hemoglobin variance. Furthermore, in patients with steatosis, body iron stores have been linked to a higher risk of metabolic complications, such as insulin resistance and diabetes^[29-31], and to the faster progression of cardiovascular diseases^[32-35].

Our group first reported an association between hyperferritinemia, NASH, and the severity of liver damage^[13], which was later confirmed by other groups, even if evidence was still controversial^[1,14,19,36]. Histological evidence of hepatic iron accumulation has also been associated with an increased risk of fibrosis in large multicenter studies in patients with NAFLD both from Europe and the NASH Clinical Research Network (NASH-CRN) in the United States^[15,37], whereas *beta-globin* mutations, the best predictor of parenchymal iron overload in the Mediterranean area, were associated with an almost double risk of severe fibrosis^[16]. Furthermore, iron overload has also been associated with HCC in Italian patients with NASH-related cirrhosis^[38]. Recent data obtained in animal models are also consistent with a synergistic interaction between liver fat and iron in the pathogenesis of liver damage, which may be related to the induction of iron-dependent cell death (ferroptosis)^[39,40]. A schematic presentation of the hypothesized mechanisms underlying hepatic iron accumulation and the role of iron in the progression of liver damage in NAFLD is shown in Figure 1.

Most importantly, iron overload represents also a treatable condition. Experimental evidence suggests that iron depletion induced by chelators induces glucose uptake and utilization in hepatocytes *in vitro* and in the liver *in vivo*, increasing insulin receptor binding activity and signaling^[41].

Several reports indicate that iron depletion, most frequently achieved by phlebotomy, may be beneficial in patients with mild iron overload associated with NAFLD. Iron depletion has been first reported to improve insulin sensitivity in a short term in patients with NAFLD with and without increased ferritin levels in two pilot studies in patients with impaired glucose tolerance^[42] and normal glucose tolerance^[43], and it led to decreased HbA1c levels, heightened insulin secretion and insulin sensitivity in a randomized controlled study in a small number of patients with type 2 diabetes and increased ferritin levels^[44]. Both venesection therapy and dietary therapy improved serum ferritin, metabolic parameters and liver function in a controlled study in patients with hepatic iron overload associated with the metabolic syndrome^[45]. However, in a matched case-control study in 128 patients with diet-resistant NAFLD, iron depletion reduced insulin resistance more than lifestyle modifications alone, independently of other confounding factors^[21]. Finally, in an observational study in 198 NAFLD patients without diabetes with adjustment for propensity score, iron depletion was associated with a higher probability of normalization not only of insulin resistance, but also of liver enzymes during follow-up compared with lifestyle modifications alone^[46].

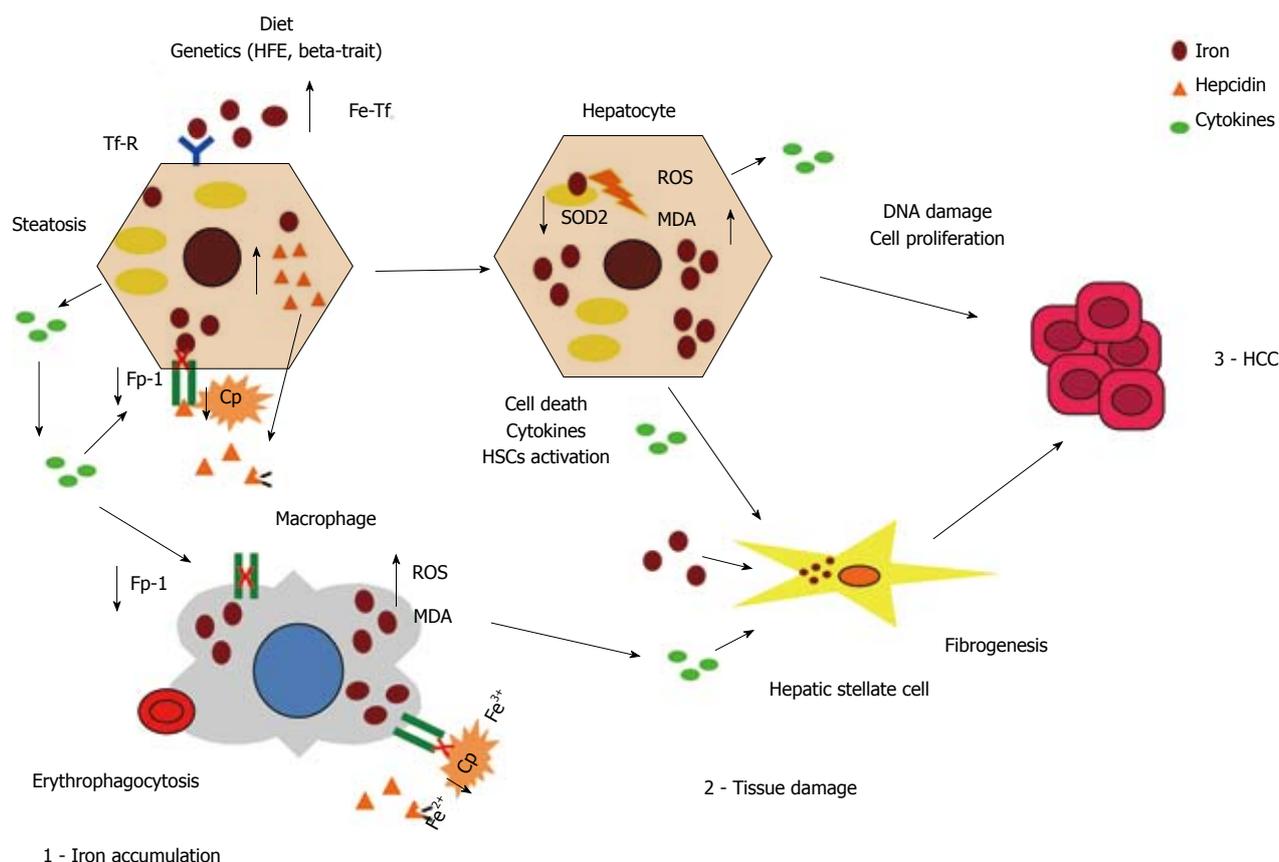


Figure 1 Proposed mechanisms explaining iron induced liver damage associated with steatosis and hepatic iron overload in hepatocytes (brown), macrophages (grey), and hepatic stellate cells (yellow). Cp: Ceruloplasmin; Fe-Tf: Ferric-transferrin; Fp-1: Ferroportin-1; HCC: Hepatocellular carcinoma; HFE: Hemochromatosis gene; HSCs: Hepatic stellate cells; MDA: Malonyl-dialdehyde; ROS: Reactive oxygen species; SOD2: Mn superoxide dismutase; Tf-R: Transferrin receptor. Modified from Dongiovanni *et al*^[9].

Within this context, the novel findings reported by Kowdley *et al*^[11] provided significant insight into this field. In this study, the authors assessed the relationship between elevated serum ferritin levels (defined as > 1.5 times the upper normal limit) and NAFLD severity in a multicenter study in 628 adult patients with NAFLD from the NAFLD-CRN database with central re-evaluation of liver histology and iron staining. Despite that the upper normal limits were specific for gender, hyperferritinemia was observed more frequently in males (25% *vs* 17%), and was strongly associated with other indices of iron stores including serum iron, transferrin saturation, and hepatic iron staining. Different from what observed in a previous study by our group^[15], hyperferritinemia was also associated with alanine and aspartate aminotransferases, but in line with our results^[15] with lower platelets, suggesting increased liver fibrosis^[47]. Histological features of NAFLD, including steatosis, hepatocellular ballooning, diagnosis of NASH, and fibrosis, were more severe in patients with increased serum ferritin, and at multivariate logistic regression analysis, hyperferritinemia remained significantly associated with advanced hepatic fibrosis [odds ratio (OR): 1.66, 95% CI: 1.05-2.62] and increased NAFLD activity score (OR: 1.99, 95% CI: 1.06-3.75). Based on these results, it is concluded that serum ferritin is associated with hepatic deposition and

worsened histological activity in patients with NAFLD, and it is a useful variable to identify NAFLD patients at risk of NASH and advanced fibrosis. An important finding, in view of the previously cited debate about significance of increased ferritin in patients without iron overload, is that hyperferritinemia was associated with enhanced histological activity even in patients without histologically detectable iron deposition.

Although a significant proportion of patients included in the NASH-CRN database had to be excluded due to the lack of ferritin measurement at the time of liver biopsy, important strengths of this study include the analysis of a large multicenter series of patients with histological evaluation, the inclusion of a large percentage of patients with moderate to severe liver fibrosis, and the availability of semiquantitative evaluation of iron stores by Perls' staining and determination of mutations in the hemochromatosis gene of hereditary hemochromatosis in the majority of patients, with concomitant exclusion of patients affected by hereditary hemochromatosis.

Therefore, adding to the previous literature^[15,19], this elegant and solid confirmation of the association between hyperferritinemia and severe hepatic fibrosis/NASH has two major implications. The first one and most scientifically grounded is that, whatever its cause (iron overload or altered compartmentalization, inflammation, cellular

stress), in patients with NAFLD, hyperferritinemia is very frequently and strongly associated with liver damage. Since serum ferritin evaluation is widely available in clinical practice and relatively inexpensive, the next step is to test its inclusion in noninvasive prognostic scores of liver damage related to NAFLD to improve their predictive power^[1,5,37]. Interestingly, a score incorporating increased ferritin levels, called the “National Association of Fraternal Insurance Counselors score” has already been developed and validated in a large series of Japanese patients, and although the evaluation of a serum marker such as the collagen IV 7S domain is required, that is not easily available outside Japan clinically, it showed a superior predictive power for NASH and severe fibrosis compared with other noninvasive scores^[48], and other authors have confirmed the predictive power of ferritin on liver damage in NAFLD^[49].

Secondly, but not less important, more clues, that link with increased hepatic iron stores or altered iron compartmentalization in macrophages with steatosis, insulin resistance, and progressive liver disease, are being found^[19,50]; and while the results of a small pilot randomized controlled trial evaluating the effect of iron depletion on the progression of histologically evaluated liver damage in patients with NAFLD and increased iron stores (NCT 00658164) are being expected, the accumulated available evidence allows to validate the effect of iron depletion on the prevention of hepatic, metabolic and cardiovascular complications of NAFLD.

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Tight glycemic control using an artificial endocrine pancreas may play an important role in preventing infection after pancreatic resection

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Abstract

It is well known that perioperative hyperglycemia is the main cause of infectious complications after surgery. To improve perioperative glycemic control, we wish to highlight and comment on an interesting paper published recently by the *Annals of Surgery* entitled: "Early postoperative hyperglycemia is associated with postoperative complications after pancreatoduodenectomy (PD)" by Eshuis *et al.* The authors concluded that early postoperative glucose levels more than 140 mg/dL was significantly associated with complications after PD. Since we recommend that perioperative tight glycemic control (TGC) is an effective method to prevent postoperative complications including surgical site infection after distal, proximal, and total pancreatic resection, we support strongly this conclusion drawn in this article. However, if early postoperative glucose control in patients undergoing PD was administrated by conventional method such as sliding scale approach as described in this article, it is difficult to maintain TGC. Therefore, we introduce a novel perioperative glycemic control using an artificial endocrine pancreas against pancreatogenic diabetes after pancreatic resection including PD.

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INVITED COMMENTARY ON HOT ARTICLES

It is well known that perioperative hyperglycemia is the main cause of infectious complications after surgery^[1]. Figure 1 shows the relationship between hyperglycemia and postoperative infection (POI). Glucose toxicity is caused by surgical stress induced hyperglycemia such as a level of more than 200 mg/dL. Glucose toxicity leads to the leukocyte deficiencies, granulocyte adherence, impaired phagocytosis, delayed chemotaxis, and depressed bactericidal capacity. These abnormalities are the principal causes of POI and they can be improved by appropriate glycemic control^[1]. However, optimal blood glucose range to prevent postoperative infectious complications

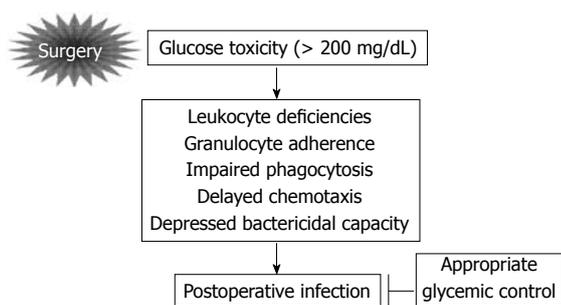


Figure 1 Relationship between hyperglycemia and postoperative infection.

remains unclear in various surgical settings^[1]. To improve the methods for perioperative glycemic control, we would like to recommend an interesting paper published recently on this topic^[2]. At the same time, we will introduce a novel perioperative tight glycemic control (TGC) using an artificial pancreas (AP) in patients undergoing pancreatic resection aimed to reduce postoperative infectious complications including surgical site infection (SSI).

We read with great interest the article published in *Annals of Surgery* entitled “Early postoperative hyperglycemia is associated with postoperative complications after pancreatoduodenectomy” by Eshuis *et al*^[2]. Among 330 consecutive patients undergoing pancreatoduodenectomy (PD), the average glucose levels were controlled at 135 mg/dL (preoperative), 133 mg/dL (intraoperative) and 142 mg/dL (early postoperative). Pre- and intraoperative glucose levels were not associated with postoperative complications. However, early postoperative glucose levels more than 140 mg/dL was significantly associated with complications after PD^[2]. Recent reports indicate that postoperative hyperglycemia increases the risk of postoperative infectious complications and prolongs hospital stay^[3-5]. Since we recommend that perioperative TGC is an effective method to prevent postoperative complications including SSI after distal, proximal, and total pancreatic resection^[6,7], we support strongly the conclusion drawn in this article^[2]. Undoubtedly, this is a significant paper in our understanding of the efficacy of strict perioperative glucose control for patients undergoing PD. However, if early postoperative glucose control in patients undergoing PD was administrated by conventional method such as sliding scale approach as described in this article^[2], it seems to be difficult to maintain strict glycemic control with less variability of blood glucose concentration recommended by the authors, including the targeting blood glucose zone of less than 140 mg/dL because pancreatogenic diabetes after pancreatic resection is likely to occur, either hypoglycemia or hyperglycemia, so called brittle diabetes^[8,9]. Therefore, we would like to share our opinions regarding more effective and safe TGC against pancreatogenic diabetes after pancreatic resection including PD.

In 2005, we reported that perioperative glycemic control using a closed-loop AP for total pancreatectomized dogs could maintain a stable blood glucose near the normoglycemia^[10]. Based on this experimental study,

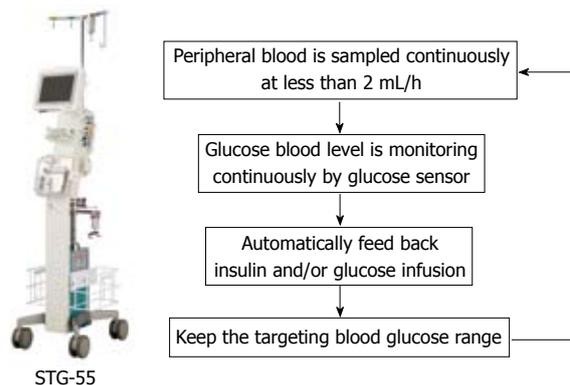


Figure 2 STG-55, a bedside-type artificial endocrine pancreas with closed-loop system.

since 2006, we have introduced clinically perioperative glycemic control using an AP^[8,11]. As described previously^[8,11,12], the Nikkiso Company (Tokyo, Japan) developed a bed-side type AP with closed-loop glycemic control system as STG-22 in conventional device^[8] and STG-55 in current device^[12] (Figure 2). Detailed mechanisms and characteristics of STG-22 and/or STG-55 were reported previously^[8,11,12]. Briefly, peripheral venous blood for glucose monitoring was sampled continuously at less than 2 mL/h. STG-55 (Figure 2)^[12] is capable of measuring continuously the blood glucose with its glucose sensor, and automatically infuses insulin and/or glucose to adjust the blood glucose level in accordance with a target blood glucose value, which is the so called closed-loop system^[13].

As a result in the clinical surgical settings, our previous report^[7] suggested that perioperative TGC using an AP (targeted blood glucose zone of 80-110 mg/dL) in patients undergoing pancreatectomy decreased significantly SSI as compared with that of conventional glycemic control by sliding scale method (targeted blood glucose zone of 150-200 mg/dL). In the sliding scale group, postoperative blood glucose levels rose initially before reaching a plateau of approximately 200 mg/dL between 4 and 6 h after pancreatectomy. The levels remained high for 18 h postoperatively. In the AP group, blood glucose levels reduced steadily, reaching the target zone (80-110 mg/dL) by 6 h after surgery. The total insulin dose administered per patient during the first postoperative 18 h was significantly higher in the AP group (mean \pm SD, 107 \pm 109 IU) than the sliding scale group (8 \pm 6 IU; $P < 0.01$). Neither group showed hypoglycemia^[7]. In addition, this novel glycemic control provided for high achievement of targeting blood glucose levels with stable blood glucose concentration^[7,8,14]. Moreover, surprisingly, we have never observed occurrence of hypoglycemia less than 40 mg/dL in more than 400 patients undergoing general surgery^[12]. Up to dates, we have performed perioperative TGC with target blood glucose levels of 80-110 mg/dL using an AP in more than 100 pancreatectomized patients including more than 50 PD and 10 total pancreatectomies. Of note, every pancreatec-

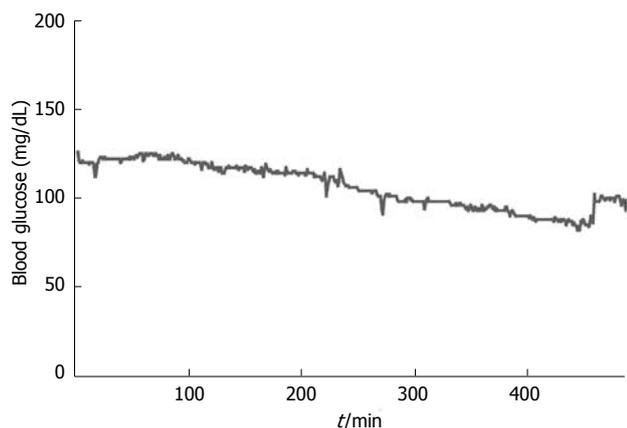


Figure 3 Continuous perioperative blood glucose levels in a case after total pancreatectomy.

tomized patient had stable perioperative blood glucose near the normoglycemia not only without hyperglycemia and/or hypoglycemia but also with less variability of blood glucose concentration, even in a total pancreatectomized patient (Figure 3) who often presented with the most serious pancreatogenic diabetes^[9]. Based on these findings from our experimental and clinical studies, we suggest that the AP helps us accomplish an effective and safe perioperative TGC in patients undergoing pancreatic resection.

Interestingly, this article^[2] suggests that an early postoperative level of at least more than 140 mg/dL is not recommended for improvement of morbidity after PD. Unfortunately, however, the ideal blood glucose range to reduce mortality and morbidity after PD remains unclear. Therefore, we promote a prospective randomized clinical trial to compared targeted blood glucose range of 80-110 mg/dL group and that of 140-180 mg/dL group in patients undergoing distal, proximal, and total pancreatic resection. Primary end point of this study is incidence of SSI and secondary end points are other postoperative complications and mortality (data not shown). We believe that this novel perioperative glucose control using AP is an easy and a reliable method to maintain targeted blood glucose zone, such as 80-110 mg/dL, 110-140 mg/dL and 140-180 mg/dL, which can be determined freely even in patients undergoing pancreatic resection. In the future perspectives, it is essential to find the optimal blood glucose range to improve morbidity and mortality in patients undergoing pancreatic resection, and then AP will be a useful device to maintain the range.

In conclusion, we suggest that the TGC using an artificial endocrine pancreas with a closed-loop system may play an important role in the effective control of in-

fection after pancreatic resection. This novel perioperative glycemic control will enable us to improve surgical outcome by reducing the postoperative infectious complications due to surgical stress induced hyperglycemia.

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Giovanni Latella, MD, Series Editor

Crucial steps in the natural history of inflammatory bowel disease

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Abstract

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are chronic, progressive and disabling disorders. Over the last few decades, new therapeutic approaches have been introduced which have led not only to a reduction in the mortality rate but also offered the possibility of a favorable modification in the natural history of IBD. The identification of clinical, genetic and serological prognostic factors has permitted a better stratification of the disease, thus allowing the opportunity to indicate the most appropriate therapy. Early treatment with immunosuppressive drugs and biologics has offered the opportunity to change, at least in the short term, the course of the disease by reducing, in a subset of patients with IBD, hospitalization and the need for surgery. In this review, the crucial steps in the natural history of both UC and CD will be discussed, as well as the factors that may change their clinical course. The methodological requirements for high quality studies on the course and prognosis of IBD, the true impact of environmental and dietary factors on the clinical course of IBD, the clinical, serological and genetic predictors of the IBD course (in particular, which of these are rel-

evant and appropriate for use in clinical practice), the impact of the various forms of medical treatment on the IBD complication rate, the role of surgery for IBD in the biologic era, the true magnitude of risk of colorectal cancer associated with IBD, as well as the mortality rate related to IBD will be stressed; all topics that are extensively discussed in separate reviews included in this issue of *World Journal of Gastroenterology*.

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Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Natural history; Clinical course; Complications; Therapy; Surgery; Mortality

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INTRODUCTION

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are chronic, progressive and relapsing inflammatory disorders of unknown etiology that may cause disability over time. Genetic, environmental and intestinal microbial factors have been reported to play a role in the etiology and pathogenesis of IBD^[1]. IBD represents a life-long disorder that may occur at any time from early childhood to late adulthood, although over 80% of cases are cur-

rently diagnosed in the second or third decade of life. UC is characterized by inflammation and ulcerations in the large bowel mucosa and submucosa, whereas CD is a trans-mural inflammatory disorder that may involve various sites of the gastrointestinal tract: in 40%-70% of cases the terminal ileum. Approximately 50% of the patients with IBD present a slight evolutive disease with a low prevalence of relapses, hospitalizations, and complications^[2,3]. Other patients have a more severe course and may develop complications that require surgery. In UC, the main complications include toxic megacolon, massive hemorrhage or colon perforation, while strictures and fistulas are uncommon. In CD, intestinal strictures, internal or perianal fistulas or abscesses are frequent, being reported in approximately one-third of patients.

A better understanding of the natural history of this chronic disabling disorder provides valid opportunities: (1) by better defining etiological factors and pathology, it may allow the set up of new strategies of disease prevention; (2) by identifying the relevant clinical subsets in which disease prognosis can be stratified by initial clinical, serological or genetic features, it may be useful in the choice of the most appropriate management of these patients; and (3) by understanding the evolution and the time course of the disease, it may help to define the best end points for clinical trials designed to test drugs modifying disease course^[4] (Figure 1).

Questions regarding the natural history and prognosis of IBD are among the most prominent concerns both for patients and clinicians. Unfortunately, most data regarding the course of IBD are obtained from a limited number of cohorts and not all studies focusing on the course and prognosis are of high quality. Adequate knowledge of the methodological requirements of studies focused on prognosis is crucial for interpreting the results of observational studies and for applying these results in clinical practice. In this issue of *World Journal of Gastroenterology* (WJG), these methodological aspects have been critically discussed by Modesto *et al.*^[5]. In particular, they stressed the criteria for an excellent cohort study of prognosis: population-based, use of standard diagnostic criteria for UC and/or CD, start of follow-up already at inception, complete follow-up (80% or more), and use of survival methods to evaluate results.

The crucial steps in the natural history of IBD include the occurrence of lesions, the manifestation and severity of symptoms, the development of complications, the need for surgery, the disability and the mortality (Figure 2). In order to achieve a favorable modification in the disease course, an effective intervention must be carried out at the right time and with a specific therapeutic endpoint (Figure 1). The main outcomes considered include disease activity and relapse, corticosteroid therapy, hospitalizations, complications, surgery, post-operative recurrence, and mortality. More recently, mucosal healing (MH) has been included^[6,8]. Early treatment is advisable, before the development of severe bowel damage and impaired functioning^[6,8]. There is probably a difference between early disease and long-lasting disease, the control of the disease process being more difficult and unstable in the latter situ-

ation^[6]. Immunological status of the patients may change over the course of the disease; a stable remission will usually be more difficult to obtain in long-lasting disease and the disease will be more treatment-dependent^[6,9].

RISK AND PROGNOSTIC FACTORS

As far as the approach to the treatment of IBD is concerned, it must take into consideration the potential impact of environmental factors (e.g., dietary factors, smoking, psychological stress, drugs and infections) on the clinical course. Clinical, serologic and genetic factors have been reported to be associated with a different clinical course, but their relevance in everyday clinical practice is still controversial^[10].

Two reviews in this issue of WJG deal with prognostic factors in IBD^[11,12].

In the first, Cabré *et al.*^[11] critically review the role of environmental and dietary factors on the clinical course of IBD. Smoking is the only well established risk and prognostic factor in IBD, with a different impact on CD and UC. In CD, there is consistent evidence that active smoking is a risk factor for post-operative disease recurrence^[13,14] but the impact of smoking on the response to therapy is still controversial. In CD patients, the beneficial effect of giving up smoking can be observed; conversely, some patients with refractory UC may even benefit from taking up smoking again^[13,15,16]. Based on these observations, therapeutic trials with transdermic nicotine have been performed but with inconclusive results.

The role of dietary habits and dietary manipulation on the clinical course of IBD is far from being well established; therefore, IBD patients are usually encouraged to follow a free diet. As far as concerns the possible therapeutic role of some dietary components, enteral nutrition appears to be effective in CD, particularly in the pediatric population and a low-fat diet seems to be particularly useful even in adult patients^[17-20].

With regard to the relationship between psychological stress and IBD, a recent systematic review of 18 prospective studies examining stress as a risk factor for disease exacerbations showed a significant association, and coping behaviors appeared to modulate the effect of stress^[21]. Furthermore, it has been reported that approximately 50% of IBD patients had experienced some type of stress; family stress was the most commonly reported form, followed by work or school and financial stress^[22].

As far as drugs are concerned, nonsteroidal anti-inflammatory drugs (NSAIDs) are generally considered to potentially affect the IBD course. This concept is not supported by consistent evidence, although in a subset of susceptible patients, NSAID-induced IBD flares appear to occur early after NSAID administration^[23]. Current evidence does not support the role of antibiotics and vaccines as a prognostic factor in IBD, albeit antibiotic use is included in the predisposing factors of IBD etiology^[24].

Intestinal infections due to enteropathogens have been associated with IBD relapse and the response to therapy^[25]. In particular, associated *Clostridium difficile* in-

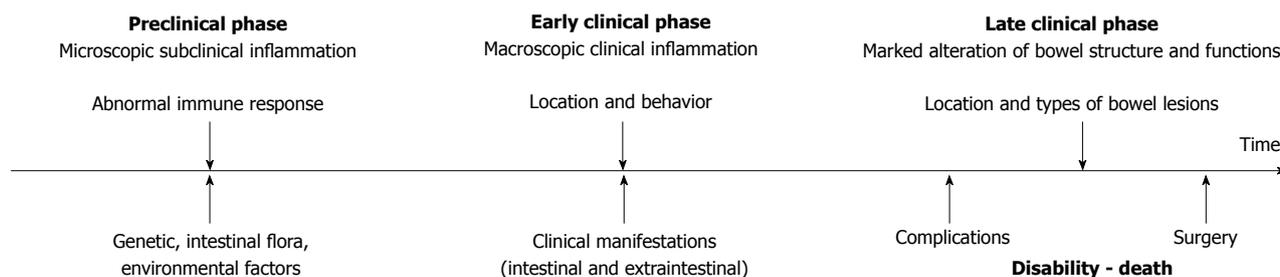


Figure 1 Longitudinal course of inflammatory bowel disease.

fection (which is more frequent in UC than CD) has been reported to have a negative effect on IBD outcome, and to lead to longer hospitalization time, as well as high rates of surgery and a high mortality rate^[26]. The role of cytomegalovirus reactivation in the colon of patients with refractory colitis remains to be fully elucidated^[27].

In the second paper, Beaugerie *et al.*^[12] review the role of clinical, serological and genetic predictors of the IBD course and discuss their potential role in clinical practice. This aspect appears to be particularly relevant: there is a need for predictors of a benign or unfavorable clinical course, in order to avoid over-treatment of patients who will experience a mild clinical course and under-treatment of those patients who will experience an aggressive and progressive disorder.

In CD, age under 40 years, perianal disease, ileal lesions and need of steroids at diagnosis have been consistently associated with an unfavorable medium- and long-term clinical course^[28-31]. In the post-operative setting, smoking, history of previous resection and severity of early post-operative endoscopic recurrence are the strongest predictors of symptomatic recurrence. In UC, extensive colitis, high disease activity, younger age and female gender are associated with poor outcome in most population-based studies^[32-34].

Genetic factors and luminal microbes, besides their role in triggering the IBD, may also be directly, or indirectly, involved in the clinical course of IBD^[35,36].

Identifying genetic prognostic factors in IBD is very attractive as they are already present at the onset of the disease and remain stable over time, which is not the case for clinical and serologic parameters. However, despite the growing number of identified susceptibility loci in both CD and UC, only very few have been associated with disease outcomes. The development of genome-wide association scanning techniques has led to the discovery of more than 100 confirmed IBD loci^[35-38]. Some of these loci, such as the Th 17 pathway genes (*IL23R*, *IL12B*, *JAK2*, *STAT3*), are shared between CD and UC, others are phenotype-specific (autophagy genes such as *ATG16L1*, *IRGM* and *NOD2* for CD; epithelial barrier genes *HNF4a*, *E-Cadherin*, *LAMB1* and *IL-10* for UC). Variants of some of these genes would be excellent prognostic factors^[39].

There is a growing body of evidence proving that in CD, the main *NOD2/CARD15* variants are closely related to ileal disease, a stenosing phenotype, an earlier need

for first surgery and a reduced post-operative disease-free interval^[10]. All of these findings provide evidence that may encourage the clinical application of *NOD2/CARD15* genotyping both as a marker of CD and as a prognostic factor of the need for early surgery due to stricturing and fibrostenosing disease^[10].

Certain genetic factors appear to influence the response to medical treatments. Polymorphisms in multi drug resistant-1, migration inhibitory factor, tumor necrosis factor (TNF) and apoptosis genes have been associated with a higher risk of treatment failure (steroids, cyclosporine, infliximab) in CD and UC^[40-45].

Antibodies directed against microbial peptides represent good serological markers that could help in the prediction of the clinical course of IBD^[10,46]. Patients with a stronger immune response to microbial peptides are associated with early disease onset of CD, fibrostenosing and penetrating disease, and need for early small bowel surgery^[10,46]. In pediatric CD patients, baseline anti-*Saccharomyces cerevisiae* antibody (ASCA) reactivity is associated with earlier complications, relapsing disease and need for additional surgery^[10,46]. The frequency of disease complications increases with reactivity to increasing numbers of microbial antigens (ASCA, anti-I2, anti-OmpC, and anti-CBir1). High levels of perinuclear anti-neutrophil antibody are associated with the risk of subsequent chronic pouchitis in UC patients undergoing ileal pouch-anal anastomosis^[47].

Overall, these data suggest that serological markers of microbial peptides may be useful predictors of IBD complications. In future, genetic and serological markers will be associated with clinical findings to obtain more reliable and useful predicting tools.

INTESTINAL LESIONS, CLINICAL MANIFESTATIONS AND DISEASE PROGRESSION

Progression of intestinal lesions may range from weeks to decades; however, it can be slowed down, stopped, or reversed spontaneously or by means of medical therapy^[2,3] (Figure 2). Superficial mucosal lesions are most prone to heal, whereas deep ulcers or transmural fissures may heal with more difficulty; fibrotic strictures are usually definitive. IBD becomes symptomatic when lesions are extensive or distal, associated with a systemic inflam-

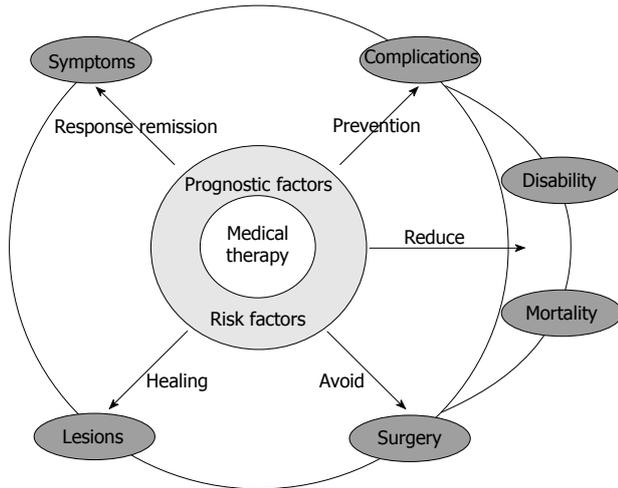


Figure 2 Potential effects of medical therapy on the natural history of inflammatory bowel disease.

matory response, or when associated with local complications such as dilatation (toxic megacolon), massive hemorrhage, strictures, perforation (abscesses and fistulas) and cancer^[2,3]. Colorectal lesions usually present more and early symptoms, whereas small bowel lesions may remain latent for several years^[2,3]. The disease course is generally characterized by a sequence of flares and remission of varying duration, while approximately one-fifth of these patients undergo a chronic, active, continuous disease course. Abdominal pain, abnormal bowel functions and rectal bleeding are the patients' main complaints that significantly alter their quality of life. Since UC is a pathological disorder that affects the mucosa and submucosa, while CD is a transmural inflammation, the anatomic evolution of the lesions and the disease progression are different, and will therefore be considered separately.

UC involves the rectum and colon and extends proximally in a continuous fashion. Upon presentation, lesions are limited to the rectum (proctitis) in 30%-35% of patients, to the splenic flexure (left-sided colitis) in 30%-45% and to the cecum (pancolitis) in 20%-25%^[2,3]. During the course of the disease, after 20 years, the rate of pancolitis may increase reaching 50% of cases. Pancolitis may be associated with inflammation of the terminal ileum ("back wash ileitis"); children do not always have rectal lesions. Mucosal lesions are usually diffuse and superficial, and deep ulcerations are present only in patients with severe disease. Perianal disease may develop in rare cases of UC. Diagnosis may change from UC to CD in 5%-10% of adult patients and in 15%-40% of pediatric patients^[2,3,32,48]. UC appears to be particularly severe in younger patients (especially in children), with a higher frequency of flares that do not respond to medical treatment. Severity of flares and their response to therapy vary and are difficult to predict. Disease activity tends to decrease over time, with 40%-50% of patients in prolonged remission and about 30% with active disease. Clinical remission is usually associated with MH^[8,49]. Extra-intestinal manifestations are observed in one-third of UC patients^[50].

In CD, the lesions can involve any segment of the digestive tract, from the mouth to the anus, but mainly affect the distal ileum and the colon. At the time of diagnosis, approximately 40% of patients present with ileocolonic disease, about 30% have isolated ileal disease, and another 30% have a pure colonic disease^[2,3]. Approximately 5%-15% of patients have associated upper gastrointestinal lesions and 20%-30% present perianal lesions^[2,3,51,52]. The localization of the lesions changes only minimally over time, with only 10%-15% of patients presenting a change in lesion localization 10 years after diagnosis^[53-55].

Although the location remains relatively stable, the clinical behavior of CD shows a dynamic evolution with striking changes over the course of the disease^[53-55]. During the first few years of CD, the non-penetrating/non-stricturing (inflammatory) form predominates, whereas most patients develop complications during follow-up and are then classified as having a penetrating or a stricturing disease. These two forms may co-exist in the same patient, since internal fistulae may complicate longstanding intestinal stenosis. Disease evolution is related to lesion localization, the development of complications (abscess, fistula, stricture) being more frequent and rapid when the small bowel is involved, whereas when the disease is localised in the colon, it may remain inflammatory and uncomplicated for many years. There is no relationship between symptoms and progression of the intestinal lesions, since strictures and fistulae may develop for several years with only mild symptoms or, in some cases, without any symptoms at all^[5]. Approximately 50% of CD patients have only a slight evolutive disease and, therefore, overtreatment should be avoided^[28-30,56]. The remaining patients present a more aggressive and evolutive disease with high rates of relapse, complications, hospitalization and surgery, all conditions that considerably affect the patients' everyday life and long-term projects. For these patients, sustained control of disease activity and progression is clearly warranted. Taken together, these data obviously indicate the need for strategies aimed at interrupting or delaying the natural evolution of this pathological condition. Current treatment options (antibiotics, steroids, immunosuppressive drugs, biological therapies) may relieve the inflammatory symptoms, but do not improve fibrostenotic obstruction^[57-63]. The results of medical treatment aimed at stricturing or penetrating CD are poor, since 64% of these patients ultimately require surgery within one year^[64]. This situation should be taken into consideration (and discussed with the patient) when planning medical treatment. It is likely that progression to a stricturing or penetrating disease phenotype is an end-stage sequel of CD associated with either irreversible fibrosis or severe inflammation that will not abate despite optimal medical therapy introduced at too late a stage^[65].

IMPACT OF MEDICAL THERAPY

The advancements in knowledge of IBD over the past few years have modified the treatment goals. While, in the

past, the aim of medical treatment was an improvement in IBD symptoms, the current objective is to achieve a deep remission, defined, both in UC and CD, as clinical remission [Mayo score for UC activity < 2 and Crohn's disease activity index (CDAI) < 150] with MH (Mayo endoscopic score for UC < 1 and simple endoscopic score for CD < 2) and cessation of steroid administration^[6-8,65-67] (Figure 2). Therefore, treatment should modify the course of the disease by avoiding the disabling condition and irreversible tissue damage. The treatment strategy in IBD should, therefore, be tailored according to the risk that each patient runs in developing a disabling disease. In this issue of *WJG*, the review by Reenaers *et al*^[68] focuses on the impact of treatment on the natural history of IBD.

Clinical and endoscopic remission is the best result that one can hope to reach and every effort should be made to maintain this condition for as long as possible. Healing of the mucosa, therefore, appears to be an obvious endpoint of treatment. MH can be considered appropriate for UC which is a disease of the mucosa, whereas the term intestinal healing would be more correct for CD which is a transmural disease^[67]. Complete assessment of intestinal healing in CD can be obtained only by using both endoscopy and cross-sectional imaging techniques (magnetic resonance, computed tomography, ultrasonography). In CD, it is not uncommon to find healed mucosa covering symptomatic intestinal stenotic segments. Another crucial point is the timing of endoscopic evaluation which was seen to differ (range: 4-52 wk) in studies evaluating the MH^[7,8,67]. Many studies have been performed investigating the relationships between clinical symptoms and intestinal lesions but, to date, MH has been evaluated as the primary clinical end point in a single randomized clinical trial^[69]. Efficacy of adalimumab (anti-TNF α antibody) for induction and maintenance of MH in 135 adults with moderate-to-severe ileocolonic CD was evaluated in this trial^[69]. Twenty-seven percent of patients receiving adalimumab had MH at week 12 (the primary end point) *vs* 13% given placebo ($P = 0.056$). At week 52, rates of MH were 24% and 0%, respectively ($P < 0.001$). Clinical remission rates (CDAI < 150) were 52% for adalimumab and 28% for placebo at week 12 ($P = 0.006$) and 28% and 3%, respectively, at week 52 ($P < 0.001$). Despite the lack of data to confirm an impact of MH induced by anti-TNFs on outcomes in CD, the issue of potential benefits is widely debated. Furthermore, scientific evidence that MH may change the natural course of IBD needs to be proved in long-term studies^[6-8,65-67].

The current medical armamentarium for treating moderate-severe IBD consists of corticosteroids, immunosuppressants and biologics (anti-TNF α antibodies), but for most of these medications it is unclear whether treating patients more aggressively will actually slow down disease progression^[67,70,71].

In UC, the percentage of patients achieving MH appears to be of the same order for treatment with immunosuppressants (53%-68%)^[8,72,73] and biologics (30%-71%)^[8,74-76]; albeit, it is obtained more rapidly with the latter agents.

Complete restoration of the mucosal architecture may be achieved when acute UC is of short duration and a prompt response to medical therapy has been reached.

In CD, anti-TNF α antibody therapy has been reported, during 54 wk trials, to reduce the need for CD-related hospitalization and surgery^[77-80]; however, the duration of these effects is unknown. Although biological therapies have shown disease-modifying characteristics in other pathological conditions, more data are necessary before it can be confirmed whether they can influence the long-term natural history of CD^[65,66,81]. There is no doubt that these agents work best when introduced early in the course of the disease, when they could reasonably be expected to change the course of CD. The fact is that they are not: the median duration of disease when this practice is adopted is almost 10 years. It is these patients, who are not frequently found in clinical practice but represent the majority, upon whom attention is focused: new therapeutic agents may add even more delay and ultimately be associated with a higher burden of disease.

It is interesting to note that the overall percentage of CD patients achieving MH with anti-TNF α antibodies (29%-73%)^[8,67,77,82-84] is of the same order as that reported with immunosuppressants (35%-73%)^[8,67,85-89]. Efficacy of infliximab monotherapy, azathioprine monotherapy, and the two drugs combined in adults with moderate-to-severe CD was evaluated by the Study of Biologic and Immunomodulator Naïve Patients in Crohn's Disease (SONIC study)^[83]. At week 26, MH had occurred in 43.9% of patients receiving combination therapy, as compared with 30.1% of patients receiving infliximab ($P = 0.06$) and 16.5% of patients receiving azathioprine ($P < 0.001$ for the comparison with combination therapy and $P = 0.02$ for the comparison with infliximab). In a recent prospective comparative study on CD, the MH rate achieved with azathioprine (50%) was not statistically different from that obtained with infliximab (60%)^[90]. On the other hand, another recent study showed that only non-complicated inflammatory CD behavior and long-term anti-TNF treatment were associated with a lower risk of the need for surgery, whereas azathioprine only slightly reduced this risk^[91].

A crucial point is the timing of commencement of early treatment. In clinical practice, early CD is usually considered as a newly diagnosed case, and this does not always correspond to onset of the early purely inflammatory form of the disease. Approximately 50% of patients already present a stricturing or penetrating disease at the time of diagnosis^[55], thus indicating a late disease which is more resistant to treatment both with immunosuppressants and biological agents.

SURGERY

Surgery plays an important role in the management of IBD. Up to 75% of CD patients will require an operation at some point in the course of the disease^[92], and, although surgery is not curative, it appears to be the most efficacious treatment in inducing prolonged remission^[93].

Therefore, surgery should not be dismissed as the end of the road after all medical options have failed, but should be considered a valid part of the overall management strategy^[63]. Nevertheless, in the biological era, avoiding surgery is becoming an emerging and interesting therapeutic endpoint. Considering that biological agents are claimed to induce MH in a large percentage of cases, a substantial reduction in the need for surgery would be expected. Although data from RCTs and observational studies^[94] suggest that biological use may reduce the need for surgery in the short term, the real impact of biologics on the lifetime risk of surgery remains to be established. Recent data from population-based cohorts have shown that in the pre-biologic era, the rate of surgery ranged between 27% and 61% within 5 years after diagnosis, and, in the era of anti-TNF α , ranged between 25% and 33%^[95], thus suggesting that the need for surgery also remains high in the era of biologics. Moreover, an analysis of secular trends of hospitalization and surgery rates in the United States, from 1990 to 2003, showed stable rates of bowel resection surgery for CD despite advances in treatment^[96]. In this issue of *WJG*, de Buck van Overstraeten *et al*^[97] discuss the need for surgery in randomized trials as well as the need for surgery in population studies (the real world).

In UC, the cumulative probability of colectomy within 10 years after diagnosis appears to be lower than previously reported^[98], but with considerable geographical variations (up to 25% in Denmark and 3.9% in Southern Europe, thus reflecting the different policies in approaching surgery)^[99]. In severe UC, colectomy is a life-saving intervention in patients refractory to intravenous steroids; prompt surgery, when necessary, is probably the major determinant of the improved outcome of severe UC in the past 30-40 years^[100,101]. During the last 20 years, medical rescue therapy with cyclosporine, and, more recently, with infliximab and tacrolimus, has received growing interest on account of its high efficacy in avoiding colectomy, in the short term, in severe steroid-refractory UC. However, the overall impact of rescue therapies on the outcome of severe UC remains to be defined: the short-term colectomy rate has remained stable over the last 30 years, despite the introduction of cyclosporine^[102] and the long-term efficacy of infliximab remains to be defined. In this issue of *WJG*, Dayan *et al*^[103] discuss, in detail, the role of surgery in severe UC in the era of medical rescue therapy.

RISK OF COLORECTAL CANCER

There is general consensus that the risk of colorectal cancer (CRC) is increased in IBD. Duration and extent of colitis, persistent inflammatory activity, family history of sporadic CRC and concomitant primary sclerosing cholangitis are well established risk factors. Although CRC risk has been studied more extensively in UC^[104], recent data suggest that CD carries a similar risk^[105]. However, the exact magnitude of the risk is controversial. In a meta-analysis published in 2001 which included

116 studies, a cumulative probability of CRC in UC of 2% by 10 years, 8% by 20 years, and 18% by 30 years has been reported^[104]. These results are in contrast with data from population-based studies from Scandinavia and the United States which report a 30-year cumulative probability of CRC in UC as low as approximately 2% with an overall risk of CRC among UC patients similar to that expected in the general population^[106,107].

Although geographical variations in the risk of developing CRC can play a role, differences in the methodology of individual studies (population-based *vs* referral center based) and different clinical approaches in the management of patients and follow-up (cumulative proctocolectomy rate, maintenance treatment with aminosalicylates, close follow-up evaluation of patients and surveillance programs) can explain the high variability in the risk. The exact definition of the risk appears to be crucial when planning “reducing-risk strategies”, for example, an endoscopic surveillance program, chemoprevention or both. In fact, the cost-effectiveness of any strategy aimed at reducing the risk of CRC is affected primarily by the baseline risk. Currently there is no strong evidence to support a chemoprophylactic role for 5-aminosalicylic acid, as well as for other drugs used in the treatment of IBD^[108-110].

An exhaustive discussion of the molecular biology and all the potential risk factors of IBD-associated CRC is reported by Dyson *et al*^[111] in this issue of *WJG*.

MORTALITY

Mortality is the most relevant clinical endpoint in studies focusing on the natural history of a chronic disease. In UC, mortality has continuously decreased over the last 50 years. This time trend probably results from improved medical and surgical management. Data from population-based studies suggest that the overall mortality in UC is not different from that of the background population. However, subgroups of patients, particularly those with extensive disease in the first few years after diagnosis, may be at greater risk of dying^[112]. Conversely, CD is associated with a small, but nevertheless significantly increased, risk of death compared to the general population^[113].

Although a slight decrease in the standardized mortality ratios has been observed over the last 30 years, this decrease is not statistically significant. This would appear to suggest that the overall prognosis of CD has not really changed despite the improvement in medical and surgical management over the last 30 years.

It will be interesting to see the trend in mortality due to CD in the near future. Preliminary data suggest that in-hospital mortality for CD is reduced in centers with a very large number of admissions for IBD^[114], thus suggesting that specialist care could improve outcomes. Besides the reduction in mortality related to the disease, we will, in the near future, also be facing more severe side-effects, including mortality, related to the more aggressive medical treatment.

CONCLUSION

Onset of IBD usually occurs in young adulthood and lasts throughout the patient's life. Despite the enormous progress that has been made in the understanding of these pathological conditions, the etiology remains unknown and no definite cure is yet available^[1-3]. The incidence of IBD is increasing worldwide, including also developing countries. UC and CD both have a negative effect on the quality of life and the capacity for work, and, furthermore, increase disability^[115]. Disease progression and prognosis have greatly benefited from the use of steroids introduced in the 1950s, immunosuppressants in the 1970s and biological agents in the 1990s. Although these treatments appear to be effective in the management of disease activity in the majority of patients, and to improve the quality of life, it is still not clear whether they are able to modify the natural history of IBD^[3,65-67]. There is evidence that new approaches aimed at optimizing immunosuppressants and biological agents by using them as early as possible could prevent disease progression and have a positive effect on the natural history of IBD.

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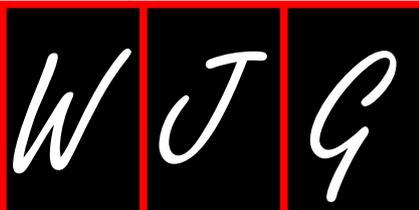
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Methodology for high-quality studies on course and prognosis of inflammatory bowel disease

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Abstract

Inflammatory bowel diseases (IBDs) are characterized by a chronic course with an alternation of relapses and remissions. Questions about prognosis are important for the patient who wants to know how the disease will affect his/her life and also for clinicians to make management decisions. Correct selection of the patients is the basis for good methodological studies on the course of IBD. A great proportion of data on the course of IBD is derived from a limited number of cohort studies. Studies help to define the endpoints for clinical trials and to identify subsets of patients in whom the prognosis of the disease can be stratified according to clinical features. Specific scientific requirements for high-quality studies on prognosis are the following: use of inception cohort, description of referral patterns, completeness of follow-up, objective outcome criteria, blind outcome assessment, adjustment for extraneous prognostic factors and statistical issues. We analyzed each of these requirements in studies on IBDs. To date, prospective and population-based cohort studies are the standard for an unbiased assessment of prognosis. A better knowledge of the

course of disease of chronic disorders ideally requires: (1) data from population-based studies, to avoid selection bias from referral centers in which patients with a more severe disease are usually treated; (2) inclusion of patients seen at the onset of the disease excluding misdiagnosed cases; and (3) follow-up from the onset of the disease to the end without dropouts.

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Key words: Methodology; Inflammatory bowel disease course; Prognosis; Population-based studies; Prospective cohort studies

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INTRODUCTION

Inflammatory bowel diseases (IBDs) are characterized by an alternate course of relapses and remissions. A better knowledge of the course of a chronic disease permits us to answer correctly the questions about the response to therapy, disability, the rate of surgery and mortality, and to identify subsets of patients in whom disease prognosis can be stratified according to clinical features. Finally, studies of the course may increase our knowledge of disease pathology and etiological factors, possibly result-

ing in the prevention of disease.

Until 1970, the course and prognosis of the IBDs were derived initially from tertiary referral centers showing high morbidity and mortality, because they concerned more severe and complicated diseases. Subsequently, several cohort studies have been carried out showing better prognosis than previously described. In fact, for prognosis studies, it is preferable to analyze an unselected group of patients, ensuring the reliability of study results.

Since 1950, drug therapies for IBDs have been introduced, so it is impossible to have any long-term natural history data even if the placebo arms of the clinical trials can be utilized as a source of data on short-term natural history.

According to Sackett *et al*¹¹, natural history is the course of a disease from its biological onset to its recovery or permanent disability or death. In the spectrum of the course of the disease, we can identify different phases: (1) biological onset, “the initial interaction between man, environment and casual factors”; actually it is not certain what the initial event is; and (2) preclinical phase, interval between biological onset and clinical manifestations. To date, we do not know of any specific markers of disease that allow early diagnosis in this phase. The importance of an early diagnosis is also questionable because of the lack of specific treatment, which can alter the natural history of the disease if initiated in a preclinical phase.

In these first two phases, we deal with natural history because patients have not been treated. However, in all the population studies it is better to use the terminology “course of disease” more than “natural history”. The following phases deal with the course of disease: (1) clinical diagnosis that does not correspond to the onset of symptoms (often there is a long gap between the onset of the disease and the time of diagnosis, which represents an important source of bias); and (2) outcome: recovery, permanent disability, mortality. In chronic disease, the interim outcomes (i.e., complications, cancer, impairment of the quality of life, the need of immunosuppression) also represent relevant endpoints in prognostic studies.

In this review, we emphasize the methodological requirements for high-quality studies on the course and prognosis of IBDs. According to Sackett *et al*¹¹, specific scientific requirements for high-quality studies on prognosis are the following: (1) use of inception cohort; (2) description of referral patterns; (3) completeness of follow-up; (4) objective outcome criteria; (5) blind outcome assessment; (6) adjustment for extraneous prognostic factors; and (7) statistical issues. We analyzed each of these requirements within the studies on IBDs.

INCEPTION COHORT

To evaluate the prognosis of a disease in a cohort of patients, it is important to start the follow-up at a common point; preferably as early as possible in the course of the

disease (i.e., onset of symptoms or clinical diagnosis). For this reason, inception cohorts, preferably prospective, now represent the standard design to minimize bias. Bias in cohort studies can create apparent differences when they do not actually exist in nature.

The most frequent selection biases in conducting studies on prognosis are as follows. (1) Prevalence-incidence bias: when mild or asymptomatic cases, for example, proctitis, as well as fatal short disease episodes, such as severe colitis, are missed when studies are performed late in the disease process. It could result in an overestimation of the severity of the disease if the patients with a mild disease are missed, and in a more favorable prognosis if the mortality is not included in the prevalent group; (2) Lead time bias: occurs when the outcomes such as survival, as measured from the time of diagnosis, may be increased not only, because the patients live longer, but because screening permits an early diagnosis (i.e., for the availability of a new diagnostic test). This results in an apparent prolongation of the time to a predefined event (i.e., death, time to surgery or time to relapse), when instead it only results in an earlier diagnosis when compared to traditional methods [i.e., detection of asymptomatic colon cancer during screening endoscopy in patients with ulcerative colitis (UC) could result in an apparent prolongation of survival]; and (3) Length time bias: screening tends to detect a disease that is destined to progress slowly and, therefore, has a good prognosis. Also, advances in diagnostic techniques allow an earlier diagnosis, in an asymptomatic phase of the diseases with less aggressive course. Length time bias occurs when the patients, whose disease is discovered by screening, may also appear to do better, or live longer, than people whose disease is clinically diagnosed with symptoms. For example in patients with IBDs, before the introduction of endoscopy, mild colonic or distal disease, which are often mildly symptomatic, were often missed. This distortion is called technical bias and is related to the length time bias; together with the therapeutic bias (concerning the advance in therapy) it concurs to determine the temporal bias.

DESCRIPTION OF REFERRAL PATTERNS

In a study of prognosis, it is of great importance to use unselected patients to obtain more realistic results and for a wider applicability of study results. However, it is important to describe the referral pattern, which occurs when the characteristics of patients differ between one setting (e.g., primary care) and another setting that includes only referred patients (e.g., secondary or tertiary care). Studies from referral centers include more severe and complicated cases and usually result in poor prognosis.

Another relevant bias is the diagnostic/therapeutic access bias that occurs when studies made between populations with a different access to diagnostic facilities or therapy are compared. For example, in a tertiary center, patients have more opportunities to access biological ther-

apy, allowing a better course of disease in severe patients.

The outcome could also be influenced by the different health insurance or government programs across countries, as is the limit which exists in some countries (i.e., the United Kingdom) on the maximum duration of anti-tumor necrosis factor (TNF) therapy. Few data are available on the relationship between the length of maintenance therapy with anti-TNF α and the natural history of the disease, or with the achievement of mucosal healing, which actually seems to be the main outcome correlated with the maintenance of remission. The best study is the population study in which all the incident cases in a well-defined area are identified and followed up regularly with a clear protocol.

COMPLETENESS OF FOLLOW-UP

According to Sackett *et al.*^[1], in an accurate study of prognosis, at least 90% of the population should complete follow-up. This statement results from the evidence that a study with many patients lost during follow-up (usually > 20%) leads to distorted results. For example, if patients are lost during follow-up, for poor compliance, it could result in a better prognosis of the cohort. If they are affected by mild disease (like proctitis in UC) and therefore omit control visits, this can result in an overestimation of poor outcomes. However, for any degree of loss during follow-up, the validity of the study could be diminished. In addition to this, the length of follow-up is also important if one is evaluating some specific outcomes like survival. In that case, the follow-up time should be long enough so that about two-thirds of the patients will have suffered the events under study at the end of the observation period. In other cases, when the outcome evaluated is more frequent and rapid in occurrence (i.e., postsurgical recurrence or response to therapy), the follow-up could be shorter.

OBJECTIVE OUTCOME CRITERIA AND BLIND ASSESSMENT

The most important outcomes to assess in a prognosis study are disease activity, intestinal complications, surgery, cancer risk and mortality. One of the most relevant problems in the study of prognosis of IBDs is the difficulty of identifying an objective outcome because of the lack of an agreement in the definition of some important outcome measures that are open to possible differences in the results.

Disease activity

For example, analyzing some of the most important studies of prognosis, the definition of disease activity is variable. Below, we report some examples of this variability in cohort studies of IBDs.

Remission or no activity: (1) In the Copenhagen study^[2-7] for Crohn's disease (CD) no activity was defined as no

more than two stools per day and no blood or pus in the stools, no abdominal pain and no systemic symptoms such as fever or weight loss; and (2) in the Olmsted County study^[8-11], remission or no medication state was defined as a patient who required no medication for CD, excluding antidiarrheals.

Mild disease: (1) In the Copenhagen study^[2-7], mild disease activity was defined as ≥ 2 and ≤ 4 bowel movements and/or blood or pus in the stools and/or mild abdominal pain less than daily and no systemic symptoms; and (2) in the Olmsted county study^[8-11], mild disease was defined according to therapy; a patient with mild disease was a patient on sulfasalazine, 5-acetylsalicylic acid, antibiotics, or topical therapy.

Severe disease: (1) In the Copenhagen study^[2-7], moderate/high activity was defined as more than four stools daily and/or blood or pus daily and or abdominal pain either severe or daily, with or without systemic symptoms; and (2) in the Olmsted study^[8-11] the authors distinguished severe disease drug responsive and severe disease drug refractory; in the former, they referred to a patient on oral corticosteroids or immunosuppressive therapy lasting > 6 mo, with documented improvements; in the latter definition they included patients on oral corticosteroids or immunosuppressive therapy with no documented improvements within 2 mo for corticosteroids or within 3 mo for immunosuppressive medications.

In another important study, as in the European collaborative study on inflammatory bowel disease^[12-17], there was not a clear definition of disease activity; the course of the disease was assessed comparing the activity in a given point during the follow-up with the initial status. Any of these definitions involves subjective judgment and blind outcome assessment, one of the requirements, is not usually feasible and the study results are difficult to compare. Probably the CD activity index^[18] and Mayo Clinic score^[19] for UC are a more objective outcome to evaluate disease activity or response to therapy in clinical trials, but owing to their complexity, they are not often used in clinical practice.

Complications

Another bias that occurs when collecting data retrospectively, in evaluating intestinal and extraintestinal complications or need of surgery, is referral bias, which occurs when the appearance of complications has triggered the visit. Thus, it is essential that data on the occurrence of complications in IBDs are collected prospectively and in unselected samples, and the diagnostic measures are well defined. It is important to define the diagnostic and therapeutic protocol for complications because a different approach among centers influences the course of the disease. For example, endoscopic dilation is an approach adopted in stricturing postsurgical recurrence in CD in some centers, whereas in others, surgery is the preferred option and this different choice may influence prognosis.

Cancer risk and mortality

In the evaluation of cancer risk, an important concern is represented by the influence of surveillance bias. Surveillance bias, what some texts call detection bias, occurs when one group is followed more closely than another. This could lead to an outcome being diagnosed more often in the more closely followed group, but not because it really occurred more often in that group. Of course, cancer risk is linked to the surgical policy of the single center. A center that proposes early intervention may have a lower risk of cancer in long-term follow-up. It is mandatory that the cancer risk is evaluated in an incident cohort and in a population study. Another relevant requirement is the presence of a cancer registry in the area where the cohort is followed-up.

Another important outcome in prognosis studies is mortality. A recognized method to assess mortality is the calculation of standardized mortality ratio (SMR). SMR is the ratio between the observed number of deaths in a study population and the number of would-be-expected deaths, based on the age- and sex-specific rates in a standard population and the age and sex distribution of the study population. If the ratio of observed/expected deaths is > 1.0 , there is said to be “excess deaths” in the study population. It is, however, a very efficient stratification method and also permits one to use retrospective data. The results of studies with good methodological requirements have been summarized in a meta-analysis^[20], and give a reliable measure of this outcome correcting for differences among centers. Of course, meta-analysis should include studies with the same methodological standards. Small differences will only be detected if the studied group is very large. At the same time, if the baseline risk of an outcome (i.e., cancer or mortality) is very low, few events in the study population can identify an apparent relevant risk difference (i.e., the risk of Hodgkin lymphoma identified in the Florence cohort^[21,22]).

ADJUSTMENT FOR EXTRANEOUS PROGNOSTIC FACTORS

Examining the effects of specific factors on prognosis, it is important to adjust for extraneous variables, for the potential effect of associated factors on the results, thus unmasking a possible erroneous association. These confounding factors can also influence data from different population-based inception cohort studies. Even population-based inception cohorts could be difficult to compare because of the presence of different sources of bias, such as temporal, diagnostic access and therapeutic. Thus, it is important always to give information about the distribution of potential confounding prognostic factors.

In UC, it is important to know the extent of the distribution of the disease (pancolitis, left-side colitis, or proctitis) at diagnosis and the duration of disease because of the known major risk of cancer in pancolitis

and in long disease duration.

In CD, many relevant prognostic factors have been identified that should always be included in a multivariate analysis, such as smoking habits, age, site of disease, and extent of disease. Prognostic factors should be evaluated in incident cohorts prospectively.

An example of a possible bias in the evaluation of the prognostic factor is the study by Beaugerie *et al.*^[23]. Among 1526 patients diagnosed with CD between 1985 and 1998, those operated upon within the first month of the disease, patients with inadequate data, and patients with severe chronic nondigestive disease were excluded. The authors identified age < 40 years, perianal disease, and initial use of steroids as predictive factors for subsequent 5-year disabling. The authors suggested that referral bias could have distorted the results and a further study in a population setting was advocated. Of course, the prognostic model identified in an incident cohort should be applied in another independent cohort (the test sample).

STATISTICAL ISSUES

To date, to evaluate survival in prognosis studies, life-table-based methods have been used to minimize the difficulty in interpreting crude rates deriving from studies with different lengths of follow-up. During a follow-up period, a decrease in the number of patients makes it easier to detect differences in the early stages of follow-up. Some problems could derive from the lack of study power, and caution should be exercised when the effects are examined over different intervals of time. Rare events, such as lymphoma, risk being overestimated because the baseline risk in the general population is low. Finally, it would be desirable that data on the number of patients under observation at a given time are reported as confidence intervals. Cox's proportional hazard analysis is a type of multivariable analysis used when the outcome is the time to obtain the event. When data on important prognostic factors are not available, sensitivity analysis is a useful tool, assuming various degrees of maldistribution between groups, and seeing how it affects the results.

CONCLUSION

The validity of prognosis studies on IBDs is based on the presence of the above-mentioned methodological requirements. An excellent cohort study must fulfill the following criteria: (1) start of follow-up at inception; (2) population-based, or near to population-based; (3) use of standard diagnostic criteria for UC and/or CD; (4) use of survival methods; and (5) complete or near to complete follow-up ($\geq 80\%$).

Better knowledge of the course of chronic disorders ideally requires: (1) data from population-based studies to avoid selection bias from referral centers where patients with more severe disease are usually treated; (2)

Table 1 Population-based prospective and retrospective studies

Study	Population size	Inception period	UC	CD	Surgery UC	Surgery CD	Mortality UC SMR (95% CI)	Mortality CD SMR (95% CI)
Prospective studies								
Copenhagen ^[2-7] , Denmark	550 000	1962-1987	1160	374	24%	61%	1.1 (1-1.2)	1.3 (1.1-1.6)
		1991-1993	89	58	24%	65%	1.5 (0.9-2.5)	2.3 (1.1-4.2)
	1 211 634	2003-2004	326	209	-	-	0.9 (0.3-2.4)	0.8 (0.02-4.2)
		1962-2004	1575	641				
EC-IBD study ^[12-17]	NA	1991-1993	1379	706	8.70%	40%-55%	1.09 (0.86-1.37)	1.85 (1.3-2.55)
		1991-2004	-	365				
IBSEN ^[23-28] , Norway	970 000	1990-1993	525	225	9.8% (7.4-12.4)	37.9% (31.4-44.4)	Survival 96%	Survival 96%
Retrospective studies								
Stockholm ^[29-31] , Sweden	1 200 000	1955-1984	1547	1251	28.00%	71%	1.37 (1.2-1.54)	1.51 (1.29-1.75)
		1955-2000	-	20 120		78% (15 yr)	(15 yr)	(15 yr)
	1 470 000	1990-2001	-	1389				
Uppsala ^[32,33] , Sweden	1 200 000	1965-1983	2509	1469	-	96% (15 yr)	1.4 (1.2-1.5)	1.6 (1.4-1.9)
Olmsted ^[8-11] , United States	110 000	1940-1993	278	225	49.00%	49%	0.8 (0.6-1)	1.2 (0.9-1.6)
	124 000	1940-2000	372	308				
Leicester ^[34,35] , United Kingdom	930 000	1972-1989	1014	610	-	-	0.9 (0.8-1.1)	0.72 (0.5-1)
Florence ^[21,22] , Italy	650 000	1978-1992	689	231	-	-	0.7 (0.56-0.88)	1.51 (1.06-2.08)
Cardiff ^[36-41] , Wales	280 000	1986-1991	-	105	-	59%	-	
		1992-1997		99		37%		
	NA	1998-2003		137		25%		
		1941-2000		394				1.29 (1.12-1.45)
Leiden ^[41] , The Netherlands	440 000	1979-1983	-	210	-	56% (15 yr)	-	2.23 (1.75-2.85)

UC: Ulcerative colitis; CD: Crohn's disease; SMR: Standardized mortality ratio; EC-IBD study: European collaborative study on inflammatory bowel disease; IBSEN: Inflammatory bowel south-eastern Norway; NA: Not available. Number of cases may vary between various reports from same centers.

inclusion of patients seen at the onset of the disease excluding misdiagnosed cases; and (3) follow-up from the onset of the disease to the end without dropouts.

The more relevant cohort studies are summarized in Table 1 (prospective and retrospective)^[2-7,12-17,23-41], which have been followed up for a long period and in which the methodological requirements listed above are satisfied. Two main outcomes are included in the table to show the variation between both types of study, despite the same methodology being adopted. Prospective cohort studies are a more relevant source of information. Although there was wide variation in the rate of surgery, which depends on the therapeutic policy adopted in different areas, mortality was homogeneous in the three main studies.

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Clinical, serological and genetic predictors of inflammatory bowel disease course

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Abstract

Patients with extensive or complicated Crohn's disease (CD) at diagnosis should be treated straightaway with immunosuppressive therapy according to the most recent guidelines. In patients with localized and uncomplicated CD at diagnosis, early use of immunosuppressive therapy is debated for preventing disease progression and limiting the disabling clinical impact. In this context, there is a need for predictors of benign or unfavourable subsequent clinical course, in order to avoid over-treating with risky drugs those patients who would have experienced spontaneous mid-term asymptomatic disease without progression towards irreversible intestinal lesions. At diagnosis, an age below 40 years, the presence of perianal lesions and the need for treating the first flare with steroids have been consistently associated with an unfavourable subsequent 5-year or 10-year clinical course. The positive predictive value of unfavourable course in patients with 2 or 3 predictors ranges between 0.75 and 0.95 in population-based and referral centre cohorts. Consequently, the use of these predictors can be integrated into the elements that influence individual decisions. In the CD postoperative context, keeping smoking and history of prior resection are the stron-

gest predictors of disease symptomatic recurrence. However, these clinical predictors alone are not as reliable as severity of early postoperative endoscopic recurrence in clinical practice. In ulcerative colitis (UC), extensive colitis at diagnosis is associated with unfavourable clinical course in the first 5 to 10 years of the disease, and also with long-term colectomy and colorectal inflammation-associated colorectal cancer. In patients with extensive UC at diagnosis, a rapid step-up strategy aiming to achieve sustained deep remission should therefore be considered. At the moment, no reliable serological or genetic predictor of inflammatory bowel disease clinical course has been identified.

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Key words: Crohn's disease; Ulcerative colitis; Inflammatory bowel diseases; Natural history; Predictors; Clinical practice

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INTRODUCTION

The lifelong risk of developing inflammatory bowel disease (IBD) exceeds one percent in industrialized countries^[1]. Such diseases are considered as chronic, with a small trend towards spontaneous long-term extinction in ulcerative colitis (UC)^[2], but not in Crohn's disease (CD)^[3]. Despite an increasing use of immunosuppressive thera-

py, the long-term risk of intestinal resection and permanent ileostomy in CD is approximately 80% and 10%, respectively^[1]. In UC, the actuarial risk of colectomy is about one percent per year in population-based cohorts of Northern Europe^[4]. To reverse these unfavourable figures that have not significantly changed during the last five decades^[5], the early use of aggressive therapy, such as the combination of thiopurines and anti-tumor necrosis factor (TNF) therapy^[6], is considered both in CD and UC, with the aim of bringing patients into deep and sustained remission. However, some CD patients present at diagnosis with localized and uncomplicated (no perforation, no stricture) disease, and some UC patients present at diagnosis without disabling symptoms, biological abnormalities or severe endoscopic lesions. In those patients, the early and prolonged use of immunosuppressive therapy, with its associated risk of serious infections^[7] and cancers^[8,9], is questionable because the spontaneous evolution of the disease could have been benign. The risk of over-treating patients could be reduced in theory with the use of clinical, biological or endoscopic factors present at diagnosis and able to predict the subsequent course of IBD. In CD, there is also a need for predictors after complete resection of intestinal lesions when considering the postoperative preventive use of immunosuppressive therapy for limiting the risk of clinical recurrence. We review here the literature on clinical, serological and genetic predictors of IBD course, and discuss which predictors can be potentially useful in clinical practice.

CD

Clinical predictors of CD course

Prediction at diagnosis of unfavourable 5-year or 10-year clinical course: This question has been specifically studied in two referral centre cohorts^[10,11] and two population-based cohorts^[12-14], with fixed 5-year or 10-year study period and complete follow-up in most patients (Table 1). Markers of unfavourable CD course were either a single event (first clinical recurrence^[14] or surgical operation^[12,13]) or the presence of at least one element of a composite definition of disabling disease^[10,11]. Whatever the definition, age below 40 years and presence of perianal disease at diagnosis were identified in most studies as predictors of subsequent unfavourable evolution. Need for steroids for treating the first flare and presence of upper gastrointestinal lesions were associated with a poor outcome in two studies. Finally, in two single studies, terminal ileal location and ileo-colonic lesions were predictive of first surgical operation and disabling disease, respectively. Of note, active smoking status, which is an established transient worsening factor of CD course^[15,16], was not shown to be an independent predictor of unfavourable short and mid-term CD course in these studies.

Other studies: As a general feature, clinical predic-

tors of unfavourable CD course identified in the above dedicated 5-year and 10-year studies have been confirmed in other studies with various design, follow-up time and endpoints. In the historical national cooperative CD study, perianal disease was an independent predictor of unfavourable outcome among 1084 patients^[17]. In a Portuguese referral centre cohort testing CD outcome according to Vienna classification and other clinical markers, earlier age at diagnosis, penetrating lesions (including perianal fistulas and abscesses) and ileo-colonic location were associated with unfavourable outcome^[18]. In the Olmstedt population-based study, young age was predictive of surgery, irrespective of smoking status^[19]. In the Maastricht population-based cohort, young age, small bowel location and stricturing lesions were predictive of first surgery in 476 patients with a mean follow-up of 7 years^[20]. In a referral centre population from Boston, early surgery (within 3 years of diagnosis) was associated with active smoking status, ileal location and need for early steroids in 345 patients followed for at least 3 years^[21].

Whether childhood- and adult-onset CD courses differ in terms of severity has been recently questioned^[22,23]. Patients with childhood-onset disease tend to have more extensive intestinal involvement, more active disease requiring more immunosuppressive therapy, and more rapid disease progression. By contrast, CD patients with a family history of IBD do not exhibit a more severe disease course in population-based studies^[24] as well as in referral centre populations^[12]. Finally, erythema nodosum and pyoderma gangrenosum, at diagnosis or later in the disease, are not predictive of more severe CD course^[25].

Clinical predictors of long-term CD course: There are very scarce data on predictors of the long-term evolution of CD. In the Saint-Antoine cohort, predictors of 15-year CD course were characterized in 600 patients^[1]. Non-severe evolution was defined as clinically inactive disease for greater than 12 years, less than one intestinal resection without permanent stoma and no death. Factors independently associated with a non-severe 15-year clinical course were non-smoking status, rectal sparing, high educational level, older age and longer disease duration.

Clinical predictors of postoperative recurrence:

Literature on clinical predictors of postoperative recurrence is abundant and has been extensively reviewed recently^[26-28]. In brief, postoperative active smoking status appears in most studies as a strong predictor of postoperative recurrence: the risk of clinical recurrence and reoperation is approximately doubled in smokers^[29], with an increase in the risk according to the number of cigarettes smoked per day among smokers^[30]. The other independent undisputed predictor of postoperative recurrence is a history of prior resection. The positive predictive value of penetrating disease behaviour, short disease duration prior to surgery, non-colonic disease location, long duration and extensive bowel resection has been evidenced

Table 1 Clinical predictors of unfavourable course of Crohn's disease and ulcerative colitis

Study	Cohort type	No. of patients	Definition or marker of unfavourable course	Independent predictors of unfavourable course, present at diagnosis
Crohn's disease				
Within the first 5 yr after diagnosis				
Beaugerie <i>et al</i> ^[10] , 2006	Referral centre (Saint-Antoine)	1188	Disabling disease ¹	Age < 40 yr Perianal disease Need for steroids for treating the first flare
Henriksen <i>et al</i> ^[12] , 2007	Population-based (IBSEN)	200	Intestinal resection within the study period	Upper gastrointestinal lesions
Loly <i>et al</i> ^[11] , 2008	Referral centre (Liège)	361	Disabling disease ¹	Perianal disease Need for steroids for treating the first flare Ileo-colonic lesions
Within the first 10 yr after diagnosis				
Wolters <i>et al</i> ^[14] , 2006	Population-based (EC-IBD)	358	First recurrence	Upper gastrointestinal lesions Age < 40 yr
Solberg <i>et al</i> ^[13] , 2007	Population-based (IBSEN)	197	First surgical operation	Age < 40 yr Strictureing and penetrating behaviour ² (including perianal fistulas and abscesses) Terminal ileal location
Ulcerative colitis				
Within the first 5 yr after diagnosis				
Henriksen <i>et al</i> ^[12] , 2007	Population-based (IBSEN)	454	Relapse during the study period	Female gender Younger age
Within the first 10 yr after diagnosis				
Langholz <i>et al</i> ^[12] , 1994	Population-based (county of Copenhagen)	1161	Relapsing or chronic active course Colectomy	Less systemic symptoms (fever, weight loss) Extensive colitis High disease activity (including systemic symptoms)
Höie <i>et al</i> ^[56] , 2007	Population-based (EC-IBD)	771	Frequent relapse	Female gender Younger age Non-smoking status
Höie <i>et al</i> ^[57] , 2007	Population-based (EC-IBD)	781	Colectomy	Extensive colitis
Solberg <i>et al</i> ^[14] , 2009	Population-based (IBSEN)	423	Colectomy	Extensive colitis

¹One or more of the following criteria: more than 2 steroid courses, steroid dependence, hospitalization for disease flare or complication, disabling chronic symptoms, need for immunosuppressive therapy, intestinal resection or surgical operation for surgical disease; ²According to Vienna classification. IBSEN: A population-based inception cohort study; EC-IBD: European collaborative study group of inflammatory bowel disease.

less consistently. Finally, the roles of young age at onset and family history remain controversial.

Serological predictors of CD course

The search for serologic markers in IBD has led to the discovery of specific antibodies. Perinuclear anti-neutrophil antibody (pANCA) is associated with UC or UC-like CD whereas anti-*Saccharomyces cerevisiae* antibody (ASCA, glycan antibody) is mostly associated with CD^[31,32]. Three other markers linked to immune response towards bacteria have been identified; antibodies to the *Escherichia coli* outer-membrane porin C (OmpC), the *Pseudomonas fluorescens* CD-related protein [anti-CD related bacterial sequence (I2)] and the CBir1 flagellin^[33,34].

Many studies have been performed to assess the predictive value of these serological markers. Reactivity to ASCA, OmpC, anti-I2 and CBir1 has been associated with early disease onset CD, fibrostenosing and penetrating disease and need for early small bowel surgery^[34-36]. In paediatric CD patients, baseline ASCA reactivity has been associated with earlier complications, relapsing disease and need for an additional surgery^[37]. The frequency of disease complications increases with reactivity to increasing numbers of antigens (ASCA, anti-I2, anti-OmpC,

and anti-CBir1)^[38]. pANCA has been shown to be associated with less severe disease, UC-like disease and to be negatively associated with small bowel complication^[36,39]. ASCA positivity has been associated with CD of the pouch after ileal pouch-anal anastomosis (IPAA)^[40].

Genetic predictors of CD course

Before the era of genome-wide association studies, the role of genetic factors in IBD severity had been looked for by comparing familial and sporadic IBD. Although having a relative with IBD increases the risk for CD, the severity of CD is unaffected by family history^[24]. A family history of CD increases the risk of subsequent CD after IPAA. Identifying genetic prognostic factors in IBD is a very attractive option as they are already present at the onset of the disease and actually even earlier. Their main advantage is their long-term stability, which is not the case for many other potential predictive factors such as clinical parameters or serologic markers. However, despite a growing number of identified susceptibility loci in both CD and UC^[41], only very few have been associated with disease outcome. The presence of *NOD2* polymorphism has been associated with a more aggressive clinical course of CD; i.e., higher risk of intestinal strictures,

Table 2 Validation of the positive predictive value of Saint-Antoine predictors

Study	Cohort type	No. of patients	Prevalence of unfavourable course ² (%)	Positive predictive value of the presence of 2 or 3 predictors ¹ for predicting 5 yr unfavourable clinical course (%)
Reference study				
Beaugerie <i>et al</i> ^[10] , 2006	Saint-Antoine referral population	1123	85.2	2 predictors: 91 3 predictors: 93
Validating populations				
Beaugerie <i>et al</i> ^[10] , 2006	Independent subsequent sample of the Saint-Antoine referral population	302	85.2	2 predictors: 84 3 predictors: 91
Loly <i>et al</i> ^[11] , 2008	Liege referral population	361	57.9	2 or 3 predictors: 67
Beaugerie <i>et al</i> ^[62] , 2009	Population-based population (Olmstedt county)	423	74.2	2 or 3 predictors: 74

¹Age < 40 years and perianal lesions at first presentation, need for steroids for treating the first flare; ²Unfavourable clinical Crohn's disease course (one or more of the following criteria: more than 2 steroid courses, steroid dependence, hospitalization for disease flare or complication, disabling chronic symptoms, need for immunosuppressive therapy, intestinal resection or surgical operation for surgical disease) within the 5 years following diagnosis.

earlier need for first surgery and reduced postoperative disease-free interval^[42-44]. Some studies have also tried to identify genetic factors associated with response to treatment. Polymorphisms in multi-drug resistant 1 (*MDR1*), *TNF* and migration inhibitory factor genes have been associated with corticosteroid refractoriness or sensitivity in CD and UC^[45-47]. *MDR1* polymorphism has also been associated with a higher risk of cyclosporine failure in patients with steroid-resistant UC^[48]. The efficacy of infliximab is partly due to the induction of apoptosis in activated T lymphocytes. A research group in Leuven has analyzed infliximab-treated patients and found polymorphisms in apoptosis genes predicting response to infliximab therapy in luminal and fistulizing Crohn's disease^[49]. Other studies suggest that genetic polymorphisms might be useful to predict anti-TNF therapeutic responsiveness in paediatric IBD^[50].

Despite their attractiveness, genetic markers will probably never be able to fully predict IBD behaviour and complications, because of the major role of environmental factors in the disease pathogenesis. On the other hand, they could be associated with other factors, such as clinical or microbiological data, in order to build relevant composite predicting tools.

Relevance and potential use of predictors in CD

Saint-Antoine clinical predictors at diagnosis and early use of immunosuppressive therapy: In the Saint-Antoine cohort, patients were considered as having an unfavourable ("disabling") clinical course in the first five years after diagnosis if they met at least one of the following criteria during the study period: more than 2 steroid courses, steroid dependence, hospitalization for disease flare or complication, disabling chronic symptoms, need for immunosuppressive therapy, intestinal resection or surgical operation for perianal disease. Among the 1123 patients studied, the rate of unfavourable course was 85%. By multivariate analysis, initial need for steroid use, age below 40 years and perianal disease at diagnosis were the three independent predictors of subsequent 5-year unfavourable course. The positive predic-

tive value of unfavourable course in patients with two or three predictors was 0.91 and 0.93, respectively.

The Saint-Antoine clinical predictors are the only predictive factors that have been tested subsequently in independent population samples (Table 2). In a subsequent group of 302 patients from the Saint-Antoine centre, the positive predictive value of unfavourable course in patients with two or three predictors was 0.84 and 0.91, respectively^[10]. The Saint-Antoine predictors were subsequently evaluated in two independent cohorts, using the same definition of unfavourable CD course as in the reference study. In the Liege referral population, the positive predictive value of unfavourable course in patients with two or three predictors was 0.67^[11]. This rate was 0.74 in the Olmstedt population^[51]. In conclusion, using the three Saint-Antoine predictors at diagnosis, the prediction of subsequent 5-year unfavourable CD course is accurate in more than two-thirds of patients from various populations. It is plausible that prediction accuracy holds true for the 5 to 10-year CD clinical course, since a strong homology has been demonstrated between the course pattern of CD in the first 3 years of the disease and the 7 subsequent years^[5,52].

In the literature, the proportion of patients exhibiting unfavourable clinical course in the first 5 years of the disease, whatever the definition (disabling disease, frequent clinical relapses, chronic disabling symptoms, need for surgery, *etc.*) exceeds 50%, with an expected trend towards higher prevalence in referral centre cohorts than in population-based studies^[53]. In clinical practice, patients presenting at diagnosis with irreversible penetrating or stricturing lesions, requiring (or not) immediate operation, can be considered as having complicated disease at diagnosis requiring early aggressive therapy (Figure 1). In the second recent European Crohn's and Colitis Organisation (ECCO) CD consensus, patients with upper gastrointestinal lesions or extensive (> 100 cm) lesions at diagnosis are also considered as having severe disease at presentation which justifies immediate use of immunosuppressive therapy^[54]. In the remaining patients, the need for treating moderate to severe flares

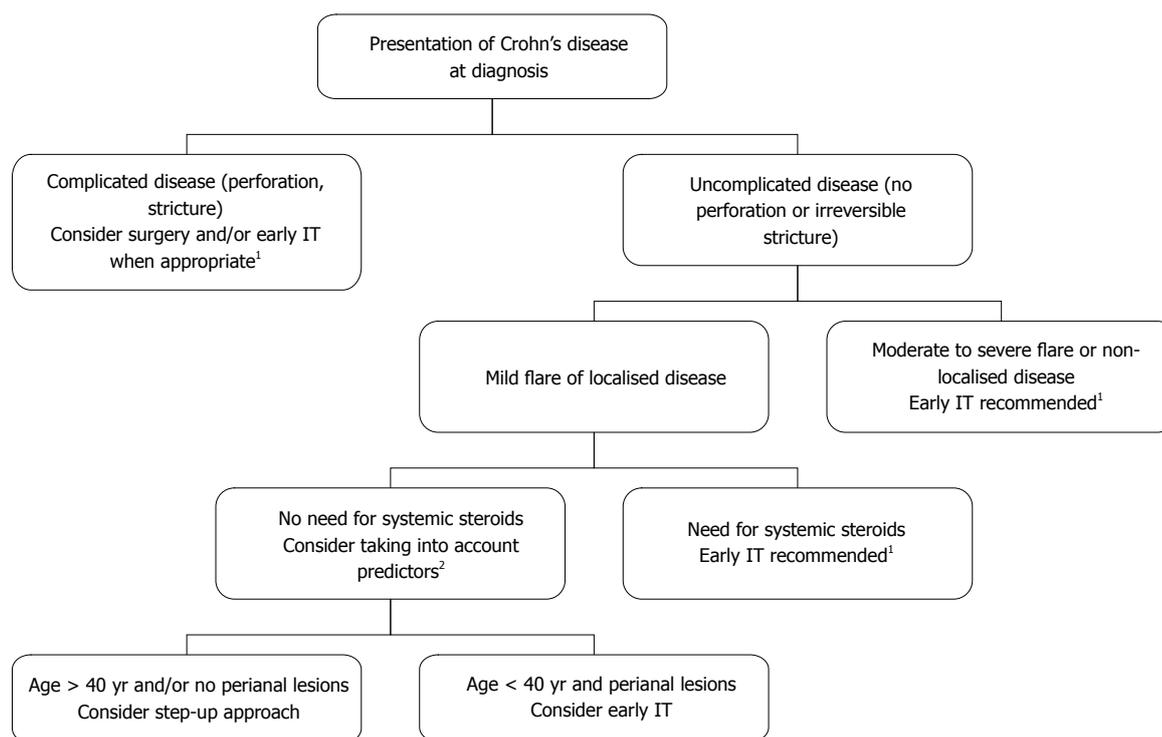


Figure 1 Indication for early immunosuppressive therapy. Thiopurines and/or anti-tumor necrosis factor therapy, according to presentation of Crohn's disease at diagnosis, ¹European Crohn's and Colitis Organisation guidelines and ²Saint-Antoine predictors (2 or 3 of the following items at diagnosis are predictive for subsequent 5-year unfavourable course: Age < 40 years, need for systemic steroids and presence of perianal lesions). IT: Immunosuppressive therapy.

with systemic steroids is increasingly considered as an indication for concurrent initiation of immunosuppressive therapy^[54], taking into account, for instance, the high rate of recurrence within the year after steroid discontinuation^[55]. Among the patients treated with systemic steroids from diagnosis, it must be noted that those who are younger than 40 years and/or have perianal lesions at diagnosis have *ipso facto* at least two predictors of unfavourable subsequent disease. Finally, patients with first disease flare not requiring systemic steroids often have localised^[28] (< 30 cm) continuous intestinal disease with mild symptoms. In this context of true initial benign disease only, Saint-Antoine predictors may be helpful for the decision: in particular, young patients with perianal lesions could be considered for early immunosuppressive therapy, given the high risk of subsequent unfavourable course. However, if early immunosuppressive therapy is not decided on and if patients go rapidly into clinical remission, subclinical inflammatory lesions can now be monitored using blood C-reactive protein, magnetic resonance imaging and faecal calprotectin evaluations, limiting the risk of subclinical progression of intestinal lesions towards irreversible damage without appropriate change in maintenance treatment.

Clinical predictors at surgery of postoperative recurrence and choice of the postoperative maintenance treatment: After intestinal resection, all previous smokers are strongly encouraged to quit smoking. For guiding the choice of the immediate postoperative treat-

ment, in the absence of a validated predictive index, the members of the ECCO consensus group have selected some of the predictors reported in the literature series^[28]. In addition to the two undisputed predictors of early recurrence (keeping smoking and prior resection), penetrating disease behaviour, perianal disease and extensive small bowel resection have been proposed as risk factors. In patients with at least one risk factor, thiopurines are proposed as the drug of choice for preventing early recurrence. However, it is also recommended to perform during the first postoperative year an ileocolonoscopy in order to assess the presence and severity of endoscopic perianastomotic lesions. These endoscopic findings are unanimously considered as the gold standard predictor of subsequent clinical evolution^[26,27], so that the definite choice of the postoperative prophylactic treatment should be based on the severity of endoscopic lesions^[28]. As a consequence, the choice of immediate therapy based on (insufficiently) validated clinical predictors *vs* tailored treatment initiated according to endoscopic findings has a limited impact since, in all patients, the final choice of the mid-term prophylactic treatment is based on endoscopic findings.

UC

Clinical predictors of UC course

Prediction at diagnosis of unfavourable 5-year or 10-year clinical course: This question has been specifically studied in five population-based studies (Table

1)^[2,4,12,56,57]. Young age at diagnosis and female gender were associated in two studies with a trend towards more frequent relapses^[12,56]. Active smoking status was associated with reduced number of relapses within 10 years after diagnosis in one cohort only^[56], but many other studies on the natural history have established the relationship between sustained non-smoking status and less active disease course^[1], with reversion of this impact when resuming smoking^[15]. However, of course, patients with UC are not encouraged to smoke. Finally, in the Danish cohort, a high level of systemic clinical symptoms at presentation (fever, weight loss) was associated at the same time with a trend towards infrequent relapses and chronic disease but higher risk of colectomy. Since proctocolectomy is no longer considered as the end of the medical problems in most operated patients, the risk of colectomy is a good marker of overall severity in patients with UC. Extensive colitis at presentation (defined as upper limit of macroscopic lesions proximal to the splenic flexure) has consistently been evidenced as an independent predictor of colectomy within the 10 years after diagnosis.

Serological and genetic predictors of UC course

Serological and genetic predictors have been far less studied in UC than in CD and only a few clinical settings have been investigated. As in CD, the severity of UC does not seem to be affected by family history of IBD^[12,58]. In the pre-colectomy situation, high levels of pANCA have been associated with the risk of subsequent chronic pouchitis in UC patients undergoing IPAA^[59]. pANCA might also be useful to predict response to anti-TNF therapy since negative status has been independently associated with early response to this treatment^[60]. Some genetic factors might also be useful to predict response to treatment in the future. A recent study by a German group suggests that homozygous carriers of IBD risk-increasing *IL23R* variants are more likely to respond to infliximab than are homozygous carriers of IBD risk-decreasing *IL23R* variants^[60]. It is also possible that a genetic scoring system taking into account several single nucleotide polymorphisms might be able to identify medically refractory UC patients^[61]. As in CD, serological and genetic markers will probably be useful in future composite predicting tools.

Relevance and potential use of predictors in UC

Compared with CD, individual natural history of patients is harder to predict than in CD^[1], and the clinical relevance of clinical predictors of 10-year UC severity has not been validated in independent cohorts. However, extensive colitis at diagnosis consistently appears as a predictor of subsequent colectomy. In addition, extensive colitis is strongly associated with the risk of long-term colorectal cancer^[62,63], and persisting colonic mucosal inflammation (macroscopic^[64] and microscopic^[65]) also contribute independently to the risk. Finally, the IBSEN group also demonstrated that obtaining mucosal

healing in the short-term in patients with UC is associated with a reduced risk of subsequent colectomy^[66]. In this context, it is reasonable to suggest considering a more aggressive therapeutic strategy (for instance, faster step-up approach) in patients with extensive colitis at diagnosis.

In conclusion, at the moment no reliable genetic or serological predictor of IBD course can be used in clinical practice. Saint-Antoine clinical predictors of subsequent unfavourable CD course may be taken into account in the decision about early immunosuppressive therapy in selected patients, and extensive colitis at diagnosis should influence individual therapeutic strategy in UC. However, we are aiming now towards the primary therapeutic goal of obtaining sustained deep remission (including mucosal healing) rather than simple sustained clinical remission. Consequently, we will need to identify predictors at diagnosis of subsequent sustained deep remission. Given the growing research in genetics and pharmacogenomics, we can imagine that sensitive and reliable predictors will be available in the near future.

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Impact of environmental and dietary factors on the course of inflammatory bowel disease

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Abstract

Besides their possible effects on the development of inflammatory bowel disease (IBD), some environmental factors can modulate the clinical course of both ulcerative colitis (UC) and Crohn's disease (CD). This review is mainly devoted to describing the current knowledge of the impact of some of these factors on the outcome of IBD, with special emphasis on smoking and diet. Although the impact of smoking on the susceptibility to develop CD and UC is firmly established, its influence on the clinical course of both diseases is still debatable. In CD, active smoking is a risk factor for postoperative recurrence. Beyond this clinical setting, smoking cessation seems to be advantageous in those CD patients who were smokers at disease diagnosis, while smoking resumption may be of benefit in ex-smokers with resistant UC. The role of dietary habits on the development of IBD is far from being well established. Also, food intolerances are very frequent, but usually inconsistent

among IBD patients, and therefore no general dietary recommendations can be made in these patients. In general, IBD patients should eat a diet as varied as possible. Regarding the possible therapeutic role of some dietary components in IBD, lessons should be drawn from the investigation of the primary therapeutic effect of enteral nutrition in CD. Low-fat diets seem to be particularly useful. Also, some lipid sources, such as olive oil, medium-chain triglycerides, and perhaps omega-3 fatty acids, might have a therapeutic effect. Fermentable fiber may have a role in preventing relapses in inactive UC.

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Key words: Environmental factors; Dietary factors; Non-steroidal anti-inflammatory drugs; Smoking; Infections; Inflammatory bowel disease

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INTRODUCTION

Despite the advances in uncovering genetic risk for Crohn's disease (CD) and ulcerative colitis (UC) over the past decade, the etiopathogenesis of inflammatory bowel diseases (IBDs) cannot be explained only in terms of genetic susceptibility. In fact, a vast number of possible environmental risk factors for the development of IBD have

been investigated, including smoking, dietary factors, psychological stress, use of non-steroidal anti-inflammatory drugs (NSAIDs) and oral contraceptives, appendectomy, breastfeeding, as well as infections and other events related to the so-called “hygiene hypothesis” in childhood^[1].

In addition to their putative effects on the development of IBD, some environmental factors can play a role in modulating the clinical course of both UC and CD. This review is mainly devoted to describing the current knowledge of the impact of some of these factors on the clinical outcome of IBD, with special emphasis on smoking and diet. The role of microbial factors (namely, the commensal microflora, and pathogens such as *Mycobacterium avium* *ssp.* paratuberculosis) in the pathogenesis of IBD will be not discussed.

SMOKING

Tobacco is the best established environmental factor affecting the susceptibility to develop IBD^[2] and maybe its clinical course, with opposite effects in CD and UC. However, most of the published studies assessing the impact of smoking on the long-term clinical outcomes of IBD are retrospective, often leading to controversial results.

Role of smoking on the clinical course of CD

Beyond the higher incidence of CD among smokers, several studies suggest that continuing to smoke leads to worse clinical outcomes^[3]. The underlying mechanisms of this deleterious effect are not well understood, but it has been reported that tobacco glycoprotein may be responsible for promoting a Th1 cell response^[4]. Moreover, tobacco increases production of reactive oxygen species and impaired antioxidant capacity has been shown in smokers^[5].

Smoking has been associated with a higher risk of relapse^[6,7] and increased need for immunomodulators^[8], but the strongest evidence of the deleterious effect of smoking upon the course of CD lies in the beneficial consequences of smoking cessation^[9]. Cosnes *et al.*^[10] demonstrated that patients who stop smoking for at least 6 mo are at a lower risk of relapse in the following 12-18 mo, as compared to non-quitters.

The negative impact of active smoking may not be the same in all CD patients and it has been suggested that it may depend, at least, on gender and disease location. The effect of smoking has been reported to be more marked in women^[8,10]. While the natural history of colonic CD seems to be the same in smokers and non-smokers, the rates of relapse^[11] and intestinal resection are higher among smokers with ileal disease^[6]. In addition, smoking has been associated with a lower prevalence of inflammatory (non-stricturing, non-penetrating) behavior of the disease, thus suggesting that tobacco facilitates progression towards complicated disease^[12-14]. Nevertheless, this may only reflect a greater proportion of smokers among patients with ileal involvement^[15].

The negative effects of tobacco seem to be dose-dependent, and some studies pointed to an increased risk

of surgery and persistent inflammatory activity in those patients smoking over 10 cigarettes/d^[12,16]. Conversely, a recent study reported non-detrimental effects of active smoking on CD course, but passive smokers needed immunosuppressants and infliximab more frequently than non-passive smokers^[17]. Although seldom assessed, genetic background may also play a role, as suggested by the lack of association between smoking and CD in Jewish patients in Israel^[18].

The worse clinical evolution among smokers might also be explained by a lesser response to drug therapies. Despite initial data suggesting a decreased likelihood of response to infliximab for luminal CD in smokers^[19,20], larger studies failed to find any association between smoking and infliximab response^[21-23]. We assessed the influence of smoking on the response to thiopurines in steroid-dependent IBD and, although no differences between smokers and non-smokers were found, CD responders who continued smoking had a higher rate of relapses during follow-up. Surprisingly, we found that smoking was an independent predictor for the need of thiopurine discontinuation because of side effects, leading to a lower treatment efficacy among CD patients (as compared to UC) when evaluated by intention-to-treat analysis^[24].

Postsurgical recurrence is the clinical scenario in which active smoking has better proven to worsen CD prognosis both in the short- and long-term^[25]. Smoking has been reported to be an independent risk factor for endoscopic, clinical, and surgical recurrence^[26-28]. We have recently reported the results of the first prospective study assessing risk factors for endoscopic recurrence in a series of 152 CD patients undergoing ileo-colic resection^[29]. Smoking was independently associated with significant postoperative recurrence as defined by the development of clinical recurrence and/or Rutgeerts grade 3 or 4 of endoscopic recurrence, whereas the only independent protective factor was the use of thiopurines^[29]. Of interest, postoperative recurrence has been reported to be much more marked among heavy smokers (> 10 cigarettes/d)^[26,29]. In spite of the fact that it has been suggested that the harmful effect of tobacco in CD might be neutralized by the use of immunomodulators^[10], this does not seem to be the case in postoperative recurrence where two different studies identified both azathioprine use and active smoking as independent predictors for both endoscopic and surgical recurrence^[26,29].

Role of smoking on the clinical course of UC

There is strong evidence pointing to a protective effect of tobacco on the susceptibility to develop UC^[2]. Early studies, performed before the widespread use of calcineurinic drugs and infliximab, suggested higher rates of flares, hospitalizations, and even colectomy for UC among non-smokers^[30-33], but these findings were not confirmed in most recent studies^[34,35]. Conflicting results have been obtained about the effect of tobacco on retrograde progression of distal forms of UC^[31,36-38].

Some authors reported a worsening in clinical out-

comes among UC patients who quit smoking^[30,39], while improvement of disease activity has been noticed in ex-smokers who returned to smoke^[40,41]. A number of trials show the efficacy of nicotine for inducing remission in active UC, although with a high rate of mild side effects^[42]. In fact, some authors still propose “mild smoking” as an alternative therapy in patients with resistant UC^[43].

DIET

Along with microbiota, dietary products are the most common luminal antigens in the bowel and may influence intestinal inflammation. Possible mechanisms include a direct antigenic effect, alteration of gene expression, modulation of inflammatory mediators (e.g., eicosanoids), changes in the composition of the enteric flora, and effects on gut permeability. Thus, the role of dietary habits on the development of IBD has been extensively investigated in case-control retrospective studies subject to different biases^[44,45]. In a recent systematic review of these studies, high intakes of total fat, omega-6 fatty acids and meat were associated with increased risk of developing IBD, while high vegetable and fruit intake decreased the risk for these diseases^[45]. A recent case-control study suggests that increased intake of refined sugars may facilitate the development of CD and UC^[46]. Prospective studies are necessary to confirm the role of dietary factors on the development of IBD.

Role of diet on the clinical course of IBD

For decades, physicians based dietary counseling for IBD patients on restrictive criteria. This was because the so-called “bowel rest” was considered as a sine qua non to induce disease remission. However, controlled trials clearly demonstrated that drug-induced IBD remission was not influenced by the type of nutritional support (i.e., enteral, parenteral or oral conventional foods)^[47-49]. Thus, the concept of “bowel rest” has been abandoned, and IBD patients are now advised to eat a diet as unrestricted as possible.

Food intolerance: Does it have a role in dietary management of IBD? IBD patients often complain of food intolerance. In a prospective study, 65% out of 130 patients who completed a food questionnaire reported to be intolerant to some food item, as compared to only 14% out of 70 healthy controls ($P < 0.0001$)^[50]. A more recent study in 187 UC patients confirms these findings: 50% of patients avoided some foodstuff (mainly dairy foods, fruits and vegetables)^[51]. However, 22% of patients ate supplemental amounts of these food items because they had the perception that these improved their symptoms^[51].

Despite its high prevalence, food intolerance is quite inconsistent in IBD patients. Pearson *et al.*^[52] sequentially introduced single conventional foods in 28 CD patients who had gone into remission with an elemental diet.

Twenty patients reported intolerance to some of these foods, but seven of them were tolerant to it after a rechallenge. Of interest, one patient who was also intolerant to this rechallenge, could tolerate the “offending” food after a second blinded rechallenge, and someone even had opposite responses to two blinded rechallenges with the same food item^[52].

These data well illustrate how difficult it is to prove food intolerances in IBD patients. From this perspective, avoiding every food that causes patient’s upset is an unwise strategy. In a large series of patients with inactive UC, dietary changes based on the patient’s self-perceptions did not have any influence on the relapse rate^[51]. Therefore, bearing in mind the fact that protein-energy malnutrition and other nutritional deficiencies are frequent in IBD, patients with UC or CD should be advised to avoid only those food items which repeatedly and systematically worsen their symptoms. In this setting, two groups of foods often raise concerns both among patients and doctors: dairy foods and dietary fiber.

None of the milk components has been proven to play a role in promoting bowel inflammation, causing the disease or triggering a flare. In contrast, it is well known that dairy foods are the main dietary source of calcium, which is necessary to prevent metabolic bone disease in these patients. However, it is also true that a significant proportion of healthy people (mainly in the Mediterranean basin) have lactase deficiency. Unabsorbed lactose reaching the colon may cause diarrhea and/or bloating in a dose-dependent manner. This phenomenon, which does not depend on the fact of suffering from IBD, may occur in lactase-deficient patients with these diseases, thus worsening their symptoms. Studies performed in our laboratory suggest that the prevalence of lactose malabsorption (as assessed by hydrogen breath test) is not higher in IBD patients than in healthy controls^[53]. Therefore, IBD patients should not limit their milk intake during flares unless it clearly worsens diarrhea. Moreover, even in these cases, dairy foods with lower lactose contents (i.e., yogurt) may be well tolerated.

Prescribing a low-residue diet - that is, devoid of insoluble fiber - may be advisable during acute flares of IBD, particularly in patients with stricturing CD or severe UC attacks. Soluble fiber generates much less residue than insoluble fiber, and is fermented by colonic microflora yielding several products such as short-chain fatty acids (SCFA) - mainly butyrate - than can be of benefit in IBD. Butyrate is the preferred fuel for colonic epithelial cells. Decreased fecal levels of SCFA have been reported in patients with UC in relation to the severity of inflammation^[54], and impaired beta-oxidation of butyrate could be demonstrated in patients with active and even inactive UC^[55,56]. Experimental work suggests that butyrate is able to down-regulate the production of proinflammatory cytokines, and also nuclear factor kappa B (NF- κ B) activation^[57].

Soluble fiber may be particularly useful in inactive UC. In a randomized controlled trial, *Plantago ovata* husks (a source of slowly fermentable soluble fiber) were as

effective as mesalazine for preventing disease relapse in patients with quiescent UC^[58]. In active UC, however, the use of soluble fiber might be potentially detrimental. The presence of intraluminal blood (and, hence, oxygen), and a lower intraluminal pH during active disease may favor the growth of lactic acid-producing bacteria (*Lactobacilli* and *Streptococci*). Lactic acid directly damages the bowel mucosa. Indeed, increased levels of fecal lactic acid have been reported in patients with active UC^[59].

The usefulness of “exclusion diets” in CD has been supported by several authors due to their potential capacity to prevent clinical relapses and spare steroids. To date, only one prospective randomized controlled trial assessing the role of exclusion diet in preventing relapse in inactive CD has been published^[60]. Seventy-eight patients, who had gone into remission with an elemental diet, were randomized to receive an exclusion diet (i.e., sequential introduction of foods, with exclusion of those that elicited symptoms) or prednisolone (40 mg/d, with tapering dose until discontinuation by week 12) (control group)^[60]. Treatment of a control group is hard to justify, since it is well-known that steroids are not useful as maintenance therapy in CD. Anyway, the two-year cumulative probability of relapse was lower in the group treated with the exclusion diet than in the control group (62% *vs* 79%, *P* = 0.048)^[60]. However, 62% is a high relapse rate, suggesting that exclusion diets benefit only a minority of CD patients.

Food components as primary treatment for CD: In the last three decades, the possibility that enteral nutrition could be used as primary treatment (i.e., able, *per se*, to induce remission) in active CD has been a matter of debate.

To date, four meta-analyses of the trials comparing enteral nutrition *vs* corticosteroids in active CD have been published^[61-64]. All of them agree that steroids are better than enteral nutrition in inducing remission but they also indicate that, as a whole, enteral nutrition is able to induce remission in about 50%-60% of patients, a remission rate substantially higher than that obtained with placebo in active CD, which barely achieves 30%. This suggests that enteral nutrition (or, at least, some enteral formulas) would have a primary therapeutic effect in active CD (or, at least, in some subsets of patients). The primary therapeutic effect of enteral nutrition in CD is particularly relevant for children, as confirmed by two meta-analyses of pediatric trials which conclude that enteral nutrition is as effective as steroids in inducing remission in children^[65,66]. In addition to its role in active CD, enteral nutrition is suggested to be useful for preventing relapse both in children^[67] and adults^[68]. Recent data suggest that it could also have a role in preventing postoperative recurrence^[69].

The mechanisms whereby enteral nutrition exerts its primary therapeutic effect in CD remain obscure. The hypothesis that elemental (i.e., amino acid-based) diets would be particularly useful by virtue of their low antigenicity was challenged by the results of meta-analyses of

randomized trials comparing elemental *vs* non-elemental (i.e., peptide- or whole protein-based) diets, which showed that both types of diets were equally effective in inducing remission^[61,64].

To date, the amount and/or the type of dietary fat are major candidates for the therapeutic effect of enteral nutrition in CD. Recent meta-analysis suggests very low fat (i.e., less than 3 g/1000 kcal) diets could be particularly effective^[64]. Early studies pointed out that olive oil-based diets were better than diets based on seed oils (corn, safflower, sunflower, soybean), suggesting that oleic acid would be better than linoleic acid in reducing inflammation^[70]. Experimental data also support this view^[71,72]. However, this hypothesis could not be confirmed in a trial comparing linoleic acid- and oleic acid-based diets, where the latter performed particularly badly^[73]. As the oleic acid source in this trial was not olive oil but synthetic triolein, it cannot be ruled out that other components of olive oil (e.g., antioxidants) could exert anti-inflammatory actions in these patients.

Although coconut oil-derived medium-chain triglycerides (MCT) are traditionally considered as a mere easy-to-oxidize energy source, recent data support the idea that they can also exhibit immunomodulatory properties. In fact, there is growing experimental evidence that MCT are able to improve bowel damage both in spontaneous and induced animal models of intestinal inflammation^[74-77]. There are also some clinical data suggesting that replacing part of dietary fat with MCT would contribute to the primary therapeutic effect of enteral nutrition in CD^[78-80].

Surprisingly, fish oil-derived omega-3 fatty acids - the paradigm of anti-inflammatory lipids - have been scarcely assessed in the setting of enteral nutrition formulas for CD. Several randomized trials have been published, however, on the role of fish oil supplements as therapy for both active and inactive CD and UC, which have been systematically reviewed^[81-83]. Overall, available data do not allow supporting the use of omega-3 fatty acid supplementation for the treatment of both active and inactive IBD. Negative results are quite consistent in trials assessing the use of omega-3 fatty acids to maintain disease remission, particularly UC, and to a lesser extent CD. Trials on their use in active disease do not allow us to draw firm conclusions, mainly because of the heterogeneity of their design (UC) or their small number (CD). In most trials, the appropriateness of the selected placebo is questionable^[83].

NSAIDs

Since it is known that NSAIDs can induce gastrointestinal mucosal inflammation, it has been suggested that they might trigger disease exacerbation in IBD patients. Several potential mechanisms for this phenomenon have been proposed such as cyclooxygenase (COX) inhibition, leukotriene shunting or inhibition of NF- κ B activity, although none of them has been firmly demonstrated^[84].

Most (but not all) retrospective, uncontrolled or cross-sectional studies evaluating the impact of NSAID use on IBD relapse agree on the potential deleterious effect of these drugs on quiescent IBD^[84,85]. In the only prospective controlled study assessing disease relapse with the use of different NSAIDs as compared to acetaminophen in IBD patients without arthritic complaints, a significantly increased risk of relapse with NSAIDs was reported^[86]. Interestingly, patients who tolerated NSAIDs for a week did not seem to be at risk for relapse, suggesting that drug-induced IBD flares occur early after starting NSAID use and only in a subset of susceptible patients. It is also still debated whether selective COX-2 inhibitors are safer than conventional NSAIDs for patients with IBD. The only prospective, randomized, double-blind, controlled trial performed to date showed no increase in UC flares as compared to placebo^[87], but most authors conclude that further randomized, double-blind trials are needed to address this issue^[84,85].

INTESTINAL INFECTIONS

Intestinal infections by enteropathogens have been associated with both IBD onset and IBD relapses, and stool microbiological studies are usually advised in patients with IBD flares. Several prospective and retrospective studies show that intestinal infections assessed by stool cultures occur in less than 10% of IBD flares, mainly in those patients with extensive colonic involvement^[88-93]. However, the clinical relevance of such infections on IBD course has not been appropriately assessed, and no study has specifically addressed the effect of adding antibiotic therapy in patients with active IBD and a positive stool culture. Baliellas *et al.*^[89] reported that only half of the patients with active IBD and positive stool cultures achieved symptomatic remission after antibiotic therapy alone, despite stool cultures becoming negative in all of them.

Clostridium difficile infection (CDI) has become in recent years a worldwide epidemic phenomenon also affecting IBD patients. In the last two decades, the prevalence of CDI increased two-fold in UC and almost three-fold in CD^[94]. As for enteropathogen intestinal infections, IBD patients with colonic involvement seem to be those at higher risk for CDI^[95-97]. In addition to the risk factors for CDI in the general population, increasing age and steroid use seem to be particularly relevant in IBD patients, with no conclusive data about the role of other immunosuppressants^[98]. Several studies have reported that CDI worsens IBD outcome, with higher rates of surgical procedures, longer hospital stay, and higher mortality, as compared to patients admitted to the hospital with IBD or CDI alone^[94,95,97,99].

Finally, many studies have been published addressing the role of intestinal cytomegalovirus (CMV) infection in IBD, mainly in UC^[100]. Two prospective studies demonstrated that this infection was a reactivation of CMV carriers that occurs almost exclusively in active, steroid-

refractory UC patients^[101,102]. However, the small number of patients included in both studies does not allow ascertaining whether CMV reactivation is the cause of refractoriness or the consequence of steroid use, and also whether it worsens UC outcome or if it is only an innocent bystander.

OTHER ENVIRONMENTAL FACTORS

Breastfeeding

Breastfeeding is a protective factor against several immunologically-based diseases. In fact, breast milk is relevant for acquiring tolerance against bacterial microflora and dietary antigens. Most studies investigating the role of breastfeeding on the development of either UC or CD have shown a protective effect, as concluded in a meta-analysis of 14 case-control studies published in 2004^[103]. Subsequent studies confirmed these results (especially for those infants breastfed for more than six months)^[46], while others suggest that breastfeeding could promote CD in childhood, rather than protecting from its development^[104].

Obesity

IBD, particularly CD, has been traditionally associated with protein-energy malnutrition and other nutritional deficiencies. However, in recent years the prevalence of obesity among IBD patients has been increasing^[105] in parallel with the obesity epidemics in the general population of developed countries. Case-control studies suggest that obese CD patients are more prone to perineal disease^[106] and early surgical treatment^[107].

Vaccinations

The role of vaccinations - mainly attenuated measles-containing vaccines - in the development of IBD is a matter of debate, with studies reporting a positive^[108], negative^[109] or no association^[110,111] with IBD. A recent case-control study reported that vaccinations against pertussis and polio increase the odds of suffering IBD^[46]. The exact role, if any, of vaccinations with regard to IBD is far from being elucidated.

Oral contraceptive pills

A meta-analysis conducted in 2008 showed that the use of oral contraceptives was associated with an increased risk of both CD and UC^[112]. The risk increased with the time of exposure and reversed after pill discontinuation^[112]. The effect of oral contraceptives on the risk of IBD appears to be related to estrogens. Estrogen acts as an immune enhancer and may increase the production of tumor necrosis factor by macrophages. Also, estrogen may induce microthrombotic phenomena in the bowel due to its thrombogenic potential.

Appendectomy

Appendectomy is associated with a lower risk of suffering from UC, particularly in children who are operated

before the age of 10, as shown in a meta-analysis of 17 case-control studies^[113]. Investigations on the relationship between appendectomy and CD are less conclusive, in spite of the fact that a recent meta-analysis showed an increased risk of CD in appendectomized subjects^[114]. However, this association was particularly strong for those appendectomies performed within one year before CD diagnosis, and almost null for those performed five years before CD^[114], suggesting that this is a circumstantial rather than causative relationship.

Psychological stress

Psychological stress has been hypothesized to play a role both in the pathogenesis of IBD and as a triggering factor for disease relapse as well. However, retrospective observational studies have yielded conflicting results^[115,116].

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Impact of medical therapies on inflammatory bowel disease complication rate

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Abstract

Crohn's disease and ulcerative colitis are progressive diseases associated with a high risk of complications over time including strictures, fistulae, perianal complications, surgery, and colorectal cancer. Changing the natural history and avoiding evolution to a disabling disease should be the main goal of treatment. In recent studies, mucosal healing has been associated with longer-term remission and fewer complications. Conventional therapies with immunosuppressive drugs are able to induce mucosal healing in a minority of cases but their impact on disease progression appears modest. Higher rates of mucosal healing can be achieved with anti-tumor necrosis factor therapies that reduce the risk of relapse, surgery and hospitalization, and are associated with perianal fistulae closure. These drugs might be able to change the natural history of the disease mainly when introduced early in the course of the disease. Treatment strategy in inflammatory bowel diseases should thus be tailored according to the risk that each patient could develop disabling disease.

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Key words: Crohn's disease; Ulcerative colitis; Inflammatory bowel diseases; Therapy; Surgery; Complications

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INTRODUCTION

The natural history of Crohn's disease (CD) is the progression to chronic complications including strictures, penetrating fistulae^[1,2] or complex perianal disease^[3], leading to the need for surgery and hospitalization^[4]. This leads to the concept of cumulative tissue damage for which a quantitative score is currently under development^[5]. Coloproctectomy due to chronic refractory disease or acute severe colitis is a major complication of ulcerative colitis (UC) and develops in 20%-25% of patients after 25 years. An increased risk of colorectal cancer (CRC) in long-standing colitis is a second major complication in UC^[6], but also in Crohn's colitis, with a relative risk of 2.5 compared to the general population. The aim of medical therapies was the improvement of inflammatory bowel disease (IBD) symptoms 20 years ago, however, the current objective is to achieve deep remission, including cessation of corticosteroids, and mucosal healing. Therefore, treatments should modify the course of the disease by avoiding disabling disease and irreversible tissue damage. This review focuses on the impact of treatment on the natural history of IBD.

MUCOSAL HEALING

Mucosal healing (MH) has become a major goal of treatment of IBD because it has been correlated with fewer complications^[7,8], fewer relapses after surgery^[9], and drug

withdrawal^[10]. There is no validated definition of MH in IBD. Mucosal healing is usually assessed by endoscopy in CD and UC and defined as the absence of ulcers^[10]. Although poorly studied, MH is achievable with thiopurine analogs in active CD. In earlier uncontrolled studies, among azathioprine (AZA) clinical responders, 74% achieved MH after a mean of 2 years^[11,12]. However, in a more recent controlled trial (SONIC) studying infliximab (IFX), AZA, or combination therapy for immunosuppressive-naïve CD patients^[13], only 16% of CD patients in the AZA arm achieved mucosal healing at week 26. Few data are available about the efficacy of methotrexate (MTX) in inducing MH. A preliminary study^[14] of 11 CD patients treated with MTX 25 mg weekly intramuscular injection showed MH and histological healing in five and four patients, respectively, after 12 wk. No MH was observed in UC, although 5/7 patients had a clinical response with histological improvement. A recent prospective study showed MH in only 11% of CD patients in clinical remission on MTX, compared to 50% on AZA and 60% on IFX^[15]. A possible bias in this study may have been the small size of the groups, the more refractory disease, and the shorter treatment duration in the MTX group. Anti-tumor necrosis factor (TNF) treatments have changed the management of IBD since the late 1990s. A subanalysis of a Crohn's disease clinical study evaluating infliximab in a new long-term treatment regimen (ACCENT1) trial demonstrated that MH on IFX was associated with fewer relapses^[16]. A retrospective single center study has shown that, among IFX responders, 68% had MH (45% complete MH) and MH was associated with fewer relapses (64% *vs* 40%)^[17]. In the step-up top-down study, 71% of patients with MH at 2 years were still in remission 2 years later, compared to patients who had endoscopic signs of activity^[18]. At week 26 of the SONIC trial, IFX was more effective to induce MH than AZA, (16.5%), either in mono- (30.1%) or combination therapy with AZA (39.5%). In the prospective ACT1/ACT2 trials studying IFX for induction and maintenance therapy in UC, IFX efficacy in inducing MH was also demonstrated with 62%/60% of MH at week 8 compared to 32%/30% in the placebo group. Adalimumab (ADA) was also more effective than placebo in inducing and maintaining clinical remission in patients with moderate-to-severe UC^[19], and MH was achieved more frequently in the ADA arm compared to placebo (25% *vs* 15% at week 52).

In CD, mucosal healing has also been consistently described as more frequently achieved when an anti-TNF was started earlier in the disease course^[20].

SURGERY AND HOSPITALIZATION

CD is a chronic condition that leads to tissue damage and complications requiring surgery in 70%-80% of patients at 20 years^[4,8]. In UC, the cumulative probability of colectomy after 25 years varies from 20% to 30%^[21,22]. In 2005, Cosnes *et al*^[23] demonstrated that immunosuppressive

drugs (AZA and MTX) were introduced more frequently and earlier in the course of the CD over the past 25 years but the percentage of patients requiring intestinal surgery each year remained stable. These results should be interpreted with caution because < 10% of the patients included in this study received AZA before surgery. Recent contradictory data have demonstrated that increased immunosuppressant prescriptions, from 11% to 45% over 25 years, have decreased the rate of intestinal resection from 59% to 25% 5 years after diagnosis^[24]. Early introduction of thiopurine was a protective factor. French results recently have demonstrated that AZA is associated with less surgery in patients newly diagnosed with CD but the benefit was modest compared to IFX^[25]. In UC, Ardizzone *et al*^[26] have demonstrated higher rates of clinical response in patients treated with AZA compared to 5-aminosalicylic acid, but the colectomy rate was similar in both groups (8%). The ACCENT 1 and 2 trials have reported a decreased risk of surgery in patients on IFX scheduled therapy at week 54 (3% *vs* 7% with IFX episodic therapy)^[27,28]. Schnitzler *et al*^[17] have demonstrated less intra-abdominal surgery (14%) and hospitalization (42%) for active CD in patients achieving MH on scheduled IFX compared to those who had endoscopically active disease (38% and 59% respectively). Lower colectomy rates in IBD were also associated with MH in a retrospective Norwegian population-based study^[29]. Recently, IFX given for at least 16 mo was reported as a protective factor against surgery in active CD^[25]. Jones *et al*^[30] have reported a stable rate of surgery in CD from 1993 to 2004, but these data should be interpreted with caution because they concern a period when IFX was mainly prescribed as episodic therapy, which is clearly a suboptimal strategy and does not represent the current practice. In the Crohn's Trial of the Fully Human Antibody Adalimumab for Remission Maintenance (CHARM) trial, CD patients treated with scheduled ADA had less hospitalization at 3 mo (1.6%) and 12 mo (5.9%) compared to placebo (7.3% and 13.9% respectively)^[31]. Surgery at 1 year also decreased from 3.8% in the placebo group to 0.6% in the ADA groups. These results were confirmed at 2 years follow-up^[32]. A sub-analysis of the ACT1 and ACT2 trials demonstrated a 10% cumulative incidence of colectomy in UC through 54 wk in the IFX group compared to 17% in the placebo group. Less UC-related hospitalization was reported in the IFX group^[33]. Moreover, the degree of MH after 8 wk IFX was correlated with less colectomy^[34].

PERIANAL COMPLICATIONS OF CD

Perianal fistulae occur in 21%-26% of CD patients after 20 years^[5]. Anti-TNF antibodies have dramatically improved the ability to heal fistula with medical therapy. After 54 wk of scheduled IFX treatment for active CD fistulae, partial response was observed in 46% of patients *vs* 23% in the placebo group and 36% had a complete response compared to 19% with placebo^[28]. After primary drainage, high rates of clinical response (85%)

and remission (74%) at week 14 were also reported in patients with severe active perianal CD treated with three infusions of IFX followed by MTX as maintenance therapy (25 mg weekly intramuscular or subcutaneous). Fifty percent of patients were still in remission from their perianal disease at 1 year, but this strategy failed to achieve a prolonged remission of luminal disease in the majority of patients^[35]. No other prospective studies have investigated the efficacy of MTX in perianal CD. In the CHARM trial that studied the long-term efficacy of ADA in CD, 33% of active fistulae achieved complete healing on ADA after 56 wk compared to 13% in the placebo group^[31]. This effect was globally maintained over 3 years follow-up^[32]. Fistula healing in a substantial proportion of patients under ADA was also confirmed in a large European open label trial mimicking routine practice^[36]. In this trial, full fistula closure was achieved in 26% of patients after 20 wk. The impact of thiopurine on fistula closure was poorly studied. A meta-analysis showed a complete closure or an improvement of the fistulae in 54% of the patients compared to 21% in the placebo group. However, these results should be interpreted with caution because fistula closure was not the primary endpoint of this study^[37].

CANCER

Chronic colitis predisposes to CRC over time, with cumulative estimated incidence rates of 2%, 8% and 18% at 10, 20 and 30 years of evolution, respectively^[6]. This risk was however reported as lower in more recent cohorts^[38], with a relative risk around 2.0 over disease course. The risk of CRC in CD has also been reported^[39]. Risk factors for CRC in chronic colitis are extensive location, long duration of the disease, familial history of CRC, and associated primary sclerosing cholangitis^[40]. Few studies have addressed the severity of colonic inflammation over time as an independent risk factor for progression to neoplasia. Rutter *et al.*^[41,42] have demonstrated a highly significant correlation between colonic inflammation scores and the risk of CRC in UC. Only association with histological inflammation was significant in the multivariable analysis [odds ratio (OR): 4.7]. In the case of normal colonoscopy, the 5-year risk of CRC was the same as that of the matched general population (OR: 0.38). Gupta *et al.*^[43] also have demonstrated that the severity of microscopic inflammation is an independent risk factor for dysplasia in patients with longstanding UC. Such data are not yet available in CD. Due to the ability of medical treatment to maintain tissue healing in IBD, we can speculate on the potential impact of these treatments on the risk of cancer. Mesalazine is effective at maintaining clinical remission in UC and remains the main drug in this disease. In retrospective studies and meta-analyses, a significant decrease in CRC in UC has been described with mesalazine. More intriguingly, this has also been suggested for ileal cancer in CD^[44]. More recently, in the Cesame cohort, a potential decrease in

CRC was also suggested in extensive longstanding UC treated with purine analogs^[45]. No data are available yet with anti-TNF agents.

CONCLUSION

In conclusion, anti-TNFs and to a lesser extent immunosuppressants, can induce MH, which is associated with long-term clinical remission, closure of perianal fistulae, less hospitalization and surgery, suggesting an impact of these medications on the natural history of the disease. The benefit might be higher if MH is achieved earlier in the course of the disease. Histological remission is also associated with a reduced risk of CRC in UC. Although immunosuppressive treatments with thiopurine or MTX are able to induce MH, their benefit on the complications of IBD appears more modest. Many questions remain open, including the degree of MH achievement (complete *vs* partial) required to improve the prognosis, when and how often in the course of the disease should this healing be assessed, and how to adapt the treatment according to MH. Prospective randomized clinical trials are ongoing to answer these questions. Furthermore, the validation and the further use of a tissue damage score in CD (Lemann score) will be an important step to assess adequately the ability of treatment strategies to change natural history.

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Surgery for Crohn's disease in the era of biologicals: A reduced need or delayed verdict?

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Abstract

Crohn's disease (CD) is a chronic inflammatory bowel disease that can affect the entire gastrointestinal tract. Ultimately, up to 70% of all patients will need surgery, despite optimized medical therapy. Moreover, about half of the patients will need redo-surgery because of disease recurrence. The introduction of anti-tumor necrosis factor (TNF) drugs (Infliximab in 1998) revolutionized the treatment of CD. Different randomized trials assessed the efficacy of anti-TNF treatment not only to induce, but also to maintain, steroid-free remission. Furthermore, these agents can rapidly lead to mucosal healing. This aspect is important, as it is a major predictor for long-term disease control. Subgroup analyses of responding patients seemed to suggest a reduction in the need for surgery at median-term follow up (1-3 years). However if one looks at population surveys, one does not observe any decline in the need for surgery since the introduction of Infliximab in 1998. The short follow-up term and the exclusion of patients with imminent surgical need in the randomized trials could bias the results. Only 60% of patients respond to induction of anti-TNF therapy, moreover, some patients will actually develop resistance to biologicals.

Many patients are diagnosed when stenosing disease has already occurred, obviating the need for biological therapy. In a further attempt to change the actual course of the disease, top down strategies have been progressively implemented. Whether this will indeed obviate surgery for a substantial group of patients remains unclear. For the time being, surgery will still play a pivotal role in the treatment of CD.

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Key words: Crohn's disease; Surgery; Biological agents; Anti-tumor necrosis factor drugs; Remission

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INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder which can affect the complete gastrointestinal tract. Only a minority of patients (10%-15%) will experience a prolonged relapse-free interval after initial diagnosis; most patients develop a mild chronic disease pattern^[1]. This relapsing inflammation results in progressive bowel occlusion and/or fistula and abscess formation. A large majority of patients (70%-80%) will require surgical treatment within a time frame of 10 years^[2,3]. The type of surgery is dictated by the anatomic location and/or the related complication(s). Depending on the localization of the disease, CD tends to have a different clinical

phenotype. Indeed, ileocolonic and small bowel involvement is more prone to develop occlusive disease than colonic affection^[2,4]. Thus, small bowel or ileocolic distribution will increase the rate of surgery compared to Crohn's colitis. Intractable inflammation is a rather seldom indication for surgery. Penetrating anal disease often leads to surgery in order to control sepsis and drain fistulas. Unfortunately, surgery in CD is not curative and the majority of patients will have early endoscopic relapse, despite clinical remission^[5]. Over time, symptomatic recurrence demands medical treatment, and up to 40% of patients will eventually need secondary surgery^[2]. This explains the tendency to avoid 'too early' surgery. If surgery is needed, the focus should be on bowel sparing and minimally invasive surgical techniques.

Progressive understanding of the pathogenesis of CD resulted in significant changes and improvements in its medical treatment. The use of immunomodulators (such as azathioprine and methotrexate) has not decreased the need for surgery, nor has it decreased hospitalization rates either^[2,6]. The introduction of anti-tumor necrosis factor (TNF) treatment in 1998 revolutionized the treatment paradigms. TNF antagonists proved to induce a rapid clinical remission in about 60% of the cases^[7,8]. In randomized controlled trials, anti-TNF therapy seemed to maintain remission in contrast to steroid regimens^[9-12]. Moreover, mucosal healing has even been obtained in a subset of patients, which could support a sustained clinical remission^[13-15]. Therefore, one could expect that, in the long run, fewer patients would need to undergo major abdominal surgery. This paper reflects on some aspects of the impact of anti-TNF treatment on the rates of surgery in CD patients.

NEED FOR SURGERY IN THE MARGIN OF LARGE RANDOMIZED TRIALS

Several randomized controlled trials have analyzed the maintenance of clinical remission in CD comparing patients who received anti-TNF agents or placebo^[9-12]. Besides an initial response rate of about 60%, a majority of patients will show sustained remission with anti-TNF therapy. Steroid discontinuation was also significantly better in the treatment groups. Moreover, an endoscopic substudy of a Crohn's disease clinical study evaluating infliximab in a new long-term treatment regimen demonstrated that about 50% of patients with a clinical response will also have mucosal healing^[14]. Considering that control of inflammation and induction of mucosal healing is predictive for long-term disease activity and bowel preservation, one could expect an effect of anti-TNF treatment on the rate of surgery^[13]. Feagan *et al*^[16] evaluated the influence of maintenance adalimumab therapy on the rate of hospital admissions and surgery in a post-hoc analysis of the Crohn's Trial of the Fully Human Antibody Adalimumab for Remission Maintenance trial. The authors came to the conclusion that adalimumab maintenance therapy significantly reduced hospitaliza-

tions and surgery for CD amongst the enrolled patients. Mucosal healing seems a promising surrogate marker of deep and prolonged clinical remission. This alteration in disease course should lead to a reduced need for surgery. More predictors are needed, not only to select those patients who will develop an aggressive and complicated disease pattern to enable early installment of immunosuppressive therapy, but also to select patients for "early" surgery to obtain a prolonged clinical remission.

One year after primary surgery, as many as 72% of the patients had already developed endoscopic recurrence, mainly at the anastomosis. Clinical manifestations of the disease are, however, often absent in this early postoperative stage^[17]. About one half of patients will need redo-surgery over a 20-year period^[2]. Although a high recurrence-rate is observed after surgery, there is no consensus about the postoperative therapy regimen. Considering the high amount of endoscopic recurrences, one could wonder if prophylactic medical therapy after surgery can play a role. Studies have been conducted to find the best prophylactic regimen. Aminosalicylates regimens seem to have modest effects on the postoperative recurrence rate. It is therefore not recommended to use them in a postoperative setting^[18,19]. Nitromidazole and ornidazole have demonstrated a significant drop in recurrence, but at the expense of important side effects, making these therapies not longer suitable for prophylactic use. Budenoside has no long term effect, but is indicated to suppress acute relapse. Azathioprine and 6-Mercaptopurine have already been used in randomized controlled trials to assess the effect on rate of recurrence after surgery. Because there was a high drop-out rate during the follow-up period, no convincing results have been found in these series^[20,21]. The advent of anti-TNF agents and their demonstrated effect on mucosal healing in the preoperative setting has given hope to care providers that relapses can be avoided when administered postoperatively. Regarding this important clinical question, two randomized controlled trials have been published so far. Twenty-four patients were randomized after ileocaecal resection to receive infliximab or a placebo for one year^[22]. The endoscopic and histologic recurrence rate after one year was significantly lower in the infliximab group. There was no significant difference in clinical recurrence rate, though more patients showed relapse after one year in the placebo group. Another study randomized 26 postoperative patients with proven endoscopic recurrence six months after receiving mesalamine in three different groups: one received infliximab, another azathioprine and the last group continued mesalamine^[23]. Control of endoscopic inflammation was improved in the infliximab group compared to the azathioprine and mesalamine group, demonstrating the clear suppressive effect of infliximab. In these two small trials, the positive impact of infliximab in avoiding postoperative recurrence has been demonstrated. These conclusions have to be interpreted with the greatest caution considering the small sample sizes and the short follow-up periods. No conclusion can be made about the usefulness of infliximab to

prevent recurrences. Large prospective randomized trials with a long follow-up have to be designed to assess the benefit of anti-TNF agents on postoperative recurrence. Moreover, one could wonder if it is reasonable to give prophylactic treatment after resection, considering the high costs and the number of patients who will be treated that would not develop recurrence. It is more likely to stratify the risk factors of every patient to assess the need of postoperative medical treatment. One of the most powerful methods for assessing patients is performing a colonoscopy six to twelve mo after resection. Rutgeerts *et al*^[51] demonstrated the predictive value of endoscopic recurrence. Indeed, patients with severe endoscopic recurrence within one year after surgery are at greater risk of developing clinical recurrence.

Approximately one third of all CD patients will develop perianal disease, including skin tags, ulcers, low and high fistulas, rectovaginal fistulas, perianal abscesses, anorectal strictures and cancer^[24]. Complex perianal fistulas are challenging to treat and can lead to destruction of the anal sphincters with intractable incontinence as a result. Twenty-five percent of patients with anal CD will eventually need a proctectomy^[25]. The classical medication used for CD, like antibiotics and immunomodulators, have not demonstrated any beneficial effect in the treatment of fistulizing CD^[26-28]. In contrast, infliximab maintenance therapy seems to reach superior durable and complete fistula closure, even in patients not responding to other medical treatments^[29,30]. This seems to have an impact on the surgery rate and hospital stay^[31]. There is, however, some concern about treatment with infliximab inducing healing of the external opening and suppressing the inflammatory reaction around the fistula tract without eradicating the tract^[32]. Magnetic resonance imaging of patients in clinical remission after infliximab treatment still showed inflammation and subsisting fistula tracts. Some are concerned about the possibility of fistula recurrence after withdrawal of treatment. More extensive investigation will be needed to test this hypothesis.

NEED FOR A WIDE SURGERY POPULATION (THE REAL WORLD)

In view of the aforementioned randomized controlled trials, it may be possible to change the course of the disease in patients treated with biologicals, perhaps leading to a decreasing need for resectional surgery. Other large population based series, however, are less convincing. Lazarev *et al*^[33] showed that, despite the increasing use of infliximab, the rate of small bowel resection has remained unchanged over the years in a large referral centre in Pittsburgh. Moreover, the relative frequency of stricturizing and penetrating disease did not change over time. Bewtra *et al*^[34] analyzed hospitalization and surgery trends for inflammatory bowel disease from 1990 to 2003. They observed a steady rate in the number of surgical interventions for CD with a significant increase in hospitalization rate, despite the introduction of inflix-

imab in 1998. Jones *et al*^[35] concluded in their series that surgery for penetrating small bowel disease increased with 60% from 1993 to 2004 despite the increasing use of infliximab.

In contrast, two population-based series reported a significant decrease in hospitalization and surgery rate^[36,37]. In a series from Wales, stoma formation and the long-term need for steroids are likely to have been influenced by the use of infliximab, but only 16% of patients had been prescribed anti-TNF agents in this series. Moreover, 614 consecutive patients responding to induction therapy with infliximab were observed to evaluate the long term clinical benefit of this anti-TNF agent^[38]. Two thirds of these patients seemed to have a sustained benefit of this therapy regimen. There seemed to be a decreased rate of surgery for patients responding to medication. Loss of response was inadvertently associated with an increased risk of surgery. This study demonstrated that infliximab could have an impact on disease course in responding patients. However, this group was not compared to patients not receiving anti-TNF agents. More recently it has been shown that the use of infliximab, and to a lesser extent of azathioprine, seems to be associated with a decreased risk of surgery^[39]. Interestingly, in this retrospective cohort study including 296 patients with CD between 2000 and 2008, the median follow-up was 57 mo, which was much longer than in the randomized controlled trials.

DISCUSSION

The introduction of anti-TNF agents significantly changed clinical practice and treatment algorithms. Optimized medical treatment should not only achieve symptomatic relief and clinical remission, but also aim to reduce the need for hospitalization and surgery. The use of these agents evolved over time and is mainly based on evidence from different large clinical trials on the efficacy for induction and maintenance treatment. The achievement of mucosal healing in a subgroup of patients raised the expectation that anti-TNF treatment could lead to a decrease in disease-related complications and the ultimate need for surgery. Biological therapies have shown to alter the natural history of psoriatic arthritis and ankylosing spondylitis^[40]. Indeed, anti-TNF therapy has been shown to reduce the need for surgery in different randomized clinical trials. Medium follow-ups of those trials have been rather limited, and the question remains whether the natural history of CD can be changed. Population-wide studies during the anti-TNF era have not yet demonstrated a decrease in the need for hospitalization and surgery. Different reasons can explain this discrepancy: firstly, the selection criterion for the randomized trials excluded patients with an imminent need for surgery and therefore contains a selection bias. Secondly, not all patients respond to anti-TNF therapy and the presence of a stricture and/or penetrating disease at the time of diagnosis is highly predictive for the need of surgery and

conservative treatment failure. Thirdly, most experience has been gained with the use of infliximab. The immunogenicity of the drug will lead to a substantial loss of response over time^[41,42]. Finally, a genuine resistance (irrespective to immunogenicity) to anti-TNF drugs has been observed. This will lead to a drop-out of 10% of patients per year. Results of recent top-down strategies clearly demonstrate the beneficial effect of early “aggressive” treatment of luminal inflammation^[43]. The medium-term benefits with regard to clinical remission and the need for surgery seem to indicate that a disease modification can indeed be obtained in a subset of patients. This concept therefore needs to be further explored and implemented into clinical practice.

The face of surgery has also evolved over time. Today, most patients can benefit from a minimally invasive approach (laparoscopy and single site laparoscopy). Furthermore, isoperistaltic stricturoplasty has demonstrated its safety and long-term efficacy in the treatment of long strictures of the small bowel and reduces the ultimate risk for intestinal failure^[44]. The implementation of enhanced recovery protocols further expedites patient rehabilitation after surgery. These aspects open a more attractive alternative to protracted medical treatment. Surgical-recurrence free survival at 5 years after primary ileocaecal resection is as high as 91% (own unpublished data). However early endoscopic and symptomatic disease recurrence hampers the enthusiasm for an early surgical approach. In patients with anorectal CD, surgery remains an essential, and often first, step in the treatment algorithm. Anal examination under anesthesia and drainage of perianal abscess precedes maintenance medical treatment. This combined approach is essential to safeguard anorectal function in the maximum number of patients, and to avoid definitive proctocolectomy and stoma formation.

The introduction of anti-TNF agents in the 1990s changed treatment algorithms in CD and has the potential to alter the natural history of the disease. Randomized data show a significant decrease in the development of complications and the need for surgery. Sustained mucosal healing seems a good predictor for fewer complications and surgery in the long-term. No reduction in the need for surgery has been documented in population-based surveys. This discrepancy is multifactorial. Further evolution and implementation of top-down treatment strategies should eventually lead to a genuine reduction in the need for surgery. For the time being, surgery still plays a pivotal role in a large subset of patients in order to obtain long-term disease remission and improvement of patient quality of life. However, the evolving concept of disease modification will certainly alter the role and need for surgery in the future. Optimal treatment of CD remains a joint effort of dedicated physicians and surgeons.

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Role of surgery in severe ulcerative colitis in the era of medical rescue therapy

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Abstract

Despite the growing use of medical salvage therapy, colectomy has remained a cornerstone in managing acute severe ulcerative colitis (ASC) both in children and in adults. Colectomy should be regarded as a life saving procedure in ASC, and must be seriously considered in any steroid-refractory patient. However, colectomy is not a cure for the disease but rather the substitution of a large problem with smaller problems, including fecal incontinence, pouchitis, irritable pouch syndrome, cuffitis, anastomotic ulcer and stenosis, missed or de-novo Crohn's disease and, in young females, reduced fecundity. This notion has led to the widespread practice of offering medical salvage therapy before colectomy in most patients without surgical abdomen or toxic megacolon. Medical salvage therapies which have proved effective in the clinical trial setting include cyclosporine, tacrolimus and infliximab, which seem equally effective in the short term. Validated predictive rules can identify a subset of patients who will eventually fail corticosteroid therapy after only 3-5 d of steroid therapy with an accuracy of 85%-95%. This accuracy is sufficiently high for initiating

medical therapy, but usually not colectomy, early in the admission without delaying colectomy if required. This approach has reduced the colectomy rate in ASC from 30%-70% in the past to 10%-20% nowadays, and the mortality rate from over 70% in the 1930s to about 1%. In general, restorative proctocolectomy (ileoanal pouch or ileal pouch-anal anastomosis), especially the J-pouch, is preferred over straight pull-through (ileo-anal) or ileo-rectal anastomosis, which may still be considered in young females concerned about infertility. Colectomy in the acute severe colitis setting, is usually performed in three steps due to the severity of the inflammation, concurrent steroid treatment and the generally reduced clinical condition. The first surgical step involves colectomy and constructing an ileal stoma, the second - constructing the pouch and the third - closing the stoma. This review focuses on the role of surgical treatment in ulcerative colitis in the era of medical rescue therapy.

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Key words: Acute severe ulcerative colitis; Colectomy; Corticosteroids; Cyclosporine; Infliximab; Tacrolimus

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INTRODUCTION

Acute severe colitis (ASC) is one of the few emergencies in gastroenterology. The mortality rate from ASC dropped from over 70% in 1933 to 20%-25% in the 1950s when the importance of timely urgent colectomy was first recognized^[1,2]. Subsequently, the mortality rate was further reduced to 7% with the introduction of corticosteroids, and eventually to about 1% nowadays^[3-5].

Truelove *et al*^[3], in their landmark clinical trial in 1955, defined severe disease as the passage of at least six daily bloody stools, erythrocyte sedimentation rate > 30, temperature > 37.8 °C, pulse rate > 90/min and hemoglobin < 10.5 g/dL. These criteria remain the most common classification of ASC in adults. In a systematic review of cohort studies on ASC, 20 of 29 studies used the Truelove and Witts classification^[5]. However, of these 20 studies, 12 required the fulfillment of all of the five items, and 8 applied various more liberal modifications. More recently, the European Crohn's and Colitis Organization (ECCO) issued guidelines on managing ASC defining severe attack as ≥ 6 bloody diarrhea per day with at least *one* of the other four bullets^[6]. In children, severe disease has been robustly defined using the Pediatric Ulcerative Colitis Activity Index (PUCAI) with a score of at least 65 points yielding high sensitivity and specificity^[7,8]. Pediatric onset ulcerative colitis (UC) is often more extensive than in adults^[9], and since disease severity has been consistently associated with disease extent, children are especially susceptible to refractory severe attacks. Nearly one third of children with ulcerative colitis will experience at least one severe exacerbation before turning into adult care^[10].

PREDICTING THE NEED FOR SALVAGE THERAPY

The only factor associated with a major surgical complication among 80 adults who underwent urgent colectomy in Oxford for ASC, was longer duration of medical therapy before colectomy^[11]. It is of great importance to recognize those who are likely to fail intravenous corticosteroid early during the admission and to introduce timely rescue therapy. This approach may reduce morbidity and mortality and avoid futile toxic medical therapy.

Several clinical predictive indices have been proven to perform well in identifying those who require salvage therapy after only 3-5 d of admission. In a prospective study, Travis *et al*^[12] from Oxford suggested that a stool frequency of > 8/d or 3-8/d and C-reactive protein (CRP) > 45 mg/dL on the third day of corticosteroid therapy should be sufficient for initiating rescue therapy. Lindgren *et al*^[13] developed the fulminant colitis index ($n = 97$) based on the same variables as the Oxford index - stool frequency/d + 0.14 \times CRP mg/L. Ho *et al*^[14] based the Scottish index on stool frequency, presence of colonic dilatation, and hypoalbuminemia. Others have also shown some predictive ability of their indices^[15,16].

In children, a predictive rule based on the PUCAI score at days three and five of steroid therapy has been validated^[10,17] and incorporated in the combined ECCO and European Society for Paediatric Gastroenterology Hepatology and Nutrition recent guidelines on pediatric ASC^[8]. A PUCAI score of > 45 points on day 3 should dictate planning of second-line therapy and > 65-70 points on day 5 should prompt execution of the planned therapy. All aforementioned indices are based on the consistently reproduced fact that the likelihood for responding to medical corticosteroids is inversely associated with the degree of disease severity even before treatment has been initiated.

Despite the significant improvement in patient care with the implementation of indices-based management schemes, their accuracy is still imperfect. The positive predictive value (PPV) of the Oxford index in predicting steroid failure is 85% for colectomy and the sensitivity and specificity of the Scottish index is 85% and 75%, respectively. The PPV of the fulminant colitis index at a cutoff score of > 8 at day 3 is 69%-72%^[13,18]. The PUCAI successfully identifies those for whom the likelihood of failing corticosteroids is 92%. These indices should be perceived as accurate enough for initiating salvage medical therapy (i.e., infliximab or calcineurin inhibitors) but probably not for early irreversible colectomy (Table 1).

SALVAGE MEDICAL THERAPY

Medical rescue therapy should be utilized as the first-line treatment in ASC before colectomy in most corticosteroid-failed patients who do not present with surgical abdomen or toxic megacolon. During the last 20 years, cyclosporine has been widely used^[19] and more recently also infliximab^[18], and tacrolimus^[20]. These medications reduced the short-term colectomy rate from 30%-70%^[3,5,21,22] to approximately 10%-20% nowadays^[17,23]. The increasing use of second-line medical therapy before colectomy has been based on the high effectiveness of these drugs and the notion that colectomy is not a cure for the disease, but rather the substitution of one large problem with several smaller problems.

Experience with cyclosporine showed that, although the short-term response rate reaches 70%-80%^[5,9], approximately 50% of responders will eventually require colectomy when the drug is discontinued, typically after 4 mo^[5,21,24-26]. The likelihood of colectomy is reduced if cyclosporine is used as a bridging medication to thiopurines^[27]. The other calcineurin inhibitor, tacrolimus (FK-506), has recently been proved effective as a second-line regimen in the clinical trial setting, while aiming at high trough levels of 10-15 ng/mL^[20]. It seems that tacrolimus is as effective as cyclosporine for salvage therapy in ASC, both in adults and children^[21,28,29]. Tacrolimus, a more expensive medication, has a better bioavailability than cyclosporine, allowing for oral treatment. The toxicity profile is more appealing but there are fewer published studies to support its use.

Table 1 Prediction rules for corticosteroid failure in patients with acute severe ulcerative colitis

Prediction rule	Study	Measure	Prediction accuracy
Oxford index	Travis <i>et al</i> ^[12] , prospective; Turner <i>et al</i> ^[10] , retrospective	Stool frequency of > 8/d or 3-8/d and CRP > 45 mg/L (on day 3 of IVCS)	Adults: PPV = 85% Children: Sens = 38%, Spec = 100%, PPV = 88%, NPV = 75%
Swedish index (the fulminant colitis index)	Lindgren <i>et al</i> ^[13] , retrospective; Järnerot <i>et al</i> ^[18] , prospective; Turner <i>et al</i> ^[10] , retrospective	CRP mg/L × 0.14 + daily stool frequency (cutoff > 8 on day 3 of IVCS)	Adults: Sens = 78%, Spec = 81%, PPV = 69%-72% Children: Sens = 64%, Spec = 92%, PPV = 88%, NPV = 75%
Seo index	Seo <i>et al</i> ^[15] , retrospective; Turner <i>et al</i> ^[10] , retrospective	60 × bloody stool + 13 × bowel movements + 0.5 × ESR - 4 × Hb - 15 × albumin + 200	Adults (cutoff > 180 on day 7 of IVCS): PPV = 52%, NPV = 97% Children: (cutoff > 240 on day 5 of IVCS): Sens = 27%, Spec = 93%, PPV = 80%, NPV = 56% Adults: Sens = 85%, Spec = 75%
Scottish index	Ho <i>et al</i> ^[14] , prospective	The score (0-9) includes: stool frequency, presence of colonic dilatation, and albumin level (cutoff > 4 on day 3 of IVCS)	Children: PUCAI > 45 on day 3 of IVCS: Sens = 92%-93%, NPV = 88%-94% PUCAI > 70 on day 5: Sens = 35%-44%, Spec = 93%-100%, PPV = 87%-100%, NPV = 63%-79%

NPV: Negative predictive value; PPV: Positive predictive value; Hb: Hemoglobin; ESR: Erythrocyte sedimentation rate; IVCS: Intravenous corticosteroids; Spec: Specificity; Sens: Sensitivity; CRP: C-reactive protein; PUCAI: Pediatric Ulcerative Colitis Activity Index.

Infliximab has been established as an effective regimen for moderate to severe ulcerative colitis, including ASC. The ACT-1 and ACT-2 randomized controlled trials assessed the ability of infliximab to induce and maintain remission in moderate to severe ulcerative colitis^[30,31]. A total of 728 adult patients received placebo or infliximab (5 or 10 mg/kg) through week 46 (ACT-1) or 22 (ACT-2). In the ACT-1 and 2 trials respectively, 61% and 69% of infliximab-treated subjects had a short-term clinical response compared with 29% and 37% of those who received placebo. In steroid-refractory ASC, infliximab is effective as salvage medical therapy in approximately 70%-80% of children and adults, reducing short- and long-term colectomy rate^[17,18,32]. In the Jarnerot trial, the colectomy-free rate was 12/24 (50%) after 3 years^[33]. Combining the data of both ACT trials has shown that the 1-year colectomy rate in the infliximab-treated arm was 10% *vs* 17% in the placebo arm^[34]. It should be emphasized that the ACT trials did not include patients who were refractory to intravenous steroid in the setting of ASC.

In the recent steroid-refractory severe attacks of ulcerative colitis trial, 116 adults with ASC who did not respond to a 5-d course of intravenous steroids were randomized to receive intravenous cyclosporine or infliximab using standard doses and protocols, both combined with azathioprine^[35]. Both the 7-d response rate (85.4% *vs* 85.7%) and treatment failure rate through day 98 (60% *vs* 54%) were similar between the cyclosporine and the infliximab arms, suggesting that the two regimens are equally viable alternatives to colectomy in steroid-refractory ASC. Similar effectiveness has also been suggested in a systematic review of non-randomized studies in children^[21]. In contrast, a nonrandomized study showed that 52% of patients receiving cyclosporine proceeded to colectomy by discharge, *vs* 18% of those administered infliximab^[36].

SURGERY

Although, in general, medical rescue therapy should be regarded as the first-line treatment in steroid-refractory ASC, colectomy is still a cornerstone of the management scheme. Colectomy is indicated in ASC not responding to medical therapy, toxic megacolon, perforation, and uncontrolled colorectal bleeding (rare)^[37,38]. Colectomy in steroid-refractory ASC cases may be considered before medical salvage therapy in chronic active UC previously resistant to thiopurines and infliximab, since no maintenance therapy would be available after discontinuing the calcineurin inhibitor. Surgery is usually the preferred therapeutic option in patients with toxic megacolon, a life threatening event. However, a 24-48 h trial of conservative treatment (i.e., bowel rest, broad spectrum antibiotics and rectal tube) may be cautiously attempted in the non-severe cases in specialized centers only while under intense monitoring. Sequential therapy of calcineurin inhibitors followed by infliximab or vice versa may be successful in approximately 25%-40% of adult patients, but is associated with significant morbidity and even mortality^[39-42]. Therefore, most recommend timely referral for colectomy after failing one medical salvage therapy, rather than attempting another regimen^[6,8]. Expected response to calcineurin inhibitors and infliximab is roughly 1-2 wk and colectomy should not be withheld in non-responders.

Although in the past ileoanal straight anastomosis has been the procedure of choice, now the ileal pouch-anal anastomosis (IPAA) (also known as "restorative proctocolectomy") is the most commonly practiced surgery, also in children. In some centers, ileo-rectal anastomosis is practiced but limited data are available to support this surgery. High early failure rates have been reported with this surgery and a life-long follow-up of the retained rectal stump is required. The ileoanal

pouch procedures are likely superior to the straight pull through (i.e., ileoanal anastomosis) as they are associated with a lower early stool frequency and better long-term continence while maintaining acceptable early complication rates. However, the IPAA procedure is associated with pouchitis in approximately 45%-60%^[43-45], of whom 60% will suffer from recurrent episodes and 5%-10% will develop chronic pouchitis^[45]. The probability of pouch failure has been found to be 9% at 10 years^[43]. Daytime and nighttime incontinence occurred in 7%-10% and 12%-24% of patients, respectively, over a 10-15 year period^[43,44]. The risk for female infertility after IPAA seems to be a major concern with an increase from approximately 10% in the average population to 25%-30%^[8,46]. The role of ileo-rectal anastomosis is controversial, but may be considered in females who are primarily concerned about the reduced fecundity associated with IPAA. The apparent advantages of the IPAA procedure must be seriously balanced against the potential adverse events which should be discussed openly with the families.

IPAA can be performed in one, two or three stages. A two-stage procedure (colectomy with pouch construction and a temporary protecting loop ileostomy to be closed in the second stage) is the most frequent procedure in stable ambulatory patients. The one step procedure (restorative proctocolectomy without protecting ileostomy) may be safe in selected ambulatory patients without any risk factors (e.g., steroids treatment, malabsorption and hypoalbuminemia) in highly trained centers^[8]. A three-stage approach (colectomy with temporary ileostomy in the first stage, pouch construction in the second stage and ultimately ileostomy closure) should be performed in patients with steroid-refractory acute severe colitis, those on high dose steroids and/or suffering from malnutrition, and those in whom Crohn's disease has not been excluded^[8]. With any chosen procedure, a laparoscopic-assisted procedure is feasible and safe^[47,48], also in children^[49,50]. The overall complication rate was higher in open surgery, compared with laparoscopic surgery (55% *vs* 39%, $P = 0.004$)^[48] with longer hospital stay. Patients who had an ileal pouch created through the laparoscopic approach had fewer occurrences of pouchitis^[49]. There were no significant differences between the two groups regarding daytime and night continence, or sexual function^[47].

In a meta-analysis, pouch failure rate was found to be 4.3% (95% CI: 3.5-6.3) and pelvic sepsis 7.5% (95% CI: 6.1-9.1)^[51]. Pouch failure was lower by 2.5% in recent studies *vs* those published prior to 2000. Functional outcome remained stable over time, with a 24-h defecation frequency of 5.9 (95% CI: 5.0-6.9), regardless of the technical aspects of the surgery^[51]. Preoperative steroid therapy (> 20 mg in adults), hypoalbuminemia and malnutrition are associated with increased surgical complications^[52]. Pre-operative high dose steroids and probably also infliximab^[53] are associated with increased surgical complications (especially in combination with other immune suppressants), while thiopurines and calcineurin

inhibitors are not.

AMBULATORY SURGERY

The most frequent indication for colectomy in ambulatory children with UC is chronic ongoing disease, at times- steroid dependent, whereas in adults- dysplasia is also a common indication^[54]. The points outlined above for ASC should also be followed in the decision-making of elective colectomy. In general, thiopurines and infliximab should be strongly considered in most cases before referral to colectomy in ambulatory mild-moderate UC. While cyclosporine should be initially administered in the hospital setting only, tacrolimus may be used in selected ambulatory patients as a bridge to thiopurines. In those losing response to infliximab, adalimumab may be considered before colectomy, given the recent evidence in adults showing its moderate effectiveness in ambulatory UC^[55,56]. Colectomy should be discussed as a viable alternative in children who suffer from ongoing symptoms despite multiple immunosuppressive medications, especially in steroid dependency.

CONCLUSION

Colectomy, a potentially lifesaving procedure in ASC, is associated with several long-term unwanted consequences. On the other hand, medical rescue therapy, including cyclosporine, tacrolimus and infliximab, are highly effective in steroid-refractory ASC. Therefore, medical salvage therapy should be offered before colectomy in most patients who do not present with surgical abdomen or toxic megacolon. Validated predictive rules can identify a subset of patients who will eventually fail corticosteroid therapy after only 3-5 d of steroid therapy with an accuracy of 85%-95%. This accuracy is sufficiently high for initiating medical therapy early in the admission without delaying colectomy if required. Families may elect to proceed to early colectomy before attempting medical rescue therapy, especially in chronic active disease. Therefore, whenever considering second-line medical therapy, colectomy should always be openly discussed. In patients failing one medical rescue therapy, colectomy should be regarded as the next therapeutic step.

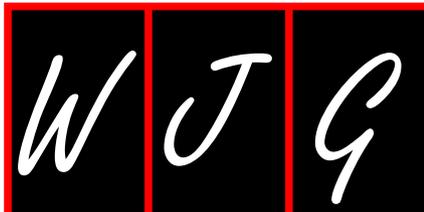
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Giovanni Latella, MD, Series Editor

Colorectal cancer in inflammatory bowel disease: What is the real magnitude of the risk?

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Abstract

The association between inflammatory bowel disease (IBD) and colorectal cancer (CRC) has been recognised since 1925 and still accounts for 10%-15% of deaths in IBD. IBD-associated CRC (IBD-CRC) affects patients at a younger age than sporadic CRC. The prognosis for sporadic CRC and IBD-CRC is similar, with a 5-year survival of approximately 50%. Identifying at risk patients and implementing appropriate surveillance for these patients is central to managing the CRC risk in IBD. The increased risk of colorectal cancer in association with IBD is thought to be due to genetic and acquired factors. The link between inflammation and cancer is well recognised but the molecular biology, immune pathobiology and genetics of IBD-CRC are areas of much ongoing research. This review examines the literature relating to IBD-CRC, focusing on the incidence of IBD-CRC and examining potential risk factors including age at diagnosis, gender, duration and extent of colitis, severity of inflammation, family history of sporadic CRC and co-existent primary sclerosing cholangitis (PSC). Confirmed risk factors for IBD-CRC are duration, severity and extent of colitis, the presence of

co-existent PSC and a family history of CRC. There is insufficient evidence currently to support an increased frequency of surveillance for patients diagnosed with IBD at a younger age. Evidence-based guidelines advise surveillance colonoscopy for patients with colitis 8 to 10 years after diagnosis, with the interval for further surveillance guided by risk factors (extent of disease, family history of CRC, post-inflammatory polyps, concomitant PSC, personal history of colonic dysplasia, colonic strictures). There is a move away from using random colonic biopsies towards targeted biopsies aimed at abnormal areas identified by newer colonoscopic techniques (narrow band imaging, chromoendoscopy, confocal microendoscopy).

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Key words: Colorectal cancer; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Risk

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INTRODUCTION

Crohn^[1] first described colorectal cancer (CRC) in association with inflammatory bowel disease (IBD) in 1925 and colorectal cancer still accounts for 10%-15% of deaths in patients with IBD^[2]. The incidence of IBD

has two peaks; between 15 and 30 years, and between 50 and 80 years^[3]. Due to this younger age peak, the mean age for developing IBD-associated CRC (IBD-CRC) is lower than that seen in sporadic CRC^[4]. A meta-analysis of 116 studies found the mean age of IBD-CRC diagnosis to be 43.2 years^[5]. Lakatos *et al*^[6] found the average age of IBD-CRC diagnosis to be 10 to 15 years younger than sporadic CRC in Eastern Europe (50.9 years *vs* 62.2 years). The prognosis for sporadic CRC and IBD-CRC is similar with a 5-year survival of approximately 50%^[7]. Identifying at risk patients and implementing appropriate surveillance for these patients is central to managing the CRC risk in IBD.

The increased risk of colorectal cancer in association with IBD is thought to be due to genetic and acquired factors^[8]. The link between inflammation and cancer is well recognised but the molecular biology, immune pathobiology and genetics of IBD-CRC are areas of much ongoing research. The role of the immune system for both tumour promotion and prevention is being examined. Studies indicate that immune cells are important in tumour promotion, with leucocyte infiltration and inflammatory mediators found in association with tumours^[9,10].

Genome-wide association studies have now identified approximately 99 susceptibility loci/genes relating to IBD. The better understanding of the genetics of IBD gives us more insight into disease pathogenesis. Some of the key themes identified are the role of interleukin (IL)-23/IL-17 signalling in IBD, defective barrier function in ulcerative colitis (UC) and defective processing of intracellular bacteria in Crohn's disease^[11]. Overlap of these loci/genes is seen with many diseases including CRC: E-cadherin (CDH1) has been associated with CRC^[12], UC^[13], and possibly Crohn's disease^[14].

MOLECULAR BIOLOGY OF IBD-CRC

IBD-CRC and sporadic CRC both have a dysplasia-cancer sequence and require multiple mutations to result in carcinoma^[7]. Sporadic CRC usually occurs as the end point of the adenoma-carcinoma sequence. Multiple molecular alterations occur within this sequence: chromosomal instability, microsatellite instability, hypermethylation^[15,16]. However, there are often differences in the timing and frequency of these events between sporadic and IBD-CRC. The molecular and genetic alterations occur more rapidly in IBD-CRC and in an unconventional sequence^[17]. It is suggested that the inflammation occurring in colitis results in a cascade of abnormal epithelial proliferation in addition to the genetic alterations that occur^[18]. In sporadic CRC, loss of the adenomatous polyposis coli gene function occurs early and p53 mutations occur late. The timing of these events is frequently reversed in IBD-CRC. It is also notable that in IBD-CRC, p53 mutations may be found within non-dysplastic mucosa. This is markedly different from sporadic CRC where p53 mutations are seen in morphologically aggressive lesions^[19].

The dysplasia-cancer sequence is a useful concept but it is more complex in IBD-CRC. There is not always a clear, stepwise transition from normal, through low and high grade dysplasia (HGD), to cancer^[17]. However, low grade dysplasia (LGD) can clearly progress to more advanced lesions and there is varying evidence as to the size of this risk. The 5-year cumulative risk has been shown to be as high as 33%-54% in a number of studies^[20-23]. This is in contrast to a 2%-10% risk of CRC over 10 years found by other groups^[24,25].

Cancer can occur without preceding detection of dysplasia, and LGD can regress or progress directly to cancer without the intermediate HGD^[26]. The high rate of synchronous and metachronous cancers associated with finding high grade dysplasia in biopsies usually leads to proctocolectomy^[27]. In a review of 10 prospective trials, 42% of patients undergoing colectomy for HGD had a synchronous CRC^[28]. Low grade dysplasia is an independent risk factor for CRC and often found in flat lesions which are difficult to see endoscopically. This is in contrast to sporadic CRC where dysplasia occurs within raised polypoid lesions. The concern is that flat LGD may be accompanied by HGD or cancer elsewhere in the colon. Studies suggest that 16%-27% of patients undergoing colectomy for LGD have synchronous CRC^[20,22,28]. Opinions differ as to how LGD should be managed: colectomy versus colonoscopic surveillance. Informed patient choice is important here. If surveillance is pursued colonoscopy should be repeated in 3 to 6 mo. The presence of multifocal LGD is a much stronger argument for colectomy.

QUANTIFYING THE RISK

It is important to quantify the risk of CRC in association with IBD. The reported risk varies widely between studies. This is partly due to the different methodology used in studies. Data comes from a mixture of tertiary referral centres, district general hospitals and population-based studies. Information from tertiary centres is likely to include patients with severe disease who are at greater risk of IBD-CRC. Early studies included patients who had already been referred with a diagnosis of CRC and those admitted to hospital with IBD, rather than gold-standard population-based studies which have a lower proportion of patients with severe or extensive colitis.

Eaden *et al*^[5] performed a meta-analysis of 116 studies including 54 478 UC patients with 1698 cases of IBD-CRC. The analysis included studies from a wide variety of centres: tertiary referral centres, population-based studies and hospital-based centres, and from different geographical areas. Studies from the United Kingdom and United States found a higher incidence [4 and 5 per 1000 person-years duration (pyd), respectively] than those from Scandinavia (2 per 1000 pyd). They found the overall prevalence of CRC in UC to be 3.7%, increasing to 5.4% in those with pancolitis.

Ekbom *et al*^[29] undertook a population-based cohort

study of 3117 patients with UC who were diagnosed between 1922 and 1983. Ninety one patients were found to have IBD-CRC giving a standardised incidence ratio of 5.7 (95% CI: 4.6-7.0) as compared with the expected incidence of CRC in the general population.

Söderlund *et al*^[30] undertook a population-based study of 7607 patients with IBD who were diagnosed between 1954 and 1989 for a total of 198 227 person-years. A total of 196 cases of CRC occurred in 188 patients, giving an overall incidence of 85 (95% CI: 82-109) cases per 100 000 person-years. This corresponds to a standardised incidence ratio of 2.3 (95% CI: 2.0-2.6) as compared with the general population.

Other population-based studies have suggested a much lower risk of IBD-CRC. Palli *et al*^[31] followed 689 patients with UC over 14 years (1978-1992) and found 10 cases of IBD-CRC, equating to an annual crude incidence of 0.13%. A study in Olmsted County followed 378 patients with UC for a total of 5567 person-years (1940-2004) and found 6 cases of IBD-CRC. They calculated the annual crude incidence to be 0.10% and the cumulative risk of CRC at 30 years to be as low as 2%. They found no statistically significant increase in the standardised mortality ratio (SMR) for CRC between IBD and non-IBD populations and concluded that the risk of CRC is only increased in patients with extensive colitis^[32]. Bernstein *et al*^[33] retrospectively examined 2672 patients with UC over a total of 19 665 person-years between 1984 and 1997. They found the annual risks to be 0.16% for colon cancer and 0.06% for rectal cancer.

Rutter *et al*^[20] followed 600 patients with extensive UC (as shown by barium enema or colonoscopy) for 5932 person-years as part of a colonoscopic surveillance programme (1970-2001). Data was gathered prospectively. Ninety-one patients (163 episodes) were found to have dysplasia or CRC. They calculated the cumulative probability of IBD-CRC to be 7.6% and a decreasing incidence over the period studied.

A Hungarian population-based study followed 723 UC patients for a total of 8564 person-years (1974-2004) and found 13 cases of IBD-CRC. They calculated the cumulative risks according to disease duration: 0.6% after 10 years, 5.4% after 20 years and 7.5% after 30 years^[6]. Hungary has a high rate of sporadic CRC and a low rate of colectomy for non-CRC reasons; factors which might have been expected to result in a higher rate of CRC.

Winther *et al*^[34] followed 1160 patients with UC over 22 290 person-years (1962-1987) and found 13 cases of IBD-CRC, giving an annual crude incidence of 0.06% and cumulative risk of 2.1% at 30 years. They found no statistically significant increase in the SMR for CRC between IBD and non-IBD populations. This study is from Denmark where the colectomy rate is one of the highest in the world: a fact that may affect the results and underestimate the risk of IBD-CRC.

The increased risk of CRC in UC has been long established. More recently, data has shown that Crohn's colitis

carries a similar magnitude of risk for the same disease extent. A Canadian cohort study matched a population-based IBD database to a cancer registry in North America between 1984 and 1997. There were 2857 cases of Crohn's disease and 2672 of UC. There was an increased incidence of CRC for patients with Crohn's [risk ratio (RR) 2.64; 95% CI: 1.69-4.12] or UC (RR 2.75; 95% CI: 1.91-3.97) as compared to the general population but no statistically significant difference between the two IBD diagnoses^[33]. They found the risk of rectal cancer to be increased in UC (RR 1.90; 95% CI: 1.05-3.43) but not in Crohn's colitis (RR 1.08; 95% CI: 0.43-2.70). A limitation of this study was the lack of definition of disease site or extent.

Ekblom *et al*^[35] also studied the risk of CRC in Crohn's disease. In a cohort study of 1655 patients in Sweden, patients with terminal ileal Crohn's had the same risk of CRC as the general population but those with colonic Crohn's had a RR of 5.6 (95% CI: 2.1-12.2).

CHANGING INCIDENCE OVER TIME

Eaden *et al*^[5] examined how the incidence of IBD-CRC is changing over time by plotting the cancer risk against the mid-point for each study. Although the incidence had increased between 1995 and 2001 the change was not statistically significant (slope 0.003, $P = 0.80$).

Rutter *et al*^[20] looked at the changing incidence of CRC over time within their 30-year colonoscopic surveillance program. They found a statistically significant decrease in CRC incidence ($r = -0.40$, $P = 0.04$), specifically for cancers proximal to the splenic flexure ($r = -0.54$, $P = 0.005$) and early cancers since 1975 (Dukes' A or B; $r = -0.4$, $P = 0.04$). However, there was not a statistically significant change in incidence for distal cancers ($r = 0.11$, $P = 0.59$) or advanced cancers (Dukes' C or D; $r = -0.05$, $P = 0.79$).

Jess *et al*^[32] examined a population-based cohort from Minnesota diagnosed between 1940 and 2001. They followed 692 patients with IBD for a total of 10 470 person-years. They analysed the incidence of CRC and the year that IBD was diagnosed. All their patients were diagnosed with CRC before 1980. The standardised incidence ratio for CRC diagnosed before 1980 was 1.6 (95% CI: 0.6-3.4) in comparison to 0 for those diagnosed after 1980. However, this difference between calendar periods was not statistically significant. They suggested that use of maintenance therapy and surveillance colonoscopy may be responsible for the absence of CRC seen in this cohort.

Söderlund *et al*^[30] examined the relative risk of CRC across calendar periods in their population-based study of 7607 patients with IBD who were diagnosed between 1954 and 1989. This cohort was followed up until 2004. After adjusting for type and extent of IBD, gender, age, and time since IBD diagnosis, the RRs were 1.7 (95% CI: 0.6-4.4) from 1960 to 1969, 1.3 (95% CI: 0.7-2.6) from 1970 to 1979, 1.2 (95% CI: 0.7-2.2) from 1980 to

1989, 1.1 (95% CI: 0.7-1.8) from 1990 to 1999, and 1.0 from 2000-2004. Although there was a trend towards decreasing incidence of CRC this did not reach statistical significance.

RISK FACTORS

Duration of colitis

A longer duration of colitis is associated with an increased risk of IBD-CRC; it is relatively rare before 8 years of colitis. However, Lutgens *et al*^[36] conducted a retrospective, nationwide database search to identify patients with IBD-CRC in the Netherlands between 1990 and 2006. For the 149 patients identified, they calculated the time interval between the diagnoses of IBD and cancer. They found that 22%-28% of patients developed cancer before the starting points for surveillance (8-10 years for extensive colitis and 15-20 years for left-sided disease). When the time interval from onset of symptoms to CRC diagnosis was used, 17%-22% would have developed cancer prior to surveillance endoscopy.

Identifying when the risk increases is central to guiding timing of surveillance strategies. Patients may also have colitis for a time before the diagnosis is made. This means they are exposed to cancer risk for a longer period if diagnosis is delayed.

In the meta-analysis by Eaden *et al*^[5], 41 studies reported colitis duration and 19 of these stratified duration into 10 year intervals. The overall incidence of IBD-CRC in any patient with UC was 3 per 1000 pyd. The cumulative incidence of IBD-CRC in patients with UC was 2% at 10 years, 8% at 20 years, and 18% after 30 years of disease. In the 61 studies that reported duration of colitis at the time of IBD-CRC diagnosis the mean was 16.3 years (95% CI: 15.0-17.6).

Lakatos *et al*^[6] retrospectively examined data relating to 723 patients within their 30-year database of IBD patients. They found 13 cases of IBD-CRC in 8564 person-years. They examined the incidence of CRC according to disease duration and found the cumulative risk to be 0.6% at 10 years (95% CI: 0.2-1.0), 5.4% at 20 years (95% CI: 3.7-7.1), and 7.5% at 30 years (95% CI: 4.8-10.2).

The prospective surveillance data from Rutter *et al*^[20] found the median duration of UC at diagnosis of CRC to be 23.5 years (range 11-48 years). The cumulative incidence of CRC was 0% at 10 years, 2.5% at 20 years, 7.6% at 30 years, 10.8% at 40 years, and 13.5% at 45 years (from onset of colitis). This lower incidence of IBD-CRC is particularly interesting given that the data comes from a specialist centre with a low colectomy rate and might have been expected to show a higher incidence. Data being obtained from a surveillance programme may in itself be responsible for the lower incidence of CRC, and regular colonic examination should prompt treatment measures to gain disease control.

Age at diagnosis

There is conflicting evidence as to whether younger age

at diagnosis of IBD is an independent risk factor for IBD-CRC. This evidence is not easy to evaluate as children tend to have more extensive and severe colitis than those diagnosed as adults, and younger people have the potential for longer colitis duration, which is itself a risk factor.

An important question is whether patients studied actually develop colitis at a younger age or whether they are diagnosed late after a period of undiagnosed inflammation. Eaden *et al*^[5] included 21 studies in their meta-analysis that examined age at onset of UC (over 20 years old). They excluded studies that reported the age at diagnosis of UC due to potential delay in diagnosis. They found a negative trend between younger age at onset (in adulthood) and increased risk of CRC but this was not statistically significant ($\chi = -1.61$, $P = 0.11$). They also analysed 12 studies looking at the incidence of IBD-CRC in children with UC (average age at onset of UC was 10 years old), only 5 of which commented on the duration of follow up. They found the overall incidence to be 6 per 1000 pyd and the cumulative risk of CRC to be 5.5% at 10 years, 10.8% at 20 years, and 15.7% at 30 years; higher than the corresponding rates for adults. However, these studies did not report incidence according to 10 yearly intervals so they assumed the log incidence rate to be constant. The small number of studies in children necessitates cautious interpretation of results.

Ekbom *et al*^[29] conducted a population-based cohort study of 3117 patients who were diagnosed with UC between 1922 and 1983 in Sweden. CRC was identified in 91 patients. They found age at diagnosis to be an independent risk factor for CRC. After adjusting for the extent of disease, they found the relative risk of CRC decreased by about half (adjusted standardized incidence ratio = 0.51; 95% CI: 0.46-0.56) for each increase in age group at diagnosis (under 15 years, 15-29 years, 30-39 years, 40-49 years, 50-59 years, and over 60 years). For those with extensive disease, after 35 years of disease, the cumulative risk for IBD-CRC was 40% if diagnosed under 15 years and 25% if diagnosed between 15 and 39 years of age.

Other studies have not confirmed this association. Greenstein *et al*^[37] found that the CRC risk was higher in patients diagnosed with IBD above 30-40 years of age compared with those diagnosed below 20 years old. Data from the 30-year study of Rutter *et al*^[20] showed that patients who developed CRC had a higher median age of onset of disease than those not developing cancer. This suggests that early onset is not an independent risk factor for IBD-CRC. Winther *et al*^[34] found the time between onset of colitis and the development of IBD-CRC to be the same in young and old patients.

Karvellas *et al*^[38] undertook a retrospective audit of adult patients with UC who were diagnosed with CRC between 1991 and 2002 in Edmonton, Alberta. They found that patients diagnosed with UC over the age of 40 years developed CRC more quickly than younger patients. The median disease duration at the time of CRC

diagnosis was 22 years in patients under 40 years, and 10 years for those over 40 years [odds ratio (OR): 11.5; 95% CI: 2.41-20.16; $P = 0.00029$].

Extent of colitis

The greater the disease extent, the greater the risk of CRC. Patients with proctitis and proctosigmoiditis are at the lowest risk, left-sided colitis carries moderate risk and those with pan-colitis are at the highest risk of CRC^[39,40].

How we measure the extent of disease is important; macroscopic versus radiological versus histological. Ekbohm *et al.*^[29] assessed IBD-CRC risk in UC according to extent of disease; proctitis versus left-sided colitis versus pancolitis. The extent was determined from barium enema and colonoscopy reports. The relative risk for CRC was 1.7 for proctitis, 2.8 for left-sided colitis, and 14.8 for pancolitis, as compared with the general population.

Söderlund *et al.*^[30] examined the risk of CRC according to extent of colitis in their population-based study. They found the relative risks of CRC to be 2.7 for all patients with UC (95% CI: 2.3-3.2), 5.6 for pancolitis (95% CI: 4.0-4.7), 2.1 for Crohn's colitis (95% CI: 1.2-3.4) and 1.7 for proctitis (95% CI: 1.2-2.4).

Severity of inflammation

IBD-CRC is thought to occur in the context of inflammation and anti-inflammatory treatments are felt to decrease the risk of CRC. Recent studies have focused on this association.

Rutter *et al.*^[20] conducted a case-control study of patients with colorectal neoplasia identified within a surveillance program between 1988 and 2002 ($n = 68$). They found a significant correlation between both colonoscopic (OR: 2.5; $P < 0.001$) and histological (OR: 5.1; $P < 0.001$) inflammation and the risk of neoplasia. When multivariate analysis was performed, histological inflammation remained a significant risk factor (OR: 4.7; $P < 0.001$). In a follow-up study from this work, Rutter *et al.*^[41] found that mucosal healing may decrease the risk of neoplasia; macroscopically normal mucosa appears to return the CRC risk to that of the general population.

Gupta *et al.*^[42] retrospectively studied a cohort of 418 patients undergoing colonoscopic surveillance for UC. They found a significant relationship between histological inflammation over time and progression to advanced neoplasia (hazard ratio 3.0; 95% CI: 1.4-6.3) which remained an independent risk factor in multivariate analysis. This is in contrast to other studies showing that those with quiescent disease have a similar risk of developing CRC as those with active disease^[6,43].

Post-inflammatory polyps have been associated with an increased risk of CRC. They do not in themselves have malignant potential^[44]. It is possible that multiple post-inflammatory polyps increase the miss rate of dysplastic lesions or that their presence is evidence of more severe previous inflammation. A retrospective study using the Mayo Clinic centralised diagnostic index identified 188 patients with UC-associated CRC and matched them

to 1528 gender and disease-extent matched controls. The presence of post-inflammatory polyps remained statistically associated with CRC even after adjusting for surveillance and anti-inflammatory treatments^[45]. In the follow-up case-control study by Rutter *et al.*^[41], cases of CRC were significantly more likely to have post-inflammatory polyps than the controls (OR: 2.14; 95% CI: 1.24-3.70).

Gender

In their population-based study, Söderlund *et al.*^[30] looked specifically at the gender-related risk of IBD-CRC by identifying those diagnosed with CRC between 1960 and 2004. They compared their results with the general population using standardised incidence ratios and data obtained from national health and census registers. There were 196 cases of IBD-CRC giving an overall incidence of 110 cases per 100 000 person-years. The relative risk in males was 2.6 (95% CI: 2.2-3.1) and in females 1.9 (95% CI: 1.5-2.4) compared to the general population. The cumulative incidence at 40 years after the diagnosis of IBD was 8.3% in males and 3.5% in females.

Ekbohm *et al.*^[29,35] found a similar relative risk for IBD-CRC in men and women whether they have UC (5.6 in men and 5.9 in women) or Crohn's disease (2.8 in men and 2.1 in women).

Family history of sporadic colorectal cancer

Patients with IBD who have a family history of sporadic CRC are at increased risk. The magnitude of the risk has been found to be a 2-3 fold increase in both case control and population-based studies. Askling *et al.*^[46] undertook a population-based cohort study of 19 876 patients with UC or Crohn's disease born between 1941 and 1995. They found that a family history of CRC was associated with a more than 2-fold risk of IBD-CRC (adjusted RR 2.5; 95% CI: 1.4-4.4) and those with a 1st-degree relative diagnosed with CRC before 50 years of age had a higher risk (RR 9.2; 95% CI: 3.7-23). In a retrospective cohort study, Velayos *et al.*^[45] also found family history of CRC to be an important risk factor for IBD-CRC in patients with UC (OR: 3.7; 95% CI: 1.0-13.2)^[47].

Co-existent primary sclerosing cholangitis

Smith *et al.*^[43] first identified a link between UC and primary sclerosing cholangitis (PSC) in 1965. The association between Crohn's disease and PSC was identified by Atkinson and Carroll in 1964^[48]. It has since been shown that the risk of IBD-CRC is greater in the presence of co-existent PSC. The adjusted relative risk for dysplasia or cancer is reported as 3.15 (95% CI: 1.37-7.27) for patients with PSC and UC as compared to those with UC alone^[49]. Kornfeld *et al.*^[50] found the cumulative risk was 33% at 20 years and 40% at 30 years from the diagnosis of UC. Causative theories include that a high concentration of bile acids in the lumen of the colon may contribute to the increased risk^[51]. Lundqvist *et al.*^[52] suggest that patients with PSC often have a longer duration of colitis and this in itself may explain the increased risk of CRC.

However, patients with PSC seem to have more quiescent colitis^[53]. Sokol *et al.*^[53], also found that patients with co-existent IBD and PSC receive more 5-aminosalicylic acid (5-ASA) treatment than patients with IBD alone. Given the importance of the severity of inflammation in the aetiology of IBD-CRC this points to the existence of PSC itself having a carcinogenic effect.

Soetikno *et al.*^[54] conducted a meta-analysis of 11 studies to establish whether the risk of CRC is increased in patients with concomitant UC and PSC. They found that patients with UC-PSC are at increased risk of colorectal dysplasia and carcinoma compared with patients with UC alone (OR: 4.79; 95% CI: 3.58-6.41) and the risk is still increased if CRC is considered alone (OR: 4.09; 95% CI: 2.89-5.76). Broomé *et al.*^[55] studied 120 patients in Stockholm; 40 with UC-PSC and 80 with UC alone. They found that those with concomitant UC and PSC have a significantly higher risk of developing CRC.

CLINICAL MANAGEMENT

Chemoprevention

As with all pathology, prevention is better than cure. By focussing on the risk factors predisposing people to IBD-CRC it is hoped we can reduce the incidence of dysplasia and cancer. Any chemoprevention strategy must be acceptable to patients and physicians, in terms of safety, efficacy and cost. Ongoing colonic inflammation has been accepted as one of the causative factors behind IBD-CRC. Studies have focussed on using maintenance anti-inflammatory medications to prevent the development of dysplasia and cancer.

5-ASA compounds are used as maintenance therapy in colitis. *In vitro* studies found that they inhibit the nuclear factor kappa B pathway which is involved in tumour survival and sustaining chronic inflammation^[4,56,57]. Due to the widespread use of 5-ASA compounds for maintenance therapy, prospective randomised trials are lacking with respect to chemoprevention. Velayos *et al.*^[58] undertook a meta-analysis of 9 studies which included a total of 1932 patients with UC. They found a protective effect of 5-ASA against colorectal cancer (OR: 0.51; 95% CI: 0.37-0.69) and colorectal cancer/dysplasia as a combined end point (OR: 0.51; 95% CI: 0.38-0.69). They did not find an association between 5-ASA use and a lower risk of dysplasia (OR: 1.18; 95% CI: 0.41-3.43) although they make the point that only 2 of the studies looked at this endpoint.

Terdiman *et al.*^[59] have reached a different conclusion with their observational study looking at the association between 5-ASA and IBD-CRC. They found that exposure to 5-ASA compounds during the 12 mo prior to the diagnosis of IBD-CRC was not associated with a decreased cancer risk (OR: 0.97; 95% CI: 0.77-1.23). They also found a non-statistically significant trend between a lower risk of IBD-CRC and an increased number of 5-ASA prescriptions in the previous year. Their conclusion was that 5-ASA compounds do not have

a protective effect against IBD-CRC when assessed over a short period. The important factor here seems to be the time period studied. Any preventative strategy has to be seen as a long term management approach. By using chemoprevention we are trying to prevent the genetic alterations which occur in the context of inflamed mucosa. The relationship between 5-ASA compounds and any protective effect must surely be assessed over a longer time period in order to discount it. The design of this study is too short and the results should be interpreted with caution.

The link between IBD-CRC and co-existent PSC is well established. The presence of additional bile acids in the colonic lumen is felt to contribute to this increased risk. Ursodeoxycholic acid (UDCA) decreases the amount of bile acids and studies have suggested treatment with UDCA may reduce the risk. Tung *et al.*^[60] found a strong relationship between UDCA and a decreased risk of colonic dysplasia (OR: 0.18; 95% CI: 0.05-0.61). The relationship remained after adjusting for gender, age at diagnosis of colitis, duration of colitis, duration of PSC, severity of liver disease, and sulfasalazine use. Pardi *et al.*^[61] also found a relative risk of 0.26 for colorectal dysplasia or cancer using UDCA when compared to placebo. These studies are in patients with colitis and PSC. The potential for using UDCA for patients with colitis only has not been explored. There are concerns about the side effects of using the medication in patients without PSC.

Given the potential benefit with suppression of inflammation, interest has been shown in other anti-inflammatory treatments. The role of steroids as chemopreventive agents has been explored. Evidence suggests a reduction in CRC risk with systemic and topical steroids^[45,62]. However, the significant complications that occur with long-term steroid treatment make this strategy unacceptable. No chemopreventive effect has been shown with azathioprine or 6-mercaptopurine^[63].

Other suggested therapies include folic acid and statins. Patients with IBD are at risk of folate deficiency. Studies have shown a protective trend against CRC but the effect is not statistically significant^[64,65]. Potack *et al.*^[66] suggest that given that folate is safe and inexpensive, supplementation should be considered for risk reduction. A study in Israel suggested statin therapy is associated with a risk reduction in sporadic CRC and a 94% risk reduction in patients with IBD^[67]. This potential benefit needs further investigation.

Surveillance

As described above, the quoted risk of developing cancer in colitis varies greatly from study to study. More recent population-based studies suggest a much lower risk than the earlier cohort studies. Methodological differences, the more recent widespread use of anti-inflammatory treatments and the advent of surveillance colonoscopy programmes may account for these differences. There is insufficient evidence to support the discontinuation

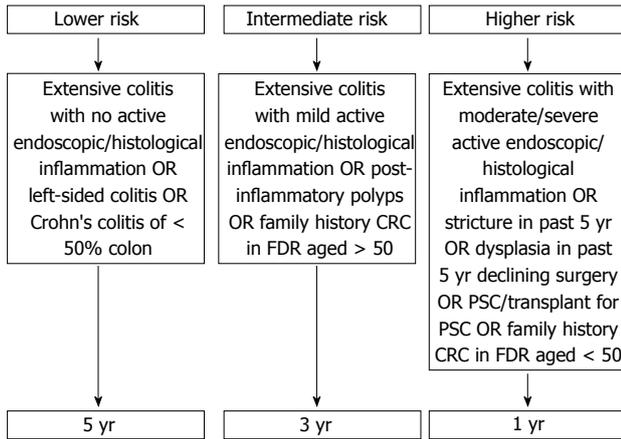


Figure 1 Surveillance recommendations for patients with colitis. OR: Odds ratio; CRC: Colorectal cancer; PSC: Primary sclerosing cholangitis; FDR: First degree relative.

of surveillance programmes and IBD is still believed to expose patients to an increased risk of CRC.

The aim of any screening or surveillance programme must be to identify early lesions to enable treatment and prevention before the development of invasive cancer. Prophylactic proctocolectomy eliminates the risk of CRC but this strategy is not acceptable to most patients or physicians. A surveillance programme must be acceptable to patients and practically possible to implement. The success of such programmes relies on patients engaging with follow up; they must understand the risks of not being tested and also that no surveillance strategy is without a miss rate.

Equally, physicians must implement the guidelines effectively. Many current guidelines advocate random quadrantic biopsies every 10 cm throughout the entire colon. This approach only visualises less than 1% of colonic mucosa. Rubin *et al.*⁶⁸ found the probability of detecting dysplasia was 90% if 33 and 95% if 56 biopsies were taken. Studies have shown that clinicians do not take sufficient biopsies^{69,70}.

The distinct differences between sporadic CRC and IBD-CRC are important for surveillance strategies. Bowel cancer screening in the general population relies on identification of adenomatous lesions which can be resected before they transform into carcinoma. IBD-CRC poses different challenges: dysplastic lesions do not follow the adenoma-carcinoma sequence, they can be difficult to see (flat lesions), difficult to resect completely, and multifocal. A meta-analysis of 1225 patients with UC found the likelihood of finding concurrent cancer at the time of colectomy for high or low grade dysplasia was 42% and 19%, respectively²⁸.

Due to the increased risk, patients diagnosed with PSC who are not previously known to have IBD should have a screening colonoscopy. For patients diagnosed with UC already, yearly surveillance colonoscopy should be performed from the point of diagnosis with PSC.

There has been increased focus on targeted biopsies and methods to improve identification of dysplastic le-

sions. Chromoendoscopy involves spraying dye (indigo carmine or methylene blue) on to the colonic mucosa to enable more detailed examination. Rutter *et al.*⁷¹ compared consecutive, random and indigo carmine targeted biopsies. Chromoendoscopy found 7 dysplastic lesions in 157 targeted biopsies, compared to no dysplasia in 2904 non-targeted biopsies. Hurlstone *et al.*⁷² conducted a prospective case-controlled study of 700 patients and also found a higher yield of dysplasia using indigo carmine chromoendoscopy as compared to conventional colonoscopy with random biopsies. They found 69 dysplastic lesions using chromoendoscopy and only 24 lesions with random biopsies ($P < 0.001$). These results are supported by Kiesslich *et al.*⁷³, who found a statistically significantly higher rate of neoplasia detection with methylene blue chromoendoscopy.

Narrow band imaging (NBI) is available on most colonoscopes. It uses optical filter technology to improve the visibility of vessels, pit pattern and other soft tissue structures. Dekker *et al.*⁷⁴ performed a prospective, randomised trial to compare NBI and conventional colonoscopy. They did not find a statistically significant difference between the two methods; a similar number of dysplastic lesions were identified and missed using both methods.

Confocal laser endomicroscopy visualises the histology of the mucosa in real time. This is useful for characterising lesions rather than finding the lesion in the first place. Kiesslich *et al.*⁷⁵ compared confocal chromoscopic endomicroscopy with conventional colonoscopy with random biopsies in a randomised controlled trial. They found the yield of neoplasia was increased 4.75 times using the new approach ($P = 0.005$) using 50% less biopsies. Hurlstone *et al.*⁷⁶ compared confocal chromoscopic endomicroscopy to chromoendoscopy alone in a prospective, randomised controlled trial. They found that endomicroscopy increased the diagnostic yield of neoplasia 2.5 times.

However, it is already well known that there is significant inter-observer variability between even expert gastrointestinal pathologists when interpreting dysplasia. The use of confocal endomicroscopy requires the endoscopist to have the ability to interpret histological findings. For this modality to be widely used endoscopists would require extensive training to enable accuracy.

The best technique for surveillance is evolving. There is a move away from using random colonic biopsies towards targeted biopsies aimed at abnormal areas identified by newer colonoscopic techniques (chromoendoscopy, confocal microendoscopy). There has been concern regarding the specialist training needed for endoscopists to use chromoendoscopy effectively. Nevertheless, the 2010 guidelines from the British Society of Gastroenterology (BSG) recommend the use of chromoendoscopy with targeted biopsies for colitis surveillance⁷⁷.

Current guidance from the BSG advises all patients with IBD should have a screening colonoscopy approximately 10 years from symptom onset (ideally when in remission) with pancolonic dye spraying and targeted

biopsies of abnormal areas. The risk of IBD-CRC is estimated based on duration and extent of disease, co-existent risk factors (PSC, family history of sporadic CRC), and the endoscopic and histological findings at colonoscopy. The surveillance intervals are based on this assessment of risk (Figure 1)^[77].

In conclusion, confirmed risk factors for IBD-CRC are duration, severity and extent of colitis, the presence of co-existent PSC and a family history of CRC. There is insufficient evidence currently to support an increased frequency of surveillance for patients diagnosed with IBD at a younger age. Evidence-based guidelines advise surveillance colonoscopy for patients with colitis 8 to 10 years after diagnosis, with the interval for further surveillance guided by risk factors (extent of disease, family history of CRC, post-inflammatory polyps, concomitant PSC, personal history of colonic dysplasia, colonic strictures)^[78-80].

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Mutual regulation between microRNA-373 and methyl-CpG-binding domain protein 2 in hilar cholangiocarcinoma

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Abstract

AIM: To investigate the reciprocal modulation between microRNA (miRNA) and DNA methylation *via* exploring the correlation between miR-373 and methyl-CpG-binding domain protein (MBD)2.

METHODS: MiR-373 expression was examined using the TaqMan miRNA assay. Methylation of *miR-373* was investigated using methylation-specific polymerase chain reaction, and recruitment of methyl binding proteins was studied using the chromatin immunoprecipitation assay. Mutation analysis was conducted using the QuikChange™ Site-Directed Mutagenesis kit. The activity of *miR-373* gene promoter constructs and targeting at MBD2-three prime untranslated region (3'UTR) by miR-373 were evaluated by a dual-luciferase reporter gene assay.

RESULTS: In hilar cholangiocarcinoma, miR-373 decreased and was closely associated with poor cell differentiation, advanced clinical stage, and shorter survival. The promoter-associated CpG island of miR-373 gene was hypermethylated and inhibited expression of miR-373. MBD2 was up-regulated and enriched at the promoter-associated CpG island of miR-373. Methylation-mediated suppression of miR-373 required MBD2 enrichment at the promoter-associated CpG island, and miR-373 negatively regulated MBD2 expression through targeting the 3'UTR.

CONCLUSION: MiR-373 behaves as a direct transcriptional target and negative regulator of MBD2 activity through a feedback loop of CpG island methylation.

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Key words: MicroRNA-373; Methyl-CpG binding domain proteins 2; Methylation; Hilar cholangiocarcinoma; Three prime untranslated region

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INTRODUCTION

Hilar cholangiocarcinoma, known as Klatskin tumor^[1], is an uncommon cancer with an incidence of 0.01% to 0.2% per year^[2]. Although it is relatively rare, hilar cholangio-

carcinoma displays a highly aggressive malignancy and is considered to be an incurable and rapidly lethal disease despite recent progress in diagnostic and therapeutic techniques. The 5-year overall survival after curative resection ranges from 15% to 35%, but the 10-year survival is almost zero^[3]. Patients with inoperable, recurrent, or metastatic disease can only be treated with palliative therapy, such as endoscopic, percutaneous biliary drainage in combination with radiotherapy and chemotherapy. However, hilar cholangiocarcinoma is not sensitive to chemotherapy and radiotherapy, and the median survival rate of those cases is only about 5.8 mo^[4].

Studies have established a direct link between aberrant DNA methylation and regulation of gene expression in human cancer^[5]. Once a given sequence becomes methylated, it can directly repress transcription by either impeding recognition of transcriptional activators to DNA sequences^[6], or by recruiting methyl-CpG binding domain proteins (MBPs) to modify chromatin compaction and control gene silencing^[7]. The MBP family consists of five isoforms, including Mecp2, methyl-CpG-binding domain protein (MBD1), MBD2, MBD3, and MBD4. With the exception of MBD4, which is primarily a thymine glycosylase involved in DNA repair^[8], all MBPs are implicated in transcriptional repression mediated by DNA methylation. Mecp2, MBD1, and MBD2 have been demonstrated to be involved in methylation-based gene repression and also affect chromatin structure^[9-11]. MBD3 lacks a functional MBD, but is an integral subunit of the histone deacetylase Mi2-NuRD complex that is recruited through MBD2^[12].

MicroRNAs (miRNAs) are non-coding, single-stranded RNAs of 18 to 24 nucleotides in length that constitute a novel class of gene regulators^[13]. In general, miRNAs negatively regulate gene expression by targeting the three prime untranslated region (3'UTR), which consequently triggers mRNA degradation or translational suppression^[14,15] on the basis of complementary value. miRNAs have recently been shown to play important roles in cancers, as more than 50% of miRNA genes reside in cancer-associated genomic regions, and their expression has been found to be dysregulated in various cancers^[16]. Depending on the target genes, miRNAs can function as tumor suppressor genes or oncogenes^[17].

Mature miRNAs are transcribed from *miRNA* genes by RNA polymerase II. Hence, the expression of miRNAs share the same genetic and epigenetic regulation of gene function including methylation^[18]. Although only subsets of miRNA genes harbor CpG islands in their promoter regions or are embedded in CpG islands, DNA methylation-mediated down-regulation of miRNAs have been reported by a number of groups^[19]. Moreover, miRNA interference with DNA methylation through DNA methyltransferases (DNMTs) 3a, 3b, and DNMT1, have been observed^[20-22]. These results suggest that miRNA and DNA methylation regulate one another. However, the literature has only revealed a one-way effect of miRNA on DNA methylation or miRNA modification by promoter methylation^[23]. We speculate that a particular miRNA may

Table 1 Relationship between miR-373 expression and clinicopathological features

Clinicopathological features	n	ΔC_t value of miR-373	P value
Age (yr)			
< 60	19	27.69 ± 3.76	0.059
≥ 60	29	25.65 ± 4.35	
Gender			
Male	33	24.43 ± 2.43	0.877
Female	15	29.01 ± 3.76	
Tumor size (cm)			
< 2	29	25.92 ± 3.64	0.606
≥ 2	31	25.49 ± 2.59	
Pathological type			
Adenocarcinoma	44	26.71 ± 3.18	0.390
Mucocellular carcinoma	2	24.63 ± 3.57	
Adenosquamous carcinoma	1	23.94	
Squamous carcinoma	0		
Undifferentiated carcinoma	1	27.91	
Cell differentiation			
Well	14	19.09 ± 3.46	0.031 ^a
Moderately	23	21.97 ± 1.74	
Poorly	11	28.43 ± 4.09	
Bismuth classification			
Bismuth I	6	24.66 ± 3.31	0.082
Bismuth II	13	27.65 ± 2.71	
Bismuth III	17	26.99 ± 4.02	
Bismuth IV	12	26.22 ± 3.96	
Lymphatic node metastasis			
Absent	19	28.59 ± 2.53	0.224
Present	29	25.01 ± 2.64	
Clinical stages			
I + II	13	18.35 ± 2.62	0.017 ^a
III + IV	35	27.95 ± 3.12	

^aP < 0.05 is significant.

act as a bidirectional regulator by not only impacting DNA methylation, but also *via* regulation by methylation itself.

In this study, we demonstrate that miR-373 functions as a negative regulator of MBD2 by targeting the 3'UTR. Inversely, miR-373 is restrained by MBD2 enrichment at the methylated promoter-associated CpG island. We functionally demonstrate the fact that miR-373 serves as a one directional transcriptional target and negative regulator of MBD2 through a feedback loop of CpG methylation in hilar cholangiocarcinoma.

MATERIALS AND METHODS

Patients and samples

A total of 48 patients with both tumor and normal bile duct tissues, which were successfully obtained from operations conducted from January 2005 to December 2008 at Tongji Hospital in the Tongji Medical College of the Huazhong University of Science and Technology (China), were used in this study. The fresh tissues were harvested immediately after surgery, washed twice with chilled phosphate buffered saline, and immediately stored in liquid nitrogen and at -80 °C in our tissue bank until further use. The detailed clinical data of these patients is provided in Table 1. Written informed consent was obtained from each patient before sample collection. Ethical approval

was obtained from the Cancer Center Research Ethics Committee of Tongji Medical College and Hospital.

Cell lines and epigenetic treatment

The QBC₉₃₉ cell line, originating from human common bile duct adenocarcinoma, was kindly provided by Dr. Shuguang Wang from Southwest Hospital of the Third Military Medical University (China)^[24]. The HEK293 cell line was purchased from the Cell Bank of Chinese Academy of Sciences (China). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco-BRL; Carlsbad, CA, United States). For the epigenetic study, QBC₉₃₉ cells were treated with 5.0 μmol 5-Aza-2-CdR for 5 d, and 200 nmol trichostatin A (TSA; Sigma-Aldrich; St. Louis, MO, United States) was added on day 5.

Taqman miRNA assay

RNA was extracted using the mirVana™ miRNA Isolation Kit (Applied Biosystems, Carlsbad, CA, United States). cDNA synthesis and analysis of miR-373 expression were performed according to the TaqMan MicroRNA Assay protocol (Applied Biosystems). U6 (RUN6B) was used as an endogenous control. Polymerase chain reaction (PCR) was conducted in three independent replicates for each sample. Expression of miR-373 was normalized to U6, and fold change was calculated based on the $2^{-\Delta\Delta C_t}$ method.

Genomic DNA bisulfate modification

Genomic DNA was isolated from tissues and cells using the DNeasy® Blood and Tissue Kit (Qiagen, Valencia, CA, United States). For DNA methylation detection, 1.5 μg of genomic DNA was modified with sodium bisulfite using the EpiTect Bisulfate Kit (Qiagen). CG Genome Universal Unmethylated DNA and CG Genome Universal Methylated DNA (mDNA) (Millipore, Darmstadt, Germany) were also modified for use as positive and negative controls, respectively (100% values).

DNA methylation analysis

Methylation-specific PCR (MSP) and MethySYBR^[25,26] quantitative methylation-specific PCR (qMSP) were performed with primers specific for fully methylated and fully unmethylated CpG island sequences (MmiR-373, UmiR-373). Primers for converted (ActB) and unconverted (ActG) β-actin special sequences containing no CpG sites were used as a control to correct *C_r* values and for efficiency of bisulfite conversion, respectively. *C_r* values of samples MmiR-373, UmiR-373, and ActB were calculated using corresponding standard curves, after which they were corrected to DNA amount with ActB values. The sum of percent of fully methylated reference (PMR) and percent of unmethylated reference (PUR) DNA sample amounts (MmiR-373 + UmiR-373 = 100%) was calculated.

Chromatin immunoprecipitation assay

The Chromatin immunoprecipitation (ChIP) assay was

performed with the ChIP-IT™ Express kit (Active Motif, Carlsbad, CA, United States) according to the manufacturer's instructions using 2 μg ChIP-validated antibodies (Mecp2, MBD2, and mouse IgG, Active Motif; MBD1, Invitrogen, Carlsbad, CA, United States). The presence of target proteins at DNA segment were validated with primers (5'-AGATCGAGACCATCCTGGCTAACA-3'; 5'-TGAGAATGAGTCTTGCTCTGTCCG-3') to a product of 201 base pairs (bp) in size, and the enrichment of RNA polymerase II on the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (116 bp in size) was quantified as the endogenous control. ChIP-qPCR reactions were performed using SYBR® GreenER™ Assay (Invitrogen) in a genomic DNA model. Protein enrichment was expressed as the ratio of immunoprecipitated DNA (IP-DNA) to the total amount of DNA in the chromatin sample (input). Fold change was calculated as the ratio of IP-DNA/input (target) to IP-DNA/input (RNA polymerase II), and was then normalized to the control.

Vectors

The promoter luciferase reporter plasmid harboring the CpG island of *miR-373* gene was constructed according to a previously described approach^[27] and designated pGL4-373-Prom. In brief, the *miR-373* gene locus was screened in the database (<http://genome.ucsc.edu/>), a 5-kb fragment upstream of the pre-*miR-373* sequence was analyzed (http://www.fruitfly.org/seq_tools/promoter) to figure out the putative promoter and transcriptional start site (TSS), and a CpG island was predicted using Methprimer software (<http://www.urogene.org/methprimer/>). The results showed 402 bases of putative CpG islands spanning -251 to +150 bp and containing 26 CpG dinucleotides in the 5'-flank region of the human *miR-373* gene (predicted TSS is recognized as +1).

A 726 bp fragment of the *miR-373* gene (GenBank accession no. NR_029866) was amplified from QBC939 cell genomic DNA using the following PCR-specific primers: 5'-CGATGGTACCTGGAAAGTGCTGCGA-CATTT-3' (sense), which contains an artificial *Kpn* I site, and 5'-TCATGCTAGCAGAGGTTGGCCTCCAAT-CAT-3' (antisense), which contains an artificial *Nhe* I site and four protective bases. The PCR-amplified fragments were digested with *Kpn* I/*Hne* I and then inserted into the pGL4.22-basic plasmid (Promega, Madison, WI, United States) to generate the *miR-373* gene promoter luciferase reporter plasmid designated pGL4-373-Prom.

Precursor miR-373 clones (miRNA Accession: MI0000781), a scrambled control clone, wild-type MBD2 3'UTR vector pEZX-MBD2-3'UTR (Gene Accession: NM_004992.3), a scrambled control, and full-length cDNA expression vector of MBD2 (pDONR™_MBD2), were purchased from GeneCopoeia company (GeneCopoeia, Rockville, MD, United States). The potential binding sequences of miR-373 on the MBD2 3'UTR were mutated using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, United States). Silencer® Select siRNA for MBD2 and the scrambled control were

purchased from Applied Biosystems (siRNA ID: s17081, Target RefSeq Number: NM_003927). The recombinants were confirmed by full-length sequencing.

siRNA knock-down study

For protein knock-down by RNAi, 5×10^6 freshly elutriated HEK293 cells were transfected with targeting MBD2-siRNA or scrambled control at a final concentration of 200 nmol, using HiPerfect Transfection Kit (Qiagen) according to the manufacturer's instructions. Seventy-two hours after transfection, the cells were harvested.

In vitro DNA methylation

pGL4-373-prom was methylated *in vitro* using M. Sss I (4 U/ μ g DNA) (New England Biolab, Ipswich, MA, United States) in the presence of 160 μ mol S-adenosylmethionine (SAM) for 16 h. The mDNA, re-designed as pGL4-m373-prom, was digested with either Sal I (blocked by M. Sss I) or Bam I (not blocked by M. Sss I) restriction enzymes. Sensitivity to Sal I and resistance to Bam I indicated efficient DNA methylation.

Establishment of stable cell line

pGL4-m373-prom or pGL4-u373-prom were transfected into HEK293 cells in 35-mm dishes using Lipofectamine™ LTX and Plus Reagent (Invitrogen). Twenty-four hours post-transfection, cells were plated into 100-mm dishes at various densities and incubated in RPMI 1640 medium containing 2.0 μ g/mL of puromycin for 7 d, followed by maintenance with 1.0 μ g/mL puromycin. The stable cell line established was designated HEK-m373-prom and HEK-u373-prom.

Luciferase reporter gene assay

For promoter luciferase activity assay, pGL4-m373-prom or pGL4-u373-prom were transfected into HEK293 cells; pRL-TK was cotransfected as an endogenous control, and methylated and unmethylated pGL4-control were also used as controls. For the 3'UTR luciferase reporter assay, vectors containing WT-3'UTR or MUT-3'UTR were cotransfected with precursor miR-373 or pre-miR-neg. Cells were harvested 72 h after transfection, and luciferase activity was quantified using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, United States) according to the manufacturer's protocol.

Western blotting

Proteins (50 μ g) were analyzed by Western blotting with primary antibodies against Meccp2 (1:500, Sigma-Aldrich), MBD1 (1:1000, Millipore, Darmstadt, Germany) and MBD2 (1:1000, Millipore), which produced a signal of approximately 75 kDa, 61 kDa, and 50 kDa, respectively. GAPDH (1:5000, abcam, Cambridge, MA, United States) was used as a loading control.

Statistical analysis

Data analysis was performed using SPSS for windows version 14.0 (Chicago, IL, United States). Student's test,

one-way analysis of variance, and Pearson were used according to the data characteristics. Duration of hilar cholangiocarcinoma recurrence and death measured from the date of surgery was referenced against disease-free survival and overall survival time. Survival duration was calculated *via* the Kaplan-Meier method. The log-rank test was employed for comparison of cumulative survival rate and disease-free survival in the patient group. *P* values < 0.05 were considered statistically significant.

RESULTS

Down-regulation miR-373 is associated with poor cell differentiation, advanced clinical stage, and shorter survival

In patients with hilar cholangiocarcinoma, significant down-regulation of miR-373 was observed in QBC₉₃₉ cells and 35 (72.92%) tumors, including seven undetectable samples (*P* < 0.01) (Figure 1A). Fold-change analysis showed a 2.94-fold decrease in the tumor group compared to the control (*P* < 0.01, Figure 1B). In regard to the correlation between miR-373 expression and clinicopathological factors, miR-373 showed low expression in specimens with poor cell differentiation (*P* = 0.031) and advanced clinical stages (stage III, IV *vs* I, II) (*P* = 0.017) (Table 1), while no association was observed with age, gender, tumor size, different pathological types, Bismuth classification, or lymphatic metastasis (*P* > 0.05). Further studies were conducted to evaluate the correlation between miR-373 expression and survival. Kaplan-Meier analysis showed that down-regulation of miR-373 correlated with decreased overall survival (Figure 1C, *P* < 0.05, log-rank test) and disease-free survival (Figure 1D, *P* < 0.05, log-rank).

Promoter-associated CpG island of miR-373 is hypermethylated in hilar cholangiocarcinoma

According to the literature, the CpG island is a region of at least 200 bp, a GC percentage greater than 50%, and an observed/expected CpG ratio greater than 60%. A 402 base canonical CpG island spanning -251 to +150 bp and containing 26 CpG dinucleotides encompasses the transcription start site (TSS, recognized as +1, Figure 2A). Methylation of the promoter-associated CpG island was investigated with standard MSP and MethylSYBR. In standard MSP, methylation was present in 38 (38/48, 79.17%) tumors, including 26 homozygous and 12 heterozygous samples, which are indicated by a single methylation band or by both methylation and unmethylation bands, respectively (Figure 2B). Heterozygous methylation was also observed in five control tissues. These results were validated by qMSP and a fluorescent signal was detected in same samples. The value of PMR and PUR was 87.4% and 14.7% in the tumor and control groups, respectively (Figure 2C, *P* < 0.01).

To determine the correlation between promoter methylation and miR-373 expression, we divided methylation into four groups according to the following PMR values:

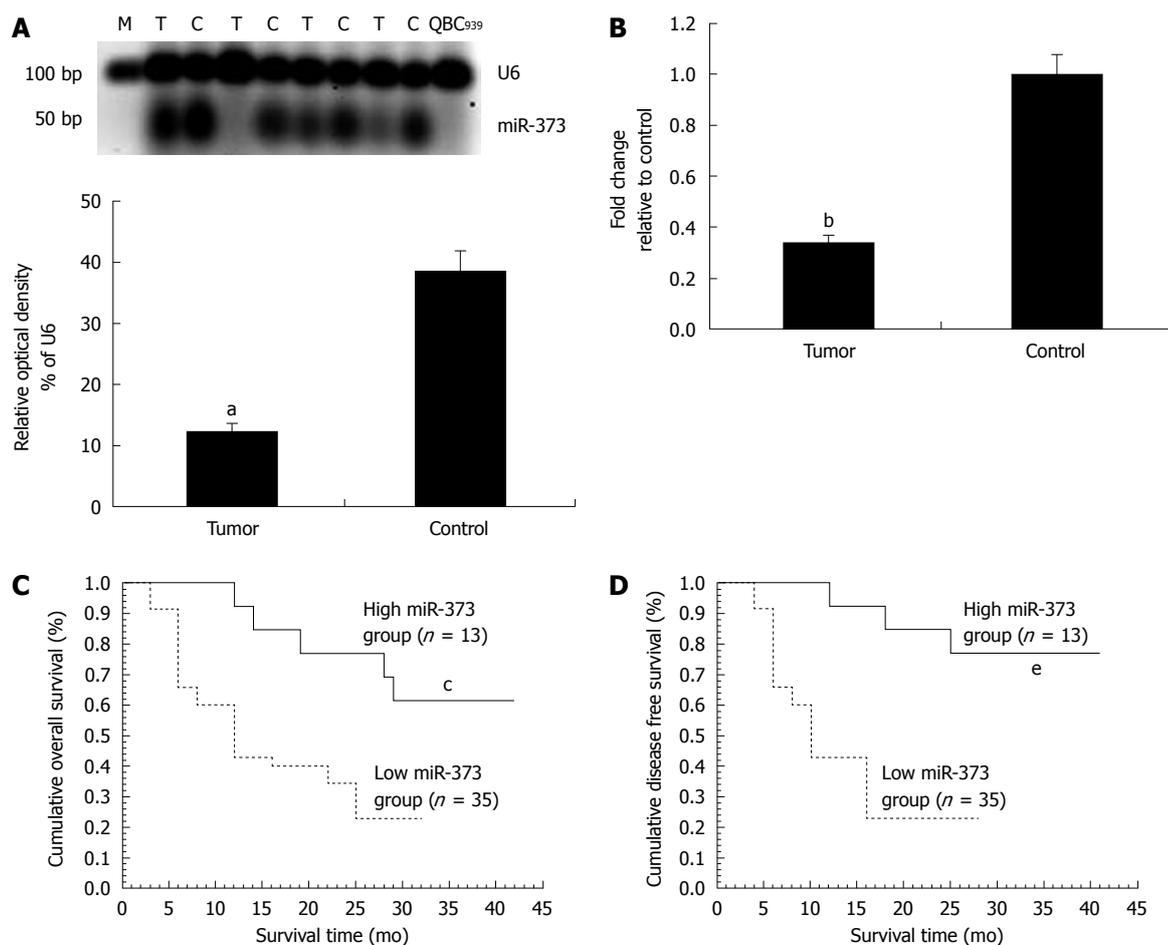


Figure 1 Expression of miR-373 and the association with clinicopathological factors in patients with hilar cholangiocarcinoma. A: Representative expression of miR-373 decreased in tumor and in QBC₉₃₉ detected by RT-PCR ($^{\circ}P < 0.05$ vs control); B: Taqman microRNA assay of miR-373 displayed 2.94-fold down-regulation in tumor group ($^{\circ}P < 0.01$ vs control); C: Relationship between miR-373 expression and overall survival in the patients with hilar cholangiocarcinoma. The median overall survival time was 16.3 mo and 29.7 mo in low- and high- miR-373 group, respectively ($^{\circ}P < 0.05$ vs high-group); D: Kaplan-Meier disease-free survival, the median disease-free survival time were 11.2 mo, 23.4 mo in low- and high- miR-373 group, respectively ($^{\circ}P < 0.05$ vs high-group).

(PMR above 90.00%), hypermethylation (PMR range from 42.00% to 89.99%), standard methylation (PMR range from 20.00% to 41.99%), and hypomethylation (PMR below 20.00%). Compared to its counterparts, miR-373 expression distinctly decreased in 88.5% (23/26) supermethylated samples ($P < 0.01$). There was a comparative reduction in seven hypermethylated samples ($P < 0.05$), and a dramatic increase was seen in 10 hypomethylated tumors and 43 control tissues ($P < 0.01$); no difference was detected in five standard methylated samples ($P > 0.05$). Interestingly, three supermethylated tumors were characterized by relatively high miR-373 expression (samples 14, 18, and 44). Despite these three extra tumors, promoter methylation demonstrated an inverse relationship with miR-373 expression in hilar cholangiocarcinoma (Figure 2D).

For further study on the contribution of promoter-associated CpG island methylation and inhibition of miR-373, pGL4-373-prom was constructed and methylated *in vitro* followed by transfection into HEK293 cells. As shown in Figure 3A, the relative luciferase activity in pGL4-u373-prom presented a higher level and decrease

significantly in pGL4-m373-prom ($P < 0.01$). This phenomenon was also proven by data showing reactivation of miR-373 with epigenetic treatment of QBC₉₃₉ cells. A 3.4-fold increase in miR-373 in cells treated with 5-Aza-2'-CdR and a 3.1-fold increase in cells treated with 5-Aza-2'-CdR and TSA were detected (Figure 3B). These results suggest that the promoter-associated CpG island acts as a cis-element of *miR-373* gene transcription, and its function can be abrogated by methylation in hilar cholangiocarcinomas and QBC₉₃₉ cells.

MBP 2 is up-regulated and enriched at the promoter-associated CpG island of miR-373

Among the MeCPs, Mecp2, MBD1, and MBD2 have been established to be involved in the methylation-dependent repression of transcription. Therefore, we explored protein expression using antibodies directed against Mecp2, MBD1, and MBD2. Compared to the control, a 2.9-fold increase in MBD2 expression was found in tumors while no difference in MBD1 and Mecp2 expression were detected (Figure 4A).

The presence of Mecp2, MBD1, and MBD2 in the

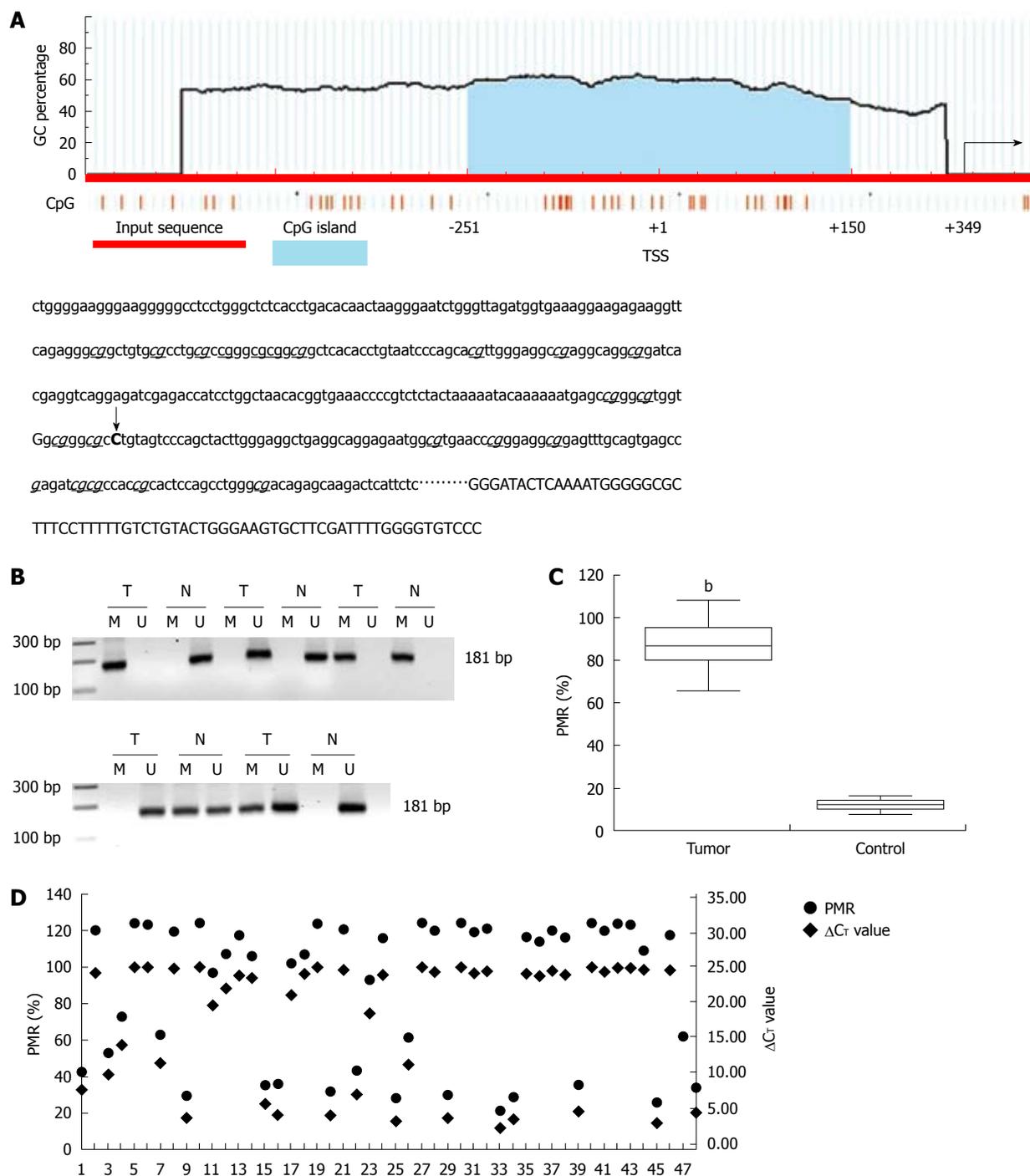


Figure 2 Methylation of miR-373 promoter-associated CpG island in hilar cholangiocarcinoma. A: Top panel, a schematic drawing of the putative CpG island in the 5'-flank region of *miR-373* gene; Bottom panel, sequence information of *miR-373* gene including partial 5'-flank region. The transcriptional start site (TSS) in boldface uppercase indicated by black arrow, 26 CpG dinucleotides are underlined and italic, pre-miR-373 sequences are capitalized; B: DNA methylation detected by methylation-specific polymerase chain reaction (MSP). Five representative cases were shown to indicate homozygous methylation (cases 1, 3), heterozygous methylation (cases 4, 5) and unmethylated (case 2), respectively. Lanes labeled M and U denote products amplified by primers recognizing methylated and unmethylated sequences; C: Percent of methylated reference (PMR) of miR-373 promoter-associated CpG island in tumor group is significant higher than control ($P < 0.01$ vs control); D: Relationship between CpG island methylation and miR-373 expression in hilar cholangiocarcinoma. A reverse correlation between PMR values and miR-373 expression could be observed (high ΔC_t value indicated low expression) excluding 3 extra samples displayed high PMR values and high mRNA expression (samples 14, 18 and 44).

region of the promoter-associated CpG island was assessed by the ChIP assay using ChIP-validated antibodies (Figure 4B). In hilar cholangiocarcinoma, the amount of CpG island fragment immunoprecipitated by MBD2 was greater than the input. ChIP-qPCR analysis showed

0.03-fold, 0.11-fold, and 0.79-fold for Mecn2, MBD1, and MBD2 compared to the endogenous control of RNA polymerase II enrichment of GAPDH, respectively ($P < 0.01$, Figure 4B, bottom panel). These findings indicate that the fraction of promoter-associated

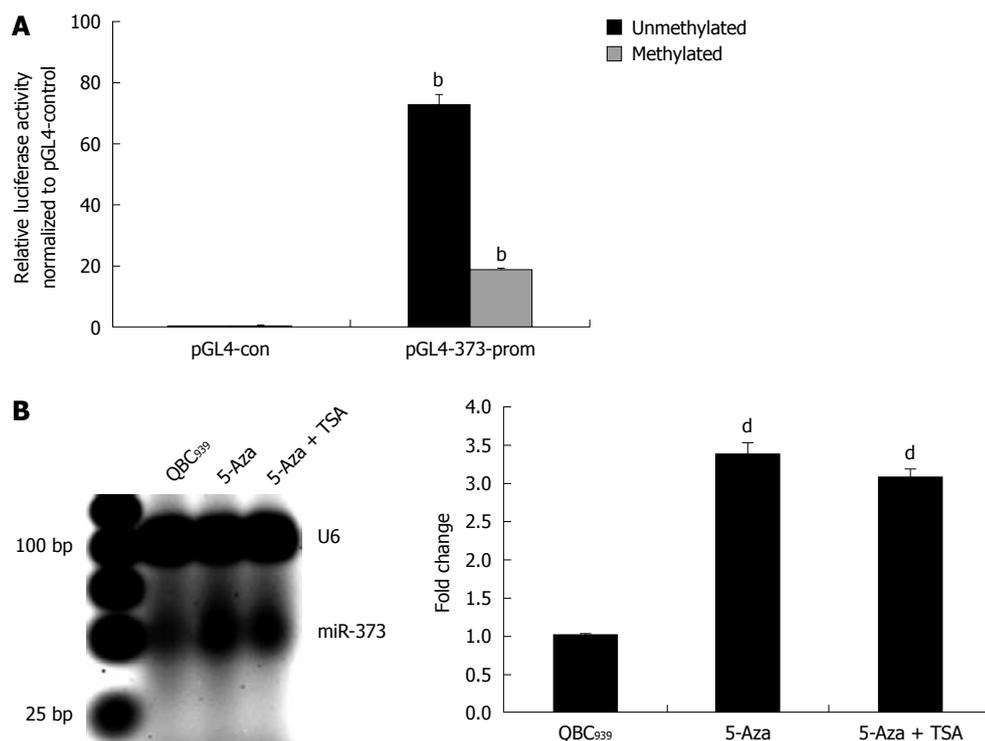


Figure 3 Methylation of CpG island regulates the expression of miR-373. A: Promoter luciferase reporter gene assay for miR-373. Prior CpG methylated resulted in dramatic decrease of luciferase activity of pGL4-m373-prom compared with pGL4-u373-prom ($^*P < 0.01$ vs control); B: miR-373 expression was reactivated by epigenetic reagents of 5'-Aza-2-CdR or combination with trichostatin A (TSA). $^{\#}P < 0.01$ vs untreated QBC₉₃₉.

CpG island is selectively immunoprecipitated by MBD2, but not by Mecp2 and MBD1. In addition, fold enrichment analysis showed a remarkable correlation between miR-373 promoter methylation and MBD2 enrichment in four groups with different frequencies of methylation ($P < 0.01$, Figure 4C).

Methylation-mediated suppression of miR-373 requires MBP 2 enrichment at promoter-associated CpG island

In QBC₉₃₉ cells and hilar cholangiocarcinoma, we evaluated whether inhibition of miR-373 was closely related to hypermethylation and enrichment of MBD2 at the promoter-associated CpG island. Specifically, to unravel whether the enrichment of MBD2 is critical for inactivation of miR-373, exogenous MBD2 expression was induced in stable cell lines of HEK-u373-prom or HEK-m373-prom. The ChIP assay was performed 72 h post-transfection, and fold change analysis showed a 6.2-fold enrichment of MBD2 in pGL4-m373-prom compared to wild-type HEK293 cells, while no change was found in the pGL4-u373-prom group (Figure 5A, $P < 0.01$).

Further knock-down studies in QBC₉₃₉ were performed to reduce endogenous MBD2 by a specific siRNA. A 4.3-fold increase in miR-373 expression was observed in knock-down cells compared to wild-type QBC₉₃₉ cells (Figure 5C). Sequential ChIP assay revealed that knock-down of MBD2 resulted in depletion of MBD2 enrichment at the CpG island (Figure 5B). Furthermore, knock-down of MBD2 was not compensated by the binding of MBD1 or Mecp2. In addition, in

MBD2 knock-down QBC₉₃₉ cells, 5-Aza-2-CdR and TSA treatments had an innocent effect on MBD2 enrichment (Figure 5D). These findings suggest that the enrichment of MBD2 is specific to the methylated region of miR-373 promoter-associated CpG island, MBD2 likely mediates CpG methylation-dependent inhibition of miR-373 in hilar cholangiocarcinomas.

miR-373 negatively regulates MBPs 2 expression by targeting the 3'UTR

One putative miR-373 binding site was predicted to have greater specificity to MBD2 3'UTR, ranging from dinucleotide 295 to 301 bp, as predicted by four algorithms (TargetScan, PicTar, miRanda, miRbase Target) (Figure 6A). To investigate whether the 3'UTR of MBD2 is a functional target of miR-373, wild-type MBD2-3'UTR vector was transfected into HEK293 cells with pre-miR-373, which led to a decrease of 55.8% reporter activity compared to the pre-miR-neg (Figure 6B, $P < 0.001$). After the conserved targeting regions for miR-373 recognition were mutated, relative luciferase activity of the reporter gene was also restored (Figure 6B). These observations suggest that the predicted complementary sequence in MBD 3'UTR is a functional element of miR-373.

On the contrary, enhanced expression of miR-373 by transfecting pre-miR-373 into QBC₉₃₉ cells resulted in a significant reduction of MBD2 protein (Figure 6C). Furthermore, reactivation of miR-373 expression in epigenetic-treated QBC₉₃₉ cells (Figure 3B) led to an inhibition

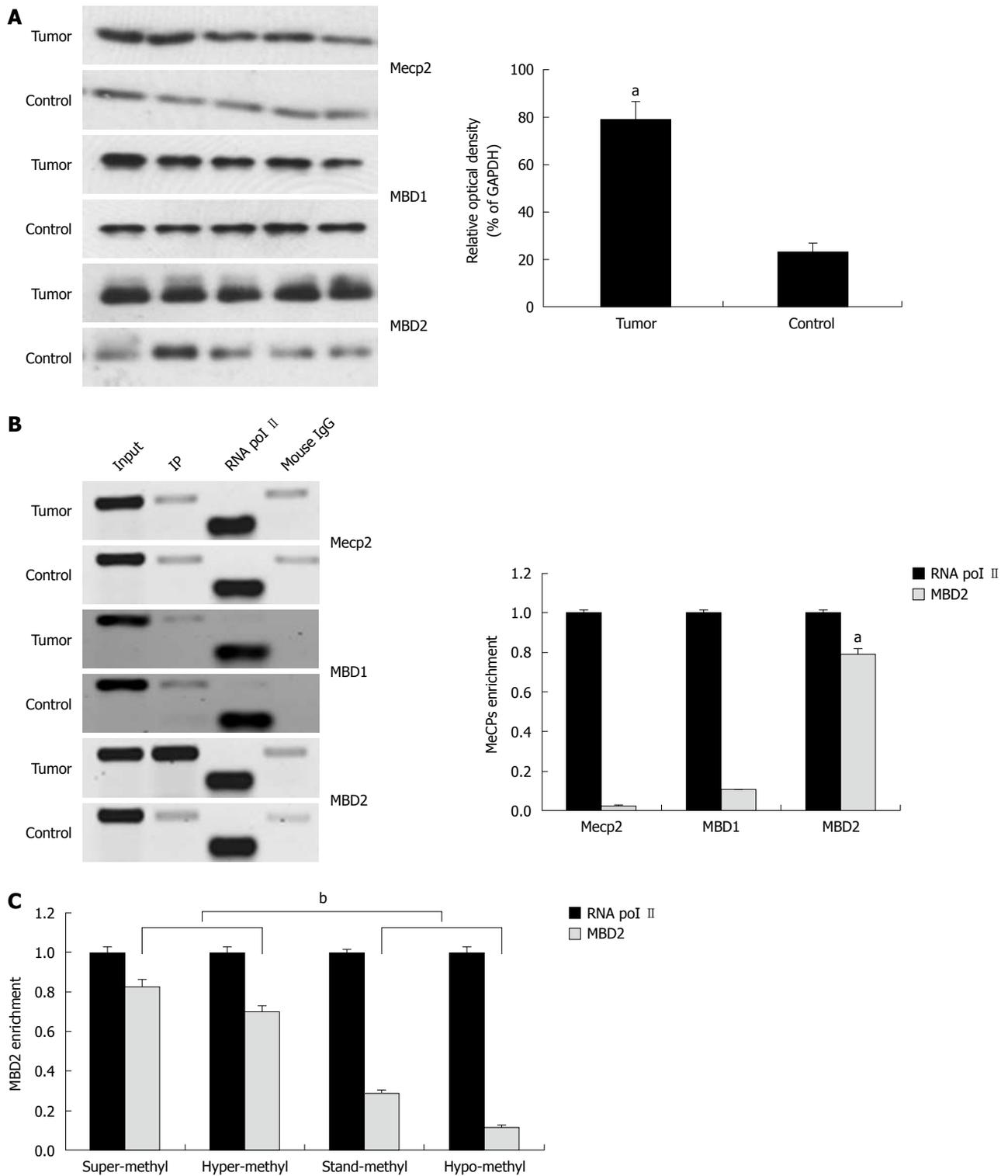


Figure 4 Methyl-CpG binding domain proteins expression and enrichment in fragment of promoter-associated CpG island. A: Expression methyl-CpG binding domain proteins (MBPs) in hilar cholangiocarcinoma. Compared to control, 2.9-fold increase of methyl-CpG-binding domain protein (MBD)2 was found while no difference of MBD1 and Mecp2 were detected; B: Chromatin immunoprecipitation (ChIP)-polymerase chain reaction analysis showed selective enrichment of MBD2 at region of CpG island; C: Correlation between MBD2 enrichment and different frequency of CpG island methylation. Remarkable difference was observed between super-/hyper-methylation and stand-/hypo-methylation group (^a*P* < 0.05, ^b*P* < 0.01 vs control). GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

of MBD2 protein (Figure 6D). Taken together, these findings suggest that MBD2 3'UTR carries a miR-373 regulatory site, and miR-373 can negatively regulate MBD2 through binding to the miRNA locus of MBD2 3' UTR.

DISCUSSION

In previous studies, miR-373 has displayed controversial characteristics in different cancers. In testicular germ cell tumors^[27], esophageal cancer^[28], and breast cancer^[29],

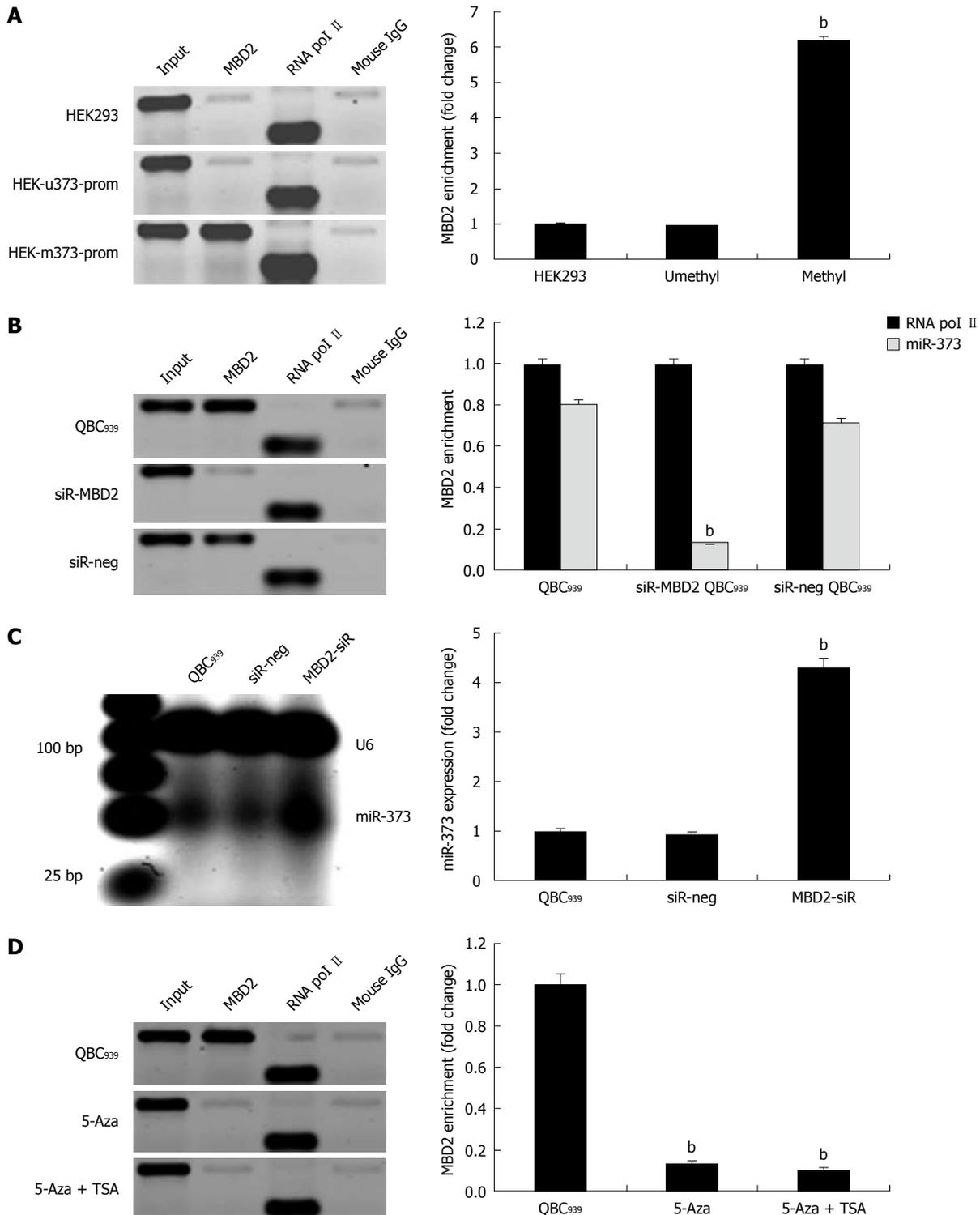


Figure 5 Enrichment of methyl-CpG-binding domain protein 2 at CpG island region is essential for methylation-mediated silencing of *miR-373* gene. A: Enrichment of methyl-CpG-binding domain protein (MBD)2 in fragment of CpG island in HEK-m373-prom stable cells showed 6.2-fold increase compared to control and no significant change was detected in HEK-u373-prom cells; B: MBD2 enrichment in MBD2-siRNA QBC₉₃₉ cells presented dramatically deduction compared to QBC₉₃₉ cells; C: Knock-down of MBD2 induced a increase of *miR-373* expression in QBC₉₃₉ cells; D: Epigenetic treatment of QBC₉₃₉ cell with 5-Aza-CdR or combination with trichostatin A (TSA) eliminated the recruitment MBD2 at the region of *miR-373* promoter-associated CpG island. ^b*P* < 0.01 vs background.

miR-373 behaves as a novel oncogene. Whereas in prostate cancer^[30] and malignant cholangiocytes, including the extrahepatic cholangiocarcinoma cell line^[31], *miR-373* shows characteristics of a tumor suppressor. Regardless of this divergence, it has been well established that

miR-373 participates in tumorigenesis, invasion, and metastasis by mediating gene expression.

In this study, we show that *miR-373* is dramatically down-regulated in hilar cholangiocarcinoma, and correlates closely with poor cell differentiation, advanced

miR-373 binding Mecp2 3'UTR

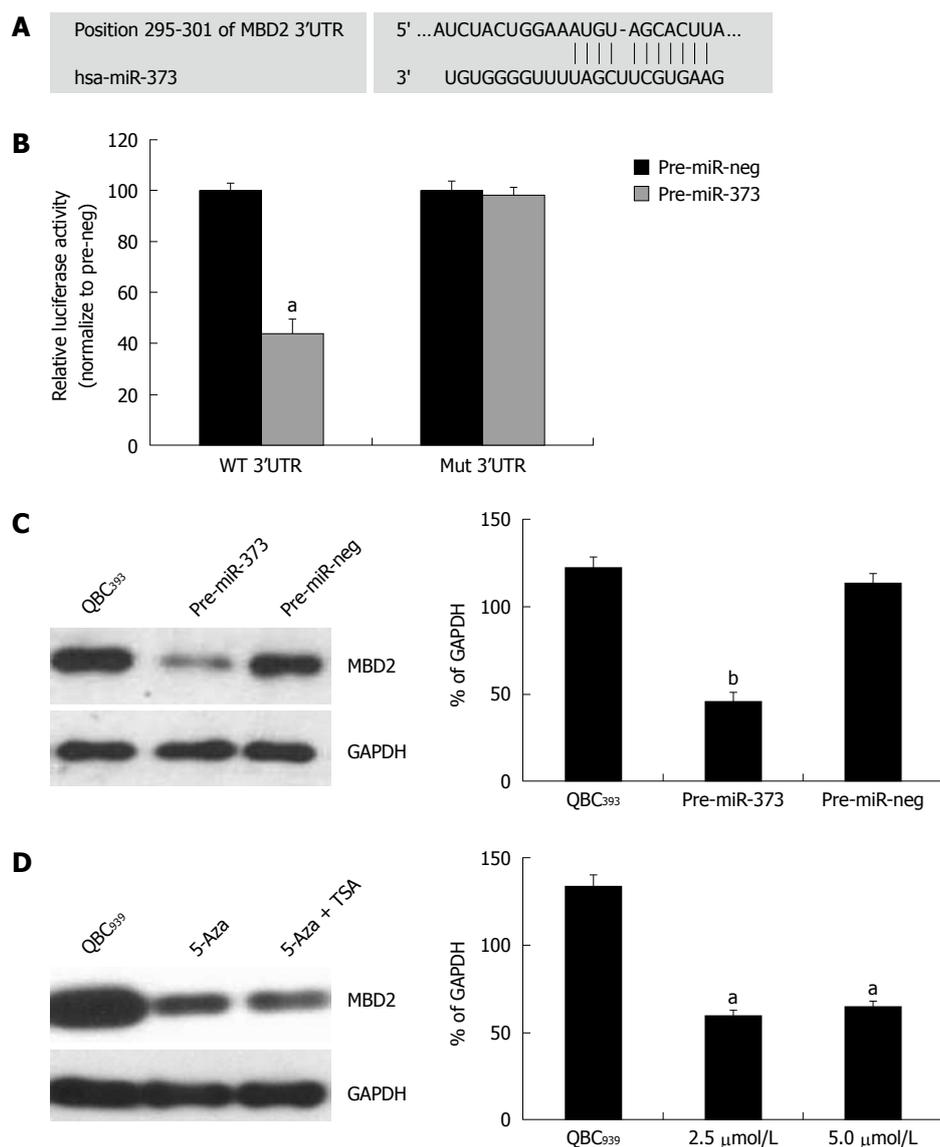


Figure 6 miR-373 negatively regulates methyl-CpG-binding domain protein expression through binding to three prime untranslated region. A: miRNA target prediction screened one computative miR-373 binding site at methyl-CpG-binding domain protein (MBD)2-three prime untranslated region (3'UTR); B: 3'UTR luciferase reporter assay showed a reduction of relative luciferase activity of wild-type MBD2 3'UTR by pre-miR-373 in HEK293 cells; C: Exogenous miR-373 down-regulates MBD2 protein in QBC₉₃₉; D: Epigenetic treatment of QBC₉₃₉ cells inhibits MBD2 protein following reactivation of miR-373 (Figure 3A). ^a*P* < 0.05, ^b*P* < 0.01 vs background. TSA: Trichostatin A; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

clinical stages, shorter overall survival, and disease-free survival. Our findings are in agreement with the last two reports. Although it is difficult to definitively explain these directly opposing results, the expression pattern of individual miRNAs with strict tissue- and clinical-feature-specificity^[32], and the different target genes involved in the unique regulation network of various cancers, could lead to these discrepancies.

Epigenetic dysregulation of miRNAs in human cancer constitutes an emerging mechanism implicated in the development of cancer^[33]. Great effort has been devoted to understanding the relevance of aberrant CpG methylation patterns, and their roles in gene transcription. The inverse correlation between miR-373 expression and hilar cholangiocarcinoma progression prompted us to study

the molecular mechanisms underlying *miR-373* gene inhibition. In the present study, miR-373 promoter-associated CpG island was found to be hypermethylated in tumor tissues and QBC₉₃₉ cells. Reciprocal assays were performed with demethylation of the CpG island by treatment of QBC₉₃₉ cells with 5-aza-CdR in the absence or presence of combination with TSA, which contributes to the reactivation of miR-373. In addition, pre-methylation of pGL4-373-prom *in vitro* inhibited luciferase activity. Together, the results presented here provide evidence that promoter-associated CpG island methylation is a major cause of *miR-373* gene suppression in hilar cholangiocarcinoma.

Promoter-associated CpG-methylation, along with MBPs and HDACs, has been identified as a major epi-

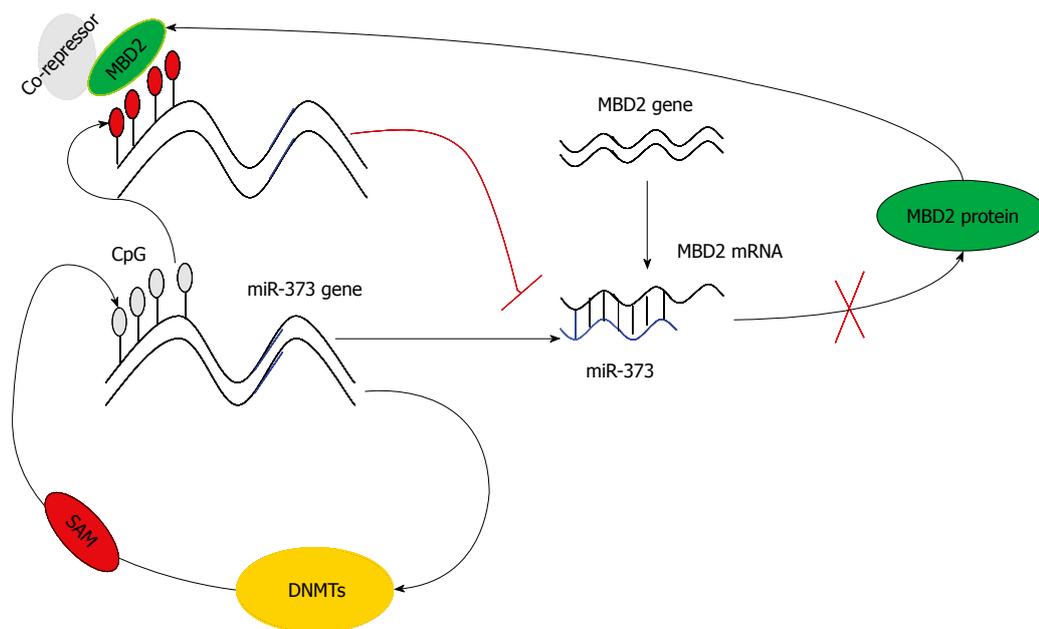


Figure 7 Dual regulation between miR-373 and methyl-CpG-binding domain protein 2. miR-373 is one direct transcriptional target and negative regulator to methyl-CpG-binding domain protein (MBD)2 through a feedback loop of CpG methylation. SAM: S-adenosylmethionine; DNMTs: DNA methyltransferases.

genetic event in the loss of gene expression during tumor progression^[34]. In this study, we further investigated the involvement of MBPs in methylation-mediated suppression of miR-373 in hilar cholangiocarcinoma. MBD2 is an exclusive MBP that is aberrantly expressed in hilar cholangiocarcinoma. ChIP assays showed that MBD2 selectively bound to methylated CpG sequences. Moreover, silencing conferred by DNA methylation and MBD2 enrichment in QBC₉₃₉ cells was reversed by treatment with 5-aza-CdR and TSA. These findings indicate that MBD2 plays an important role in recruiting transcription-repressive machinery to the methylated promoter, thereby suppressing transcriptional activation of miR-373. Confirming these findings, siRNA knock-down of MBD2 triggered a stimulation of miR-373 in QBC₉₃₉ cells. Taken together, these data suggest that MBD2 is an important factor in methylation-mediated inhibition of miR-373.

The reciprocal correlation of expression between miR-373 and MBD2 encouraged us to explore whether miR-373 is a negative regulator of MBD2. To this end, four algorithms were used to predict the alignment of miR-373 with MBD2 3'UTR. In consensus, the seed region of miR-373 matched nucleotides 295-301 of the MBD2 3'UTR, suggesting the ability of miR-373 to directly bind to MBD2 mRNA. However, not all miRNAs identified in this manner are likely to be functional since factors such as steric hindrance may render them inaccessible to the mRNA^[35]. Hence, functional validation experiments including transfection of miR-373 precursors showed that miR-373 can down-regulate the relative luciferase activity of MBD 3'UTR reporter vectors and MBD2 protein in QBC₉₃₉ cell lines. These findings indicate that miR-373 functionally regulates the expression of MBD2 by targeting the 3'UTR.

In this study, several interesting observations were made. First, the heterozygous methylation of miR-373, as indicated by both methylated and unmethylated bands in MSP, was detected in 12 cancers and five normal bile duct tissues. This is contrary to the 'all-or-none' manner of DNA methylation in regulating gene expression. Although the mechanism is unclear, there are several points to keep in mind: (1) *miR-373* gene exhibits allele-specific DNA methylation (ASM) which means that only one allele is methylated and the other one is unmethylated^[36]. ASM has been documented in a number of cancer cases except imprinted regions and X chromosomes; (2) samples contain normal and malignant cells although multiple efforts have been adapted to obtain purified tissues; and (3) miR-373 displays discrepant methylation in various differentiated cells. Secondly, in primary samples, three supermethylated tumors were characterized with relatively high miR-373 expression. The relevant mechanism underlying transcription that escapes methylation-mediated suppression is unknown, but whether MBPs bind effectively to the methylated CpG dinucleotides may determine the expression level.

Based on these findings, we conclude that due to the hypermethylation of the promoter-associated CpG island and enrichment of MBD2, the function of miR-373 is restrained rendering it unable to inhibit MBD2. As a result, the expression of MBD2 is predominantly enhanced, leading to a strong inhibitory effect on miR-373 (Figure 7). This dysregulation ultimately results in the promotion of tumorigenesis and the development of hilar cholangiocarcinoma. In conclusion, our study proves that miRNA-373 behaves as a direct transcriptional target and negative regulator of MBD2 through a feedback loop of CpG methylation in hilar cholangiocarcinoma.

COMMENTS

Background

Both DNA methylation and microRNAs (miRNAs) are epigenetic and play vital roles in tumorigenesis and development of human malignance. DNA methylation represses transcription by impeding recognition of transcriptional activators to DNA sequences or recruiting methyl-CpG binding domain proteins (MBPs) to modify chromatin compaction and control gene silencing. miRNAs regulate gene expression mainly by binding to the three prime untranslated region of target mRNAs, leading to mRNA degradation or translation inhibition. Many studies have reported that the expression of miRNAs gene is regulated by DNA methylation, and in addition, DNA methyltransferases and MBPs are regulated by miRNAs.

Research frontiers

Hilar cholangiocarcinoma displays highly aggressive malignancy. Many studies have reported miRNA expression and DNA methylation in hilar cholangiocarcinoma. In this study, the authors report evidence of the role of miR-373 in hilar cholangiocarcinoma. In particular, they show that miR-373 behaves as a direct transcriptional target and negative regulator of methyl-CpG-binding domain protein (MBD)2 through a feedback loop of CpG methylation in hilar cholangiocarcinoma.

Innovations and breakthroughs

It has been well established that miR-373 participates in tumorigenesis, invasion, and metastasis by mediating gene expression. In this study, the authors demonstrate that miR-373 is dramatically down-regulated in hilar cholangiocarcinoma, and closely correlates with poor cell differentiation, advanced clinical stage, shorter overall survival, and disease-free survival. The authors show that due to the hypermethylation of the promoter-associated CpG island and enrichment of MBD2, function of miR-373 is restrained, rendering it unable to inhibit MBD2. As a result, MBD2 expression is predominantly enhanced and has a strong inhibitory effect on miR-373. This dysregulation finally promotes the tumorigenesis and development of hilar cholangiocarcinoma.

Applications

This study provides the first evidence showing that miR-373 behaves as a direct transcriptional target and negative regulator of MBD2 through a feedback loop of CpG methylation in hilar cholangiocarcinoma. These results shed light on the mutual regulation between miRNA-373 and MBD2, which may eventually serve as useful biomarkers as well as therapeutic targets.

Peer review

This study demonstrates the role of miR-373 in cholangiocarcinoma. In particular, the authors showed that miR-373 acts through a feedback loop of CpG methylation. This study is well designed and performed, and is of great interest for its novelty and impact in the field.

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Moro orange juice prevents fatty liver in mice

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Abstract

AIM: To establish if the juice of *Moro*, an anthocyanin-rich orange, may improve liver damage in mice with diet-induced obesity.

METHODS: Eight-week-old mice were fed a high-fat diet (HFD) and were administered water or *Moro* juice for 12 wk. Liver morphology, gene expression of lipid transcription factors, and metabolic enzymes were assessed.

RESULTS: Mice fed HFD displayed increased body weight, insulin resistance and dyslipidemia. *Moro* juice administration limited body weight gain, enhanced insulin sensitivity, and decreased serum triglycerides and total cholesterol. Mice fed HFD showed liver steatosis associated with ballooning. Dietary *Moro* juice markedly improved liver steatosis by inducing the expression of peroxisome proliferator-activated receptor- α and its target gene *acylCoA-oxidase*, a key enzyme of lipid oxidation. Consistently, *Moro* juice consumption suppressed the expression of liver X receptor- α and its target gene fatty acid synthase, and restored liver glycerol-3-phosphate acyltransferase 1 activity.

CONCLUSION: *Moro* juice counteracts liver steatogenesis in mice with diet-induced obesity and thus may represent a promising dietary option for the prevention of fatty liver.

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Key words: Liver steatosis; Anthocyanins; Lipogenesis; Lipid oxidation

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a chronic

metabolic disorder with significant impact on cardiovascular and liver-related mortality^[1,2]. NAFLD is closely associated with obesity, dyslipidemia, diabetes and the full spectrum of the metabolic syndrome^[3,4], with insulin resistance as a common pathophysiological determinant^[5]. Lifestyle, that is, dietary habits and physical activity, plays a pivot role in the pathogenesis of the metabolic syndrome^[6]. Likewise, dietary factors have been shown to exert a major role in the development of fatty liver^[7]. Recently, the consumption of foods and beverages containing fructose has been identified as a risk factor for NAFLD^[8]. Nonetheless, despite their fructose content, fruits are also rich in different polyphenolic compounds that exert several beneficial effects on health, mainly by modulating expression of key enzymes involved in glucose sensitivity and lipid homeostasis^[9].

Anthocyanins (ACNs) are water-soluble plant polyphenolic pigments that confer a typical blue, red or purple color, and are contained especially in berries, blood oranges and pigmented corn and potato^[10]. ACNs have been attributed several putative therapeutic roles, including beneficial effects on obesity and related metabolic complications^[11]. In this respect, Tsuda *et al.*^[12] firstly showed that ACN from purple corn prevented obesity, dyslipidemia and visceral fat inflammation in mice fed a high-fat diet (HFD). Recently, a further demonstration of the putative hepatoprotective properties of ACN has been provided by Liu *et al.*^[13], who observed that administration of protocatechuic acid, the major *in vivo* metabolite of the main ACN cyanidin-3-O- β -glucoside, reduced gene expression of lipogenic enzymes in the liver, thus leading to a decreased hepatic lipid accumulation. *In vitro* and *in vivo* experiments suggest that ACNs can modulate gene expression of several adipocytokines and regulate the pathways involved in lipogenesis and fat accumulation^[12,14]. ACNs are contained also in blood oranges, a variety of sweet orange (*Citrus sinensis*) with an intense red pigmentation^[15]. A recent study has demonstrated that blood orange consumption inhibits fat accumulation in mice^[16] and may represent a promising dietary tool for the treatment of obesity.

In the current study, we aimed at clarifying whether consumption of the juice of *Moro*, a blood orange cultivated in Sicily, Italy, improved liver steatosis in mice fed HFD; a physiological model of NAFLD and metabolic syndrome.

MATERIALS AND METHODS

Animals and treatments

All procedures fulfilled the Italian Guidelines for the Use and Care of Laboratory Animals. Eight-week-old male C57BL6/J mice were purchased from Charles River Laboratories (Calco, Italy). Animals were maintained in a temperature- and light-controlled facility for 12 wk. Diets were obtained by Harlan Teklad (Madison, WI, United States). The standard diet (SD) provided 3.3 kcal/g with 60% carbohydrates, 23% proteins and 17%

fat. The HFD provided 5.2 kcal/g with 60% fat, 20% proteins and 20% carbohydrates. Mice were distributed in three groups: group I included six mice fed SD and permitted *ad libitum* consumption of water (SD + water); group II comprised six mice fed HFD and permitted *ad libitum* consumption of water (HFD + water); group III comprised six mice fed HFD and permitted *ad libitum* consumption of *Moro* orange juice instead of water (HFD + *Moro*). *Moro* fruits were collected in the experimental farm of the Research Center for Citric Culture and Mediterranean Crops (Acireale, Italy). Fruits were immediately stored at 4 °C and squeezed a few days later; the juice obtained was pre-filtered and stored at -20 °C in aliquots of 0.5 L. Every 2 d, frozen juice aliquots were thawed, filtered and put in the bottle of each cage. Fruit juice analysis was performed as previously described^[16]. Food and beverage consumption was recorded twice weekly; body weight was recorded weekly. After sacrifice by CO₂ asphyxiation, blood and liver samples were obtained, processed and stored for further analysis.

Histopathology

Formalin-fixed paraffin-embedded liver sections were stained with haematoxylin-eosin and Masson's trichrome, using standard procedures. Liver injury was blindly evaluated according to the NAFLD activity score.

Biochemical analysis

Serum glucose, total cholesterol, triglycerides and alanine aminotransferase (ALT) were measured using Reflotron Plus system from Roche Diagnostic (Milan, Italy). Liver triglycerides content was measured using a serum/tissue triglyceride colorimetric kit (Biovision, Mountain View, CA, United States). Insulin tolerance test (ITT) was performed on 5-h starved mice, and glycemia was measured immediately before and 15, 30 and 60 min after intraperitoneal injection of 0.4 U/kg recombinant human insulin. The total amount of ACN in *Moro* juice was determined as previously described^[16]. Glycerol-3-phosphate acyltransferase 1 (GPAT1) activity was assayed as previously described^[17].

RNA extraction and real-time polymerase chain reaction

Total RNA was extracted by homogenizing snap frozen liver samples in TRIzol reagent (Invitrogen, Milan, Italy). Quantitative real-time polymerase chain reaction (PCR) was performed in 7900HT Fast Real-Time PCR System Applied Biosystems (Applied Biosystems, Foster City, CA, United States), using the EXPRESS SYBR GreenER™ qPCR SuperMix with Premixed ROX (Invitrogen).

Reactions were performed in a 20 μ L mixture containing cDNA, specific primers of each gene, and the SYBR GreenER™ qPCR SuperMix. The specific PCR products were detected by the fluorescence of SYBR Green, the double-stranded DNA binding dye. The relative mRNA expression level was calculated by the threshold cycle (Ct) value of each PCR product and normalized with that of glyceraldehyde-3-phosphate de-

Table 1 Primer sequences for real-time polymerase chain reaction

Gene name	Forward	Reverse
PPAR- α	5'-AGTCAAGGTGTGGCCCAAGGT-3'	5'-TGTCTATCGGACACTAGCGGAGGC-3'
AOX	5'-CTTGTTCCGGCAAGTGAGG-3'	5'-CAGGATCCGACTGTTTACC-3'
LXR- α	5'-TGCCATCAGCATCTTCTCTG-3'	5'-GGTCACCAGCTTCATTAGC-3'
FAS	5'-AGCCCACGTCGTAGCAAACCA-3'	5'-GCAGGGGCTCTTGACGGCAG-3'
HMG-CoA reductase	5'-CCTGACACTGAACTGAAGCG-3'	5'-TCTTTCCAG AACACAGCACG-3'
GAPDH	5'-ACCACCATGGAGAAGGCCGG-3'	5'-CTCAGTGTAGCCCAAGATGC-3'

PPAR: Peroxisome proliferator-activated receptor; AOX: Acyl-CoA oxidase; LXR: Liver X receptor; FAS: Fatty acid synthase; HMG: Hydroxy methylglutaryl; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

hydrogenase by using comparative $2^{\Delta\Delta Ct}$ method. Primer sequences are shown in Table 1.

Statistical analysis

Statistical analysis was performed by GraphPad Prism software (San Diego, CA, United States). Data are the results of three independent experiments. All results were expressed as mean \pm SE. One-way analysis of variance with Bonferroni post-hoc analysis was used for parametric data. Kruskal-Wallis test was used for non-parametric data. *P* values < 0.05 were considered significant.

RESULTS

Moro juice improves dyslipidemia and enhances insulin sensitivity

Moro juice had an ACN content of 85 mg/L. Food intake was identical among the three experimental groups (SD + water, 3.1 ± 0.5 g/d *vs* HFD + water, 3.0 ± 0.8 g/d *vs* HFD + *Moro*, 3.1 ± 0.7 g/d). The amount of *Moro* juice intake (4.1 ± 0.75 mL/d) did not differ from the amount of water intake in the control groups (4.0 ± 1.2 mL/d). All mice had similar body weight at the start of the experiment, and after 12 wk, mice fed HFD + water had higher body weight compared with SD mice (Figure 1A). Strikingly, mice fed HFD + *Moro* had the same body weight gain as mice fed SD. This effect on body weight gain occurred despite the fact that mice fed HFD + *Moro* received a 10% higher energy intake, due to the sugar content of the juice, as compared to mice fed HFD + water (Figure 1A). Obese mice had increased serum total cholesterol, triglycerides and ALT as compared to the SD group (Figure 1B-D). *Moro* juice decreased serum total cholesterol and triglycerides, and reduced serum ALT to the levels of lean controls (Figure 1B-D). Furthermore, orange juice consumption significantly enhanced insulin sensitivity, as demonstrated by the area under curve derived from ITT, which was 8685 ± 516 in mice fed HFD + water and was reduced to 7225 ± 718 in the HFD + *Moro* group (Figure 2).

Moro juice induces liver peroxisome proliferator-activated receptor- α and inhibits liver lipogenesis

Liver sections of mice fed HFD + water showed moderate steatosis with a panacinar pattern (Figure 3B), mild lobular inflammation, and diffuse hepatocyte ballooning.

In the HFD + *Moro* group, steatosis was almost absent (Figure 3C). Consistently, lobular inflammation and ballooning degeneration was less pronounced throughout the hepatic parenchyma. Fibrosis, assessed by Masson's trichrome, was not found in any mice after 12 wk HFD (data not shown). Biochemical analysis confirmed that *Moro* juice induced a marked decrease of liver triglyceride content in mice fed HFD (Figure 3D).

HFD was associated with impaired expression of key transcription factors and metabolic enzymes involved in lipid homeostasis. In particular, we found a decrease in the gene expression of peroxisome proliferator-activated receptor (PPAR)- α (Figure 4A) and acyl-CoA oxidase (AOX) (Figure 4B) and a significant increase in the expression of liver X receptor (LXR)- α (Figure 4C), fatty acid synthase (FAS) (Figure 4D) and hydroxy methylglutaryl (HMG)-CoA reductase (Figure 4E). *Moro* juice markedly decreased the mRNA levels of LXR- α , FAS and HMG-CoA reductase but augmented the mRNA levels of PPAR- α and AOX (Figure 4A-E). Consistent with gene expression findings, GPAT1 activity was restored to the levels of lean animals by *Moro* juice consumption (Figure 4F).

DISCUSSION

In this study, we explored the effect of the consumption of the juice of *Moro*, an ACN-rich orange cultivated in the Mediterranean region, on liver steatosis in mice with diet-induced obesity. In previous experiments in mice fed SD, we observed that C57BL6 mice and other strains tolerated the substitution of drinking water with orange juice as the only drinking source, had similar food intake, and did not show any behavioral abnormality. Here, we demonstrated that *Moro* juice drinking reversed most of the metabolic abnormalities exhibited by obese mice, including fatty liver.

Our results are in agreement with previous experimental data suggesting that ACNs from different fruits and vegetables are able to exert beneficial effects on several metabolic aspects related to obesity^[11]. One common effect of ACN administration both in diet-induced and genetic models of obesity is the reduction of body weight and of visceral fat. In this respect, Kwon *et al*^[18] showed that ACN extracted from black soybean led to improvement in dyslipidemia and a decrease in visceral

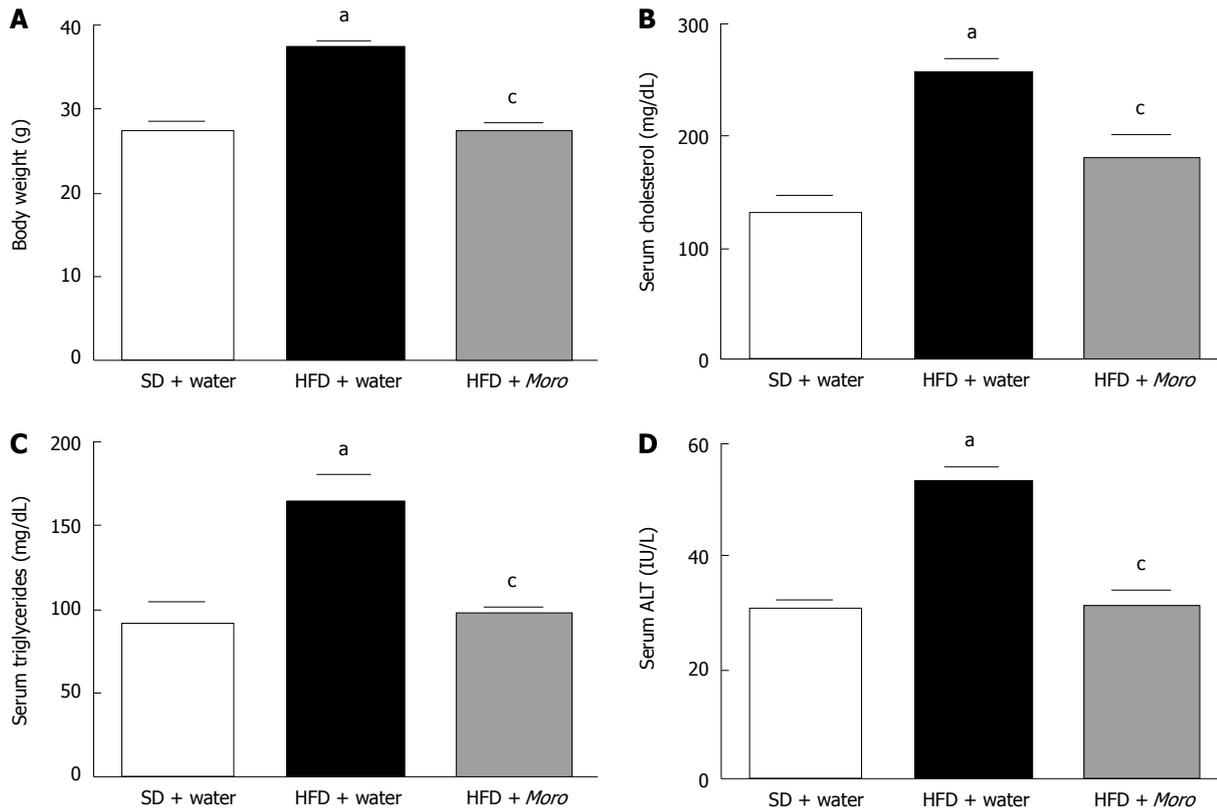


Figure 1 Effects of *Moro* juice on body weight and serum parameters. A: Body weight was markedly increased in mice fed high-fat diet (HFD) and was decreased to the levels of lean mice by *Moro* juice; B: Serum total cholesterol was significantly reduced in mice drinking *Moro*; C and D: Serum triglycerides (C) and alanine aminotransferase (ALT) (D) were restored to the levels of lean mice. ^a $P < 0.05$ vs standard diet (SD) + water; ^c $P < 0.05$ vs HFD + water.

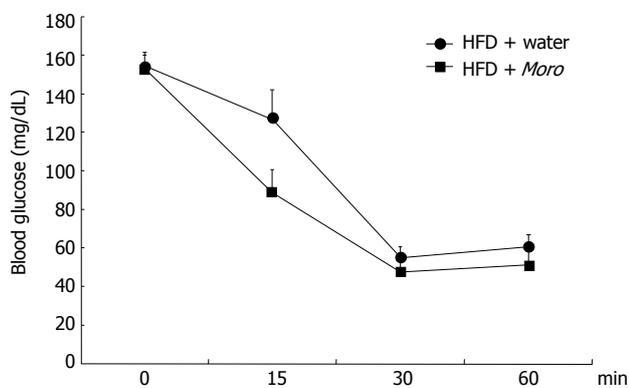


Figure 2 Effects of *Moro* juice on insulin sensitivity. Insulin tolerance test was performed with 0.4 U/kg recombinant human insulin in the two groups of mice fed high-fat diet (HFD); area under curve of blood glucose was 8685 ± 516 in mice fed HFD + water and was reduced to 7225 ± 718 in HFD + *Moro* ($P < 0.05$).

adiposity in rats fed HFD. Similarly, extracts from tart cherries ameliorate dyslipidemia and decrease liver fat content in genetically insulin-resistant rats^[19], as does cyanidin 3-glucoside in db/db mice^[20].

As regards the molecular events underlying the effects of *Moro* on liver lipid metabolism, we demonstrated that a major mechanism is induction of PPAR- α . PPAR- α is a key transcription factor promoting lipolysis and lipid oxidation in different tissues^[21]. Mice lacking PPAR- α develop obesity and liver steatosis^[22]; similarly,

the hepatic levels of PPAR- α are decreased in patients with NAFLD^[23], and pharmacological agonists are able to improve liver steatosis^[24]. Previous studies have suggested that PPAR- α induction is involved in the antisteatotic effect of extracts containing ACNs in different models of obesity^[19,20]. Along with the promotion of lipid oxidation, *Moro* juice consumption induced the inhibition of lipogenesis. In this respect, a major mechanism for the antisteatotic effect is the reversion of LXR- α expression. LXR- α is a nuclear hormone receptor that promotes lipogenesis^[25], which is increased in the liver of patients with NAFLD^[26]. LXR- α stimulates lipogenesis through upregulation of enzymes of *de novo* lipogenesis such as FAS^[25]. Suppression of LXR- α expression and activity exerts potent antisteatotic effects in the liver^[27]; some flavonoids have been shown to inhibit LXR- α ^[28].

Besides the effects on lipid homeostasis, the beneficial impact of *Moro* on insulin sensitivity is also noteworthy. Insulin resistance is the metabolic hallmark of patients with NAFLD^[29]. Studies using the hyperinsulinemic-euglycemic clamp coupled with infusion tracers have demonstrated that hepatic fat content is directly related to insulin resistance in the liver, skeletal muscle, and adipose tissue of obese subjects^[5]. In contrast, recent findings have demonstrated that liver triglyceride content, not visceral adipose tissue volume, predicts the impairment of insulin sensitivity and of very low density lipoprotein secretion in patients with obesity^[30,31].

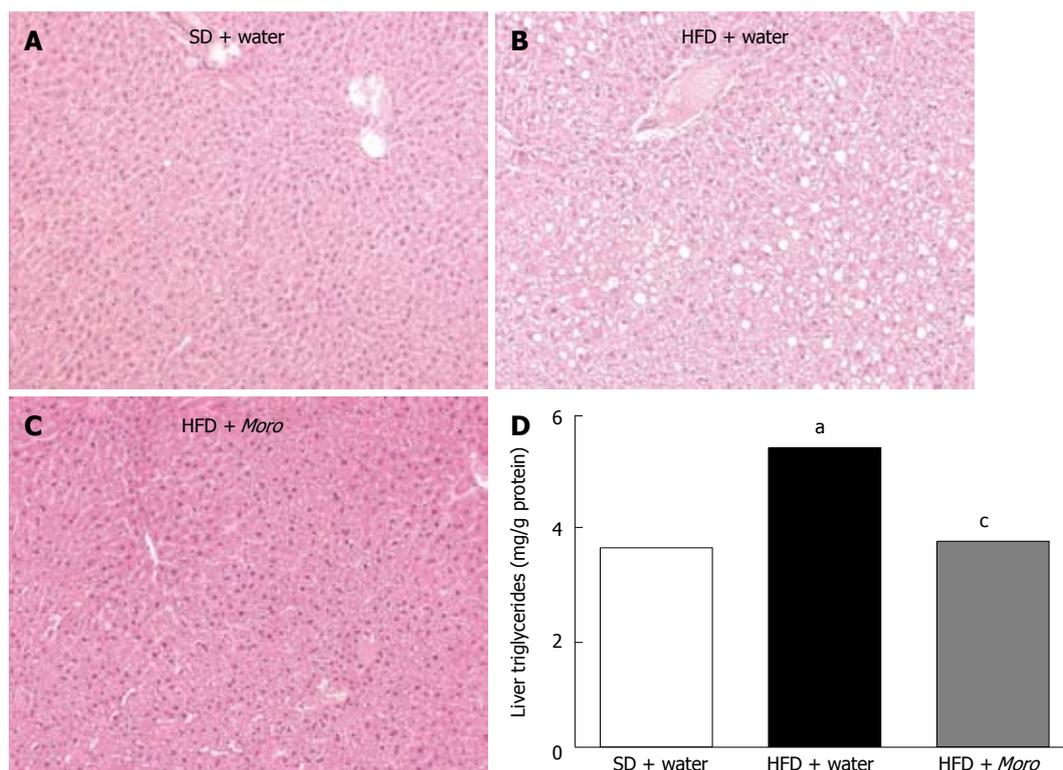


Figure 3 Effects of *Moro* juice on liver histology and liver triglycerides content. A: Hematoxylin-eosin-stained liver sections showing normal histology in lean mice; B: Moderate panacinar steatosis and hepatocyte ballooning in mice fed high-fat diet (HFD); C: Liver sections of mice fed HFD + *Moro* showing absence of steatosis and ballooning; D: Liver triglycerides content was significantly decreased in mice drinking orange juice. ^a*P* < 0.05 vs standard diet (SD) + water, ^c*P* < 0.05 vs HFD + water. Magnification: 10 ×.

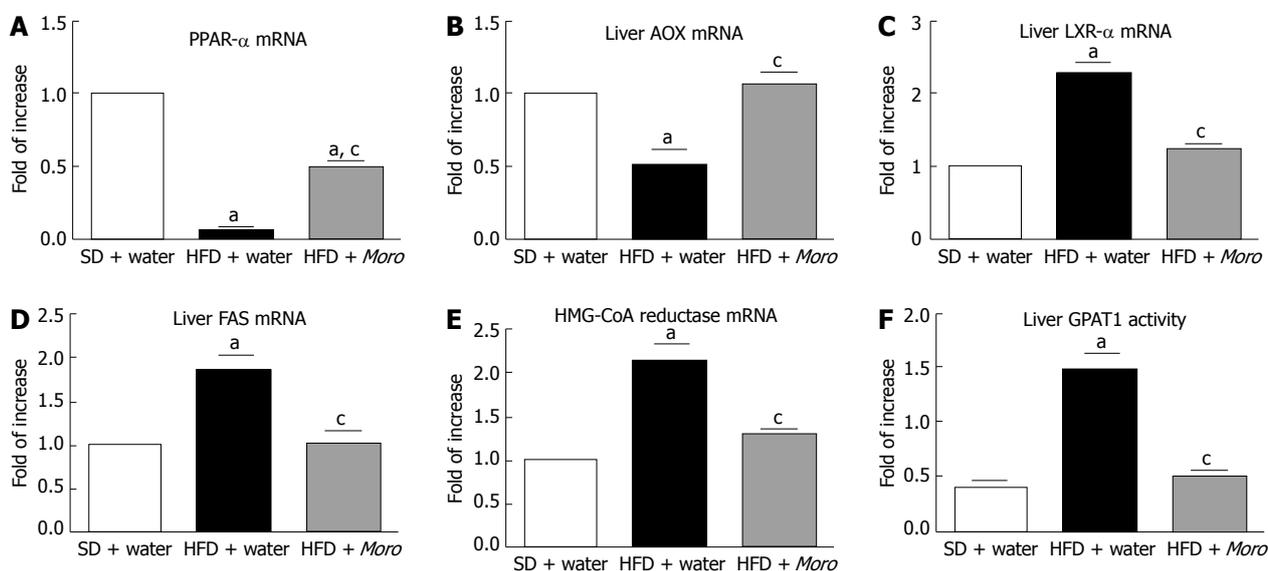


Figure 4 Effects of *Moro* juice on liver lipid homeostasis. Gene expression of (A) peroxisome proliferator-activated receptor (PPAR)-α and (B) acyl-CoA oxidase (AOX) was significantly increased in mice fed high-fat diet (HFD) + *Moro*, whereas gene expression of (C) liver X receptor (LXR)-α, (D) fatty acid synthase (FAS) and (E) hydroxy methylglutaryl (HMG)-CoA reductase was markedly reduced by orange juice; Liver glycerol-3-phosphate acyltransferase 1 (GPAT1) was increased in mice fed HFD + water and was restored to control levels in mice drinking *Moro* juice (F). ^a*P* < 0.05 vs standard diet (SD) + water, ^c*P* < 0.05 vs HFD + water.

Although mice drinking *Moro* juice remained hyperglycemic, probably because of the sugar content, the ITT unequivocally revealed that *Moro* juice exerted insulin-sensitizing activity. An insulin-sensitizing effect has been

demonstrated for ACN-rich extracts from bilberry in genetically obese mice^[32], and for dietary blueberry in mice fed HFD^[33].

In conclusion, in this study, we demonstrated that the

juice of *Moro* exerts metabolic hepatoprotective effects due to changes in the expression of several enzymes involved in lipid homeostasis. Thus, the dietary administration of this food may be effective in preventing liver steatosis, and may be considered as a nutritional approach for the prevention of NAFLD. Clinical trials are now warranted.

COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) is a chronic metabolic disorder with significant impact on cardiovascular and liver-related mortality. Lifestyle, that is, dietary habits and physical activity, plays a pivot role in the pathogenesis of NAFLD.

Research frontiers

Anthocyanins (ACNs) are water-soluble plant polyphenolic pigments that confer a typical blue, red or purple color, and are contained especially in berries, blood oranges, and pigmented corn and potato. ACNs have been attributed several putative therapeutic roles, including beneficial effects on obesity and related metabolic complications. Diet enriched with ACNs may provide a useful tool to counteract liver steatogenesis.

Innovations and breakthroughs

ACNs are contained in blood oranges, a variety of sweet orange (*Citrus sinensis*) with an intense red pigmentation. A recent study has demonstrated that blood orange consumption inhibits fat accumulation in mice. Furthermore, the administration of protocatechuic acid, the major *in vivo* metabolite of ACN, reduces the activity of lipogenic enzymes in the liver, thus leading to decreased hepatic lipid accumulation. The data in this article demonstrated for the first time that *Moro* juice counteracted liver steatogenesis in mice with diet-induced obesity, through modulation of enzymes involved in lipogenesis and lipid oxidation. Thus, *Moro* juice consumption may represent a promising dietary option for prevention of fatty liver.

Terminology

ACNs are water-soluble pigments that belong to the family of flavonoids. They are contained in berries, blood oranges, and pigmented corn and potato. They are able to modulate lipid and glucose metabolic pathways in humans.

Peer review

It is interesting to readers and may be effective dietary supplements for NAFLD. The manuscript described an interesting finding about the protective effect of this orange juice on the development of fatty liver induced by a high-fat diet in mice.

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A totally mini-invasive approach for colorectal laparoscopic surgery

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Abstract

AIM: To study the short-term outcome of patients treated with laparoscopic right colectomy and how intracorporeal anastomosis has improved the outcome.

METHODS: We retrospectively examined all patients affected by colorectal cancer who underwent a laparoscopic right colectomy between January 2006 and December 2010 in our department. Our evaluation criteria were: diagnosis of colorectal carcinoma at presurgical biopsy, elective surgery, and the same surgeon. We excluded: emergency surgery, conversions from laparotomic colectomy, and other surgeons. The endpoints we examined were: surgical time, number of lymph nodes removed, length of stay (removal of nasogastric tube, bowel movements, gas evacuation, solid and liquid feeding, hospitalization), and major complications. Seventy-two patients were divided into two groups: intracorporeal anastomosis (39 patients)

and extracorporeal anastomosis (33 patients).

RESULTS: Significant differences were observed between intracorporeal vs extracorporeal anastomosis, respectively, for surgical times (186.8 min vs 184.1 min, $P < 0.001$), time to resumption of gas evacuation (3 d vs 3.5 d, $P < 0.001$), days until resumption of bowel movements (3.8 d vs 4.9 d, $P < 0.001$), days until resumption of liquid diet (3.5 d vs 4.5 d, $P < 0.001$), days until resuming a solid diet (4.6 d vs 5.7 d, $P < 0.001$), and total hospitalization duration (7.4 d vs 8.5 d, $P < 0.001$). In the intracorporeal group, on average, 19 positive lymph nodes were removed; in the extracorporeal group, on average, 14 were removed ($P < 0.001$). Thus, intracorporeal anastomosis for right laparoscopic colectomy improved patient outcome by providing faster recovery of nutrition, faster recovery of intestinal function, and shorter hospitalization than extracorporeal anastomosis.

CONCLUSION: Short-term outcomes favor intracorporeal anastomosis, confirming that a less traumatic surgical approach improves patient outcome.

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Key words: Anastomosis; Cancer; Colorectal disease; Surgery; Laparoscopy

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INTRODUCTION

Over the last decades, colorectal laparoscopic surgery has outachieved traditional surgery for safety and oncological radicality. Some studies such as COST^[1], CLAS-ICC^[2], Barcelona^[3], and COLOR^[4] have shown short-term postoperative advantages without compromising long-term oncological results. For right colectomy, laparoscopic surgery was established later because of technical difficulties related to anatomic and vascular variability^[5]. The re-establishment of intestinal transit with ileocolic anastomosis can be performed with two techniques: extracorporeal anastomosis and intracorporeal anastomosis. In extracorporeal anastomosis, also called “laparoscopic-assisted colectomy”, stitching (manual or mechanical) and vascular resection are done extracorporeally by externalizing the bowel through a cutaneous mini-incision. On the other hand, the other type of anastomosis is totally intracorporeal, and the stitching is often performed mechanically. A case-control study showed that laparoscopic right colectomy performed with intracorporeal anastomosis is considered one of the most difficult surgeries after transverse resection, rectum resection, and recanalization after Hartmann’s resection. However, the ligation of ileocolic vessels and the medial-to-lateral dissection of the right mesocolon intracorporeally are more oncologically safe in terms of the number of lymph nodes removed and cancer management. Nevertheless, extracorporeal anastomosis is the most popular as the literature shows, although it has some limitations in terms of the number of accesses and complications after bowel externalization^[6-11].

We evaluated the short-term outcome of patients with colorectal cancer who underwent a laparoscopic right colectomy, and we showed that intracorporeal anastomosis improved patient outcome (shorter hospitalization, fewer postoperative complications, and better oncological radicality). Finally, we considered the influence of the “learning curve” on these surgical techniques.

MATERIALS AND METHODS

We retrospectively examined all patients with colorectal cancer who underwent a laparoscopic right colectomy between January 2006 and December 2010 in our department. Our evaluation criteria were elective surgery and the same surgeon as the first operator. We excluded patients undergoing emergency surgery, conversions from laparotomic colectomy, and other surgeons as the first operator. Patients were divided into two groups: the first included those who underwent a right colectomy with intracorporeal anastomosis, and the second included those who underwent a right colectomy with extracorporeal anastomosis. Patient data included age, gender, body mass index (BMI), American Society of Anesthesiology (ASA) class, and surgical history. We also obtained other data concerning the operation including the surgical time, preoperative diagnosis, and number of lymph nodes removed. We noted parameters of post-

Table 1 Homogeneous groups

Patients		Intracorporeal <i>n</i> = 39	Extracorporeal <i>n</i> = 33	
Age (yr)	Median	74.5	74	NS
	Min-max	53-89	45-96	
Gender	Male	24	20	NS
	Female	15	13	
	Ratio M/F	1.6	1.5	
Weight (kg)	Median	71	77	NS
	Min-max	50-90	51-120	
Height (cm)	Median	165	167	NS
	Min-max	148-182	146-183	
BMI (kg/m ²)	Median	26.3	28.1	NS
	Min-max	20-37	19.9-37	

BMI: Body mass index; M: Male; F: Female; NS: Not significant.

surgery hospitalization including removal of nasogastric tube, resumption of bowel movements, resumption of gas evacuation, time to consumption of solid and liquid feeding. We also considered major complications in terms of post-surgery time and hospitalization.

We obtained data from medical records, surgical cards, and databases. We used JMP software 7a Version [SAS Institute Inc. (1989-2007), Cary, NC, United States] for electronic data processing. Descriptive variables were expressed as mean, standard deviation, mode, median, number of events, patients, and percentage. According to the different features of these variables, we used the χ^2 test, *F* test, and Student’s *t* test as appropriate, and considered *P* < 0.05 to indicate statistical significance.

RESULTS

In this study, 72 patients were divided into two groups: intracorporeal anastomosis (*n* = 39) and extracorporeal anastomosis (*n* = 33). There were no significant differences in age, gender, BMI (Table 1), or ASA class between the two groups (*P* = 0.8645 for ASA).

Twenty patients in the intracorporeal group had a positive abdominal surgical history (51.3%), whereas 21 patients in the extracorporeal group had such a history (63.6%; *P* = 0.8433, Table 2). There were also no significant differences in the diagnosis from the pre-surgery biopsy (Figure 1).

In the intracorporeal group, we performed additional surgical procedures in seven patients during surgery, i.e., one nefrectomy, five colecistectomies, and one intraoperative coloscopy, whereas in the extracorporeal group, we performed two colecistectomies, one intraoperative coloscopy, and one polypectomy.

In the intracorporeal group, we removed an average of 19 lymph nodes (range: 7-36), whereas in the extracorporeal group we removed an average of 14 lymph nodes (range: 2-29, *P* < 0.0001).

The average surgical time was 186.8 min (range: 105-280 min) in the intracorporeal group and 184.1 min in the extracorporeal group (range: 115-285 min, *P* = 0.6549).

Gas evacuation was shorter in the intracorporeal

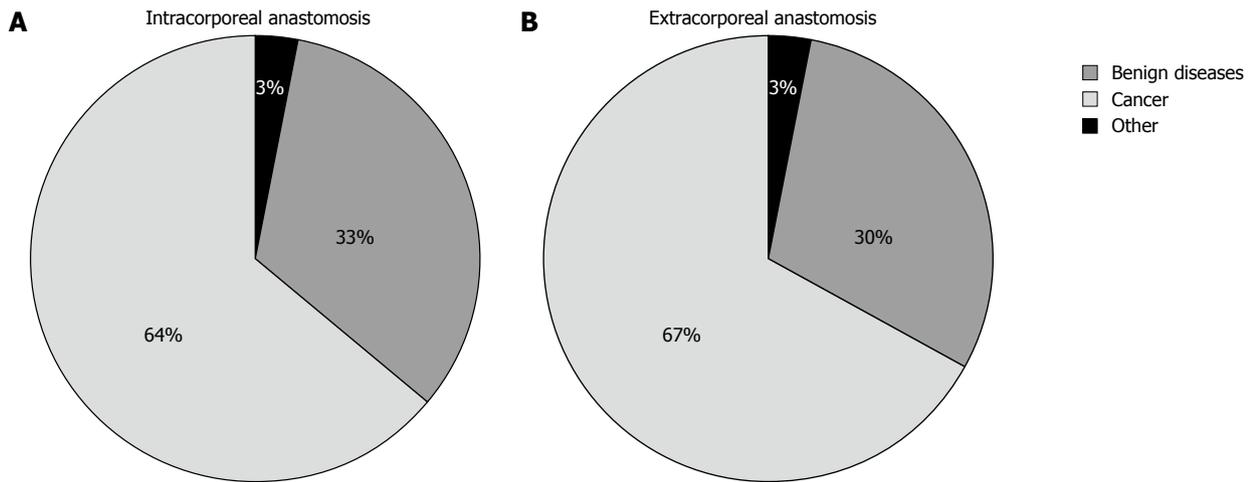


Figure 1 Intracorporeal anastomosis and extracorporeal anastomosis were no significant differences in the diagnosis from the pre-surgery biopsy. A: Intracorporeal anastomosis; B: Extracorporeal anastomosis.

Intracorporeal anastomosis		Extracorporeal anastomosis	
20/39 (51.3%)		21/33 (63.6%)	
One operation	11	One operation	14
Two operations	7	Two operations	7
> Two operations	2	> Two operations	7
Appendectomy	9	Appendectomy	15
Colic resection	2	Colic resection	1
Isteroannesectomy	4	Isteroannesectomy	2
Cholecystectomy	5	Cholecystectomy	1
Urologic surgery	4	Urologic surgery	1
Hernioplasty	2	Hernioplasty	9

		Intracorporeal anastomosis	Extracorporeal anastomosis	P value
Time until resumption of gas evacuation (d)	Median	3	3.5	< 0.0001
	Min-max	1-6	1-6	
Time until resumption of bowel movements (d)	Median	3.8	4.9	< 0.0001
	Min-max	1-7	2-7	
Time until removal of nasogastric tube (d)	Median	1.8	3	< 0.0001
	Min-max	0-11	0-6	
Time until resumption of liquid diet (d)	Median	3.5	4.5	< 0.0001
	Min-max	2-12	2-10	
Time to resumption of solid diet (d)	Median	4.6	5.7	< 0.0001
	Min-max	2-12	3-11	
Discharge (d)	Median	7.4	8.5	NS
	Min-max	4-19	5-25	0.5424

group than in the extracorporeal group (3 ± 1.05 d vs 3.5 ± 1.1 d, range: 1-6 d, $P < 0.0001$). Bowel movements occurred earlier in the intracorporeal group (3.8 ± 1.4 d, range: 1-7 d vs 4.9 ± 1.5 d, range: 2-8 d, $P < 0.0001$). In addition, the NGT was removed sooner in the intracorporeal group than in the extracorporeal group (1.8 d and 3 d, respectively, $P < 0.0001$).

Resumption of a liquid diet occurred an average of (3.5 ± 2.2 d, range: 2-12 d, $P < 0.0001$) after intracorporeal anastomosis and (4.5 ± 1.7 d, range: 2-10 d, $P < 0.0001$) after extracorporeal anastomosis. Resumption of a solid diet occurred (4.6 ± 2.1 d, range 2-12 d) and after intracorporeal and extracorporeal anastomosis, respectively (5.7 ± 1.7 d, range: 3-11 d, $P < 0.0001$).

Total hospitalization time was significantly less after intracorporeal anastomosis (average of 7.4 ± 3.2 d, range: 4-19 d) than after extracorporeal anastomosis (average of 8.5 ± 3.9 d, range: 5-25 d, $P < 0.0001$; Table 3).

Major complications occurred in 10.2% of patients undergoing intracorporeal anastomosis, i.e., three patients: one with severe anemia, one with anastomotic dehiscence, and one with enterocutaneous fistula. In the extracorporeal group, 12.1% of patients had major complications, i.e., five patients: two with severe anemia, one with occlusion, one with anastomotic dehiscence, and one with enterocutaneous fistula.

NS: Not significant.

DISCUSSION

Our study shows that intracorporeal anastomosis for right laparoscopic colectomy improved patient outcome compared with patients who underwent extracorporeal anastomosis. With intracorporeal anastomosis, we found faster recovery of nutrition, faster recovery of intestinal function, and shorter hospitalization. However, there was no difference in average surgery time between the two groups.

According to the inclusion and exclusion criteria, we obtained two homogeneous and comparable groups without significant differences in age, gender, BMI (Tables 1 and 4), ASA class, or abdominal surgical history (Table 2). In laparoscopic right colectomy with extracorporeal anastomosis (laparoscopic-assisted colectomy), the bowel is externalized through a lateral mini-incision. With this approach, bowel mobilization and ligation of vessels is usually laparoscopic, whereas resection of the specimen and creation of the anastomosis is extracorporeal. On the other hand, in laparoscopic right colectomy with intracorporeal anastomosis (totally laparoscopic

Table 4 Patient distribution according to age and body mass index, number of removed lymph nodes and duration of hospital stay *n* (%)

	Intracorporeal	Extracorporeal
Age (yr)		
< 65	13.6	12.1
65-80	43.2	48.5
> 80	43.2	33.4
BMI (kg/m ²)		
< 25	44.4	26.9
25-30	40.7	53.1
> 30	14.9	23.0
Number of lymph nodes removed		
< 12	7 (21.2)	14 (46.7)
12-15	6 (18.2)	6.6 (2)
> 15	20 (60.6)	14 (46.7)
Discharge from hospital (d)		
< 6	15 (42.8)	8 (24.4)
7	10 (28.6)	10 (30.3)
8-9	5 (14.3)	8 (24.2)
> 10	5 (14.3)	7 (21.1)

BMI: Body mass index.

colectomy), bowel mobilization, ligation of vessels, resection of the specimen, and creation of the anastomosis are totally intracorporeal.

In our experience, right colectomy with intracorporeal anastomosis has been standardized step by step: first, ileocolic vessels are isolated, secured between clips, and divided near their origin. Then, the right mesocolon is dissected medial-to-lateral, and the small bowel mesentery is divided to reach the edge of the terminal ileum. Then, the specimen is resected with an Endo-GIA stapler. The end the Endo-GIA stapler is deployed through the bowel openings to form a side-to-side anastomosis. The last step is specimen extraction and wound closure. We did not standardize the right colectomy with extracorporeal anastomosis; in 36.4% of cases, the ligation of vessels was performed after partial bowel mobilization, whereas in 63.6% of cases, it was the first step of the surgical procedure. Finally, the anastomosis was realized manually, lateral-to-lateral, in a double layer.

Both techniques are oncologically safe; according to the latest Union for International Cancer Control Tumor Node Metastasis classification, removal of at least 12 lymph nodes is fundamental to guarantee sufficient oncological radicality^[12]. To achieve this goal, the arterial vessels must be ligated at the origin from the superior mesenteric artery. When vascular ligation is extracorporeal, it is very difficult to obtain an adequate number of lymph nodes^[13]. Bergamaschi *et al*^[14] showed that extracorporeal vascular oncologic ligation is very difficult through a small cutaneous incision, and the bowel undergoes a hard traction with this technique. Hellan *et al*^[15] emphasized that the limitations of extracorporeal vascular ligation include poor exposure of the ileocolic pedicle through the small incision. Difficult exposure of the base of the mesentery could compromise the oncological result. That is why some surgeons propose the technique of

intracorporeal high-vessel ligation combined with extracorporeal anastomosis^[16-19].

Regarding oncological radicality, we found significant differences in the number of lymph nodes removed. We removed an average of 19 lymph nodes from the intracorporeal group and 14 lymph nodes from the extracorporeal group. In particular, in the first group we removed more than 15 lymph nodes in 60% of patients, 12 to 15 lymph nodes in 18.2% of patients, and fewer than 12 lymph nodes in 21% of patients. In the extracorporeal group, we removed more than 15 lymph nodes in 46.7% of patients, 12 to 15 lymph nodes in 6.6% of patients, and fewer than 12 lymph nodes in 46.7% of patients (Table 4). Thus, our experience shows that there is an important difference in the number of positive lymph nodes removed in the intracorporeal group, and also on the percentage of patients in which more than 12 lymph nodes were removed ($P < 0.0001$). The explanation for this difference is the missed ligation of vessels before the mobilization of the right colon. We believe that is very difficult to obtain an adequate number of lymph nodes when vessel division is not the first step in laparoscopic right colectomy.

In the literature, some authors have reported no differences in safety, whereas others noted that the only advantage was a smaller incision^[20,21]. On the other hand, other studies affirmed the safety of intracorporeal anastomosis, with the same complication rate as for extracorporeal anastomosis^[22,23].

Because intracorporeal anastomosis is considered more difficult, only a few surgeons have used this kind of technique; however less mobilization is required, and less tension is applied to the bowel and mesentery because the bowel does not need to reach the anterior abdominal wall for externalization. Furthermore, the excessive tension on the mesentery during the mobilization is associated with an increased risk of mesenteric or portal vein thrombosis^[24].

Concerning surgical times, we did not find a significant difference in surgical time between the two groups.

Patients in the intracorporeal group had a shorter hospitalization duration. In some cases, the hospitalization duration was longer possibly because of age (43.2% of patients in the intracorporeal group and 33.4% in the extracorporeal group were over 80 years old). Our results showed a significantly shorter average hospitalization stay in the intracorporeal group (Table 4). These data agree with a recent Spanish study^[25], although this difference was not significant ($P = 0.5424$) because hospitalization duration is influenced by many patient factors. On the other hand, we found that 71.4% of patients in the intracorporeal group went home within 7 d, and 54.7% of patients in the extracorporeal group went home within this period ($P = 0.0001$, Figure 2).

Concerning the recovery of intestinal function, our results found significantly shorter average times for resumption of gas evacuation after 3 d in the intracorporeal group compared to after 3.8 d in the extracorporeal

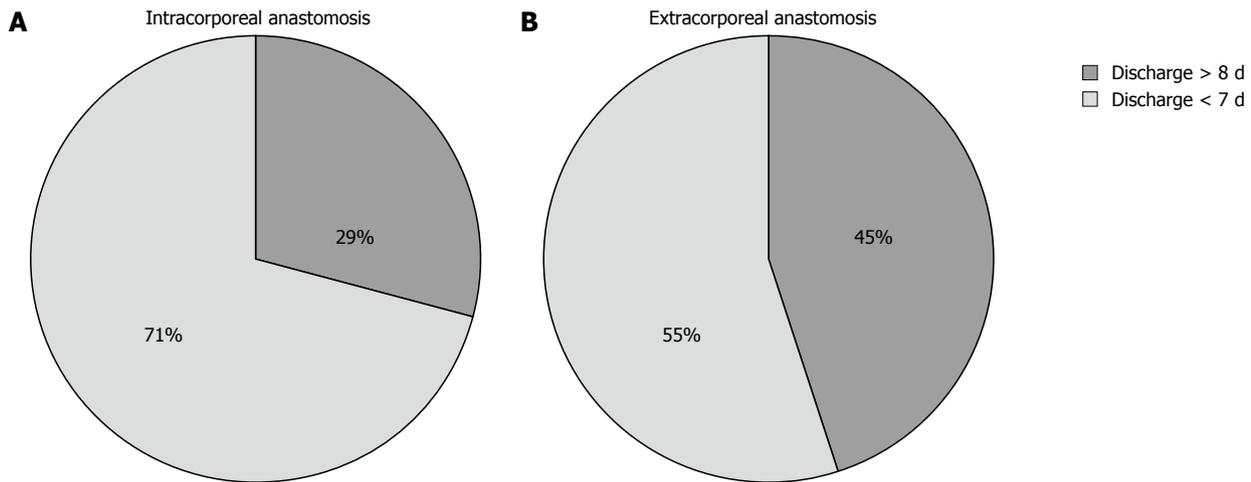


Figure 2 Patients in the intracorporeal group and extracorporeal group went home within 7 d. A: Intracorporeal group; B: Extracorporeal group.

group. Bowel movements occurred after an average of 4.9 d in the intracorporeal group. In the intracorporeal group, the nasogastric tube was removed after 1.8 d, whereas it was removed after 3 d in the extracorporeal group. This difference can be explained by an increased percentage of paralytic ileum in the second group, which is due to the traction of the right colon and terminal ileum through the mini-incision on the pancreas and duodenum^[26]. This approach allowed a more rapid recovery of liquid and solid nutrition consumption.

We analyzed major complications, which included severe anemia, occlusion, anastomotic dehiscence, and enterocutaneous fistulae. There were no significant differences between the two groups.

In conclusion, our study clearly shows that laparoscopic right colectomy with intracorporeal anastomosis improves patient outcome. We found that intracorporeal anastomosis resulted in faster recovery of nutrition consumption, faster recovery of intestinal function, and shorter hospitalization duration. The higher number of lymph nodes removed seems to be related to vascular division as the first surgical step as a rule. This confirms that a mini-invasive approach improves patient outcome.

COMMENTS

Background

A lot of studies have demonstrated the benefits of laparoscopic right colectomy, proving the short-term outcome of patients. The aim of this study is to show how intracorporeal anastomosis in laparoscopic right colectomy has further improved patient outcome.

Research frontiers

In the area of mini-invasive surgery, intracorporeal anastomosis, confirm that a less traumatic surgical approach improves patient outcome.

Innovations and breakthroughs

Based on a large series, this study describes the outcome of patients treated with laparoscopic right colectomy and is a reference for comparison in future studies.

Applications

The study results show how laparoscopic right colectomy with intracorporeal anastomosis improves patients outcome. This study suggests that all patients treated with intracorporeal anastomosis have faster recovery of nutrition consumption,

faster recovery of intestinal function, and shorter hospitalization duration.

Peer review

This paper demonstrated the outcomes of patients treated with laparoscopic right colectomy with intracorporeal anastomosis.

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A novel animal model for *in vivo* study of liver cancer metastasis

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Abstract

AIM: To establish an animal model with human hepatocyte-repopulated liver for the study of liver cancer metastasis.

METHODS: Cell transplantation into mouse livers was conducted using alpha-fetoprotein (AFP)-producing hu-

man gastric cancer cells (h-GCCs) and h-hepatocytes as donor cells in a transgenic mouse line expressing urokinase-type plasminogen activator (uPA) driven by the albumin enhancer/promoter crossed with a severe combined immunodeficient (SCID) mouse line (uPA/SCID mice). Host mice were divided into two groups (A and B). Group A mice were transplanted with h-GCCs alone, and group B mice were transplanted with h-GCCs and h-hepatocytes together. The replacement index (RI), which is the ratio of transplanted h-GCCs and h-hepatocytes that occupy the examined area of a histological section, was estimated by measuring h-AFP and h-albumin concentrations in sera, respectively, as well as by immunohistochemical analyses of h-AFP and human cytokeratin 18 in histological sections.

RESULTS: The h-GCCs successfully engrafted, repopulated, and colonized the livers of mice in group A (RI = 22.0% ± 2.6%). These mice had moderately differentiated adenocarcinomatous lesions with disrupted glandular structures, which is a characteristics feature of gastric cancers. The serum h-AFP level reached 211.0 ± 142.2 g/mL (range, 7.1-324.2 g/mL). In group B mice, the h-GCCs and h-hepatocytes independently engrafted, repopulated the host liver, and developed colonies (RI = 12.0% ± 6.8% and 66.0% ± 12.3%, respectively). h-GCC colonies also showed typical adenocarcinomatous glandular structures around the h-hepatocyte-colonies. These mice survived for the full 56 day-study and did not exhibit any metastasis of h-GCCs in the extrahepatic regions during the observational period. The mice with an h-hepatocyte-repopulated liver possessed metastasized h-GCCs and therefore could be a useful humanized liver animal model for studying liver cancer metastasis *in vivo*.

CONCLUSION: A novel animal model of human liver cancer metastasis was established using the uPA/SCID mouse line. This model could be useful for *in vivo* testing of anti-cancer drugs and for studying the mechanisms of human liver cancer metastasis.

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Key words: Urokinase-type plasminogen activator/severe combined immunodeficient mouse; Mouse with humanized liver; Liver cancer metastasis; Alpha-feto-protein-producing gastric cancer cells

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INTRODUCTION

Tumor metastasis, which is defined by a process in which tumor cells originating from an organ invade another anatomically distant organ, is the leading cause of cancer-related mortality^[1,2]. One of the major target organs for cancer metastasis is the liver^[1-3], and therefore there is increasing need for animal models that accurately mimic the pathophysiological situations in human liver and are suitable for investigating the mechanisms of hepatic cancer metastasis. In fact, several studies have attempted to transplant metastatic h-tumor cells into the livers of the immuno-compromized mice, such as athymic nude mice^[4], which cannot generate T cells, severe combined immunodeficient (SCID) mice that lack mature B and T cells^[5-7], and NOD/SCID/ c^{null} (NOG) mice^[8,9], which are deficient in T, B, and natural killer cells, and have impaired dendritic cells. In these animal models, the transplanted h-tumor cells invade the hepatic parenchyma, which is composed of mouse hepatocytes that are phylogenetically distant from h-hepatocytes and are known to exhibit biological and pathological features that are different from the human counterpart.

Heckel *et al*^[10] established transgenic mice expressing urokinase type plasminogen activator (uPA) under the control of the albumin (Alb) enhancer/promoter and found that the m-hepatocytes were constitutively damaged due to constant exposure to the expressed uPA. In another study, a mouse line possessing a humanized liver (chimeric mouse) was generated by transplanting healthy and normal h-hepatocytes into the liver of the immuno- and liver-compromized mouse, which was created by mating the uPA-Tg mouse with the SCID mouse (uPA/SCID mouse)^[10,11].

We previously developed chimeric mice where the liver was stably and reproducibly replaced with h-hepatocytes and found that the occupancy ratio or replacement index (RI) in the parenchyma was quite high (> 90%) in best cases^[12]. Human hepatocytes in the chimeric m-liver have been intensively and extensively characterized based on normal hepatic phenotypes, such as expres-

sion profiles of cytochrome P450, the major xenobiotic-metabolizing enzymes, drug-metabolizing capacities, and hepatitis virus infectivity^[11,13-15]. Based on these studies, which indicate that a chimeric m-liver can appropriately recapitulate the characteristics of h-liver, we hypothesized that the chimeric mouse as an animal model can be used to investigate the underlying mechanisms of tumor metastasis into the liver where the parenchyma is largely composed of normal and healthy h-hepatocytes.

In the present study, we established a chimeric mouse as a novel experimental model that sufficiently mimics the pathophysiological micro-environment in h-liver for studying liver cancer metastasis.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the National Hospital Organization, Nagasaki Medical Center, the Hiroshima Prefectural Institute of Industrial Science and Technology Ethics Board, and the Phoenix-Bio Ethics Board. This study was conducted in accordance with their guidelines.

Animals

The uPA/SCID mice were generated and used as transplant hosts once they reached an age of 24-32 d old as previously described^[14,15]. The mice were maintained in the laboratory in a specific pathogen-free environment in accordance with the guidelines of the Hiroshima Prefectural Institute of Industrial Science and Technology Ethics Board as well as the PhoenixBio Ethics Board.

Cancer cells

Human gastric cancer cells (h-GCCs) were purchased from the Japanese Collection of Research Biosources (Osaka, Japan) and used as liver metastatic cancer cells. These cells are adenocarcinoma cells derived from human gastric cancer cells that produce alpha-fetoprotein (AFP) and have a high affinity for liver tissue^[16-18]. The cells were maintained in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO, United States) containing 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO, United States) in an atmosphere of 95% air and 5% CO₂ at 37 °C.

Cell transplantation into the uPA/SCID

Human GCCs were suspended at a concentration of 1×10^7 cells/mL and placed on ice until transplantation. Cryopreserved h-hepatocytes derived from a 6-year-old African female were purchased from BD Biosciences (San Jose, CA, United States), thawed in a 37 °C water bath, rapidly diluted with culture medium at 4 °C, and washed twice to remove the cryopreservation solution. The cell viability was assessed by a trypan blue exclusion test. The uPA/SCID mice were anesthetized with ether and then were intrasplenically injected with the h-hepatocytes as previously described^[12]. Blood samples, 5 μ L each, were periodically collected from the host tail-vein for

Table 1 Serum concentrations of human albumin and human alpha-fetoprotein in host mice at 56 d post-transplantation

Experimental groups	Transplanted cells	No. of animals	Serum concentration	
			h-Alb (mg/mL)	h-AFP (mg/mL)
A	h-GCCs	4	UD	7.1-324.2 (211.0 ± 142.2)
B	h-GCCs and h-hepatocytes	6	0.03-9.1 (3.1 ± 3.5)	0.3-126.1 (54.3 ± 60.7)

The numerals represent the range of the concentrations and those in the parentheses indicate the mean ± SD. h-GCCs: Human gastric cancer cells; h-Alb: Human albumin; h-AFP: Human alpha-fetoprotein; h-hepatocytes: Human hepatocytes; UD: Undetectable.

determining concentrations of human albumin (h-Alb) and human AFP (h-AFP) using an h-Alb enzyme-linked immunosorbent assay quantification kit (Bethyl Laboratories Inc., Montgomery, TX) and an h-AFP enzyme immunoassay test kit (Hope Laboratories, Belmont, CA, United States), respectively.

Histological and immunohistochemical evaluation of the m-liver

Liver tissue specimens were removed from the transplanted mice, paraffin-embedded, sectioned at a 4 μm thickness, and stained with hematoxylin and eosin (H and E). Human hepatocyte-colonies were identified by staining the sections with mouse monoclonal antibodies against human-specific cytokeratin 18 (h-CK18) (DAKO, Glostrup Denmark). Human GCCs in the m-liver were identified by h-AFP staining with a polyclonal Ab (Novocastra Laboratories Ltd, United Kingdom). The sections were treated with a biotinylated, goat anti-rabbit IgG for h-CK18 and rabbit anti-m-IgG (DAKO, Glostrup Denmark) for h-AFP. All of the tissue specimens or cells were counterstained with H and E.

Determination of h-hepatocytes and h-GCCs repopulation of the uPA/SCID m-liver

Serial liver sections were double immunostained for h-CK18 and h-AFP to identify h-hepatocytes/h-GCCs and h-GCCs, respectively. The extent of repopulation of h-hepatocytes and h-GCCs in the chimeric mouse liver was determined as the RI, which is the occupational ratio of the transplanted cells in the examined area of histological sections, as previously described^[12]. The RI of h-hepatocytes (RI_{h-hepatocytes}) in the uPA/SCID m-liver was determined using h-CK18 as a maker to histologically identify h-hepatocytes. When appropriate, the RI for h-GCCs (RI_{h-GCCs}) was referred to as the metastatic index (MI_{h-GCCs}) in this study. Human hepatocytes and h-GCCs were identified on histological sections as the h-CK18-positive (h-CK18⁺) and h-AFP-negative (h-AFP⁻) cells and the h-CK18⁺ and h-AFP⁺ cells, respectively. The RI_{h-hepatocytes} and MI_{h-GCC} of the m-livers were calculated as the ratio of the “h-CK18⁺/h-AFP⁻” and “h-CK18⁺/h-AFP⁺” areas to the entire examined area of the sections, respectively.

Experimental groups

The uPA/SCID mice were divided into two groups (A and B groups). Four uPA/SCID mice in group A were each injected with 1×10^6 h-GCCs. Six mice in group B were co-transplanted with 7.5×10^5 h-hepatocytes and h-GCCs each. The blood h-Alb and h-AFP concentrations were periodically monitored after cell transplantation. The mice were euthanized at the termination of the experiments and their livers, spleens, and lungs were microscopically examined to identify any metastasis of h-GCCs.

RESULTS

Group A experiment

Human GCCs were transplanted into the livers of uPA/SCID mice and euthanized 56 d after transplantation. Human GCC colonies were macroscopically distinguishable from the host m-liver cells as brown colored regions (Figure 1A). Histological examinations showed that these areas contained h-GCC colonies and host m-liver cells composed of m-parenchymal and m-nonparenchymal cells (Figure 1B). The whitish or pale regions observed in Figure 1A were composed of only m-liver cells. The specimens were also stained for h-AFP to define h-GCCs (Figure 1C and D). Human GCCs formed colonies with well-developed glandular structures, which is a characteristic feature of gastric cancer. The serum concentrations of h-AFP increased to 211.0 ± 142.2 g/mL (range 7.1-324.2 g/mL, Table 1), which reflected the repopulation of h-GCCs in the liver, since serum h-AFP was undetectable in uPA/SCID mice without transplantation of h-GCCs (data; not shown). The MI of h-GCCs (MI_{h-GCC}) was $22.0\% \pm 2.6\%$ at the termination of the experiment 56 d post-transplantation.

Group B experiment

Both h-hepatocytes and h-GCCs were simultaneously transplanted into six uPA/SCID mice. The serum concentrations of h-Alb and h-AFP monitored after the cell transplantation (Figure 2). These protein levels were variable among individual mice, and three mice (No. 1-3) had substantially elevated h-Alb levels over the 56-d study. In addition, these mice exhibited RI_{h-hepatocytes} > 70% based on the correlation graph between h-Alb concentrations and RI_{h-hepatocytes}^[12]. These hosts also had markedly elevated h-AFP concentrations. In particular, mice No. 1 and 2 showed the highest h-Alb levels (approximately 9.1 mg/mL) and h-AFP concentrations (approximately 126.1 mg/mL) at 56 d post-transplantation (Table 1; Figure 2). As shown in Figure 3A, mouse 1 had the highest h-Alb and h-AFP levels, and the liver was composed of brown and whitish regions indicated by the thick and the thin arrows, respectively, which corresponded to the colonies composed of both h-hepatocytes and h-GCCs or m-liver cells, respectively. The brown region in the liver shown in Figure 3A was sectioned and stained with H and E (Figure 3B), anti-h-CK18 Abs to identify both h-hepatocytes and

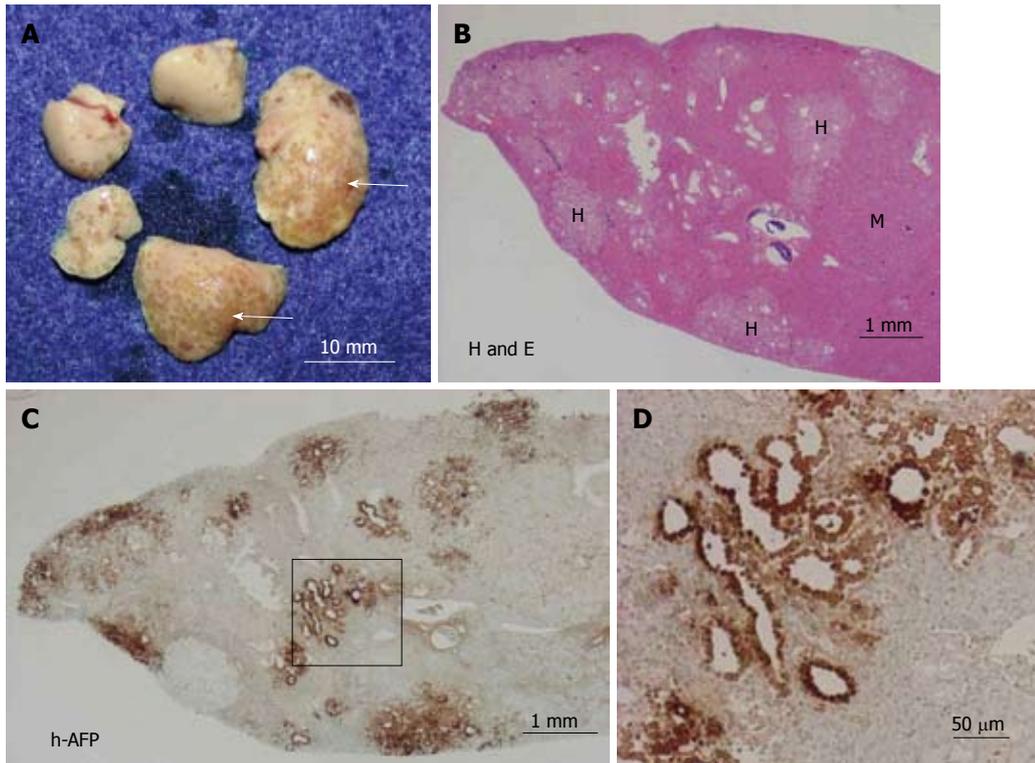


Figure 1 Macro- and microscopic images of the liver from group A mice. A: The urokinase-type plasminogen activator/severe combined immunodeficient mouse were transplanted with human gastric cancer cells (h-GCCs) and euthanized 56 d later, at which time the livers were isolated and photographed; B: The arrows in A point to concentrated regions of h-GCC colonies, and the sections were stained with hematoxylin and eosin (H and E). H and M in B represent h-GCC colonies and m-liver cell regions, respectively; C: The sections were stained with anti-human alpha-fetoprotein (h-AFP) antibodies; D: The square region in C is enlarged and shown.

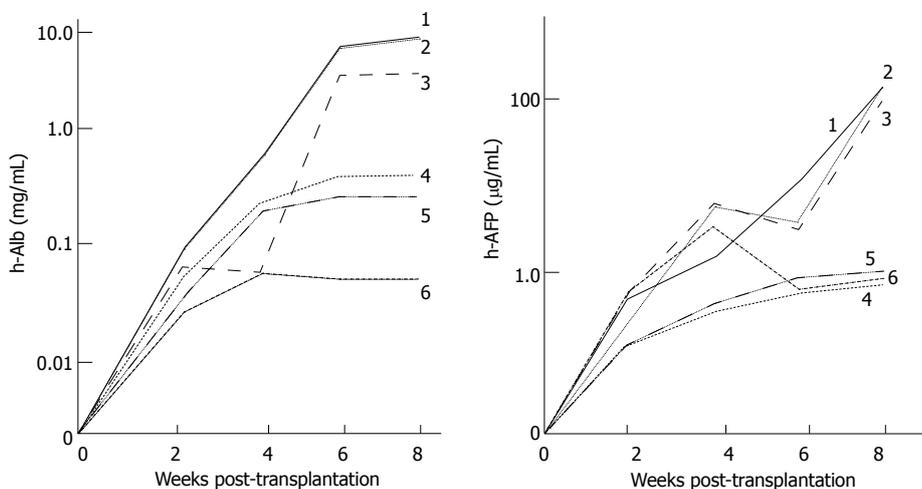


Figure 2 Changes in the serum concentrations of human albumin and human alpha-fetoprotein in group B-mice. Six mice (No.1-6) were co-transplanted with h-hepatocytes and human gastric cancer cells. The serum levels of human albumin (h-Alb) (left panel) and human alpha-fetoprotein (h-AFP) (right panel) were periodically monitored after the cell transplantation.

h-GCCs (Figure 3C), and the anti-h-AFP Ab to identify h-GCCs (Figure 3D). A comparison of Figure 3B and C showed that most of the section from Figure 3B was occupied with h-CK18⁺ cells, which corresponded to the cells in the less eosinophilic areas of the H and E section. Human CK18⁺ m-liver cells were located in eosinophilic areas in the H and E section, which were sporadically distributed as clusters with variable forms among large engrafted h-cell colonies. Human-AFP⁺ h-GCC-colonies were distinguished by comparing Figure 3B-D. These colonies were surrounded with less eosinophilic

h-hepatocytes (Figure 3D) that were swollen and clearer (Figure 3B and C). Magnified views of the brown area obtained from another serial sections of the liver shown in Figure 3A are shown in Figure 4A (H and E) and Figure 4B (h-AFP-stain). Human GCCs formed moderately differentiated adenocarcinomas with disrupted glandular structures, which is a characteristic feature of gastric cancer. Morphometric analyses using these h-CK18- and h-AFP-stained serial sections indicated that the RI_{h-hepatocyte} and MI_{h-GCC} in group B mice was 66.0% ± 12.3% (*n* = 6) and 12.0% ± 6.8% (*n* = 6), respectively. The mice in

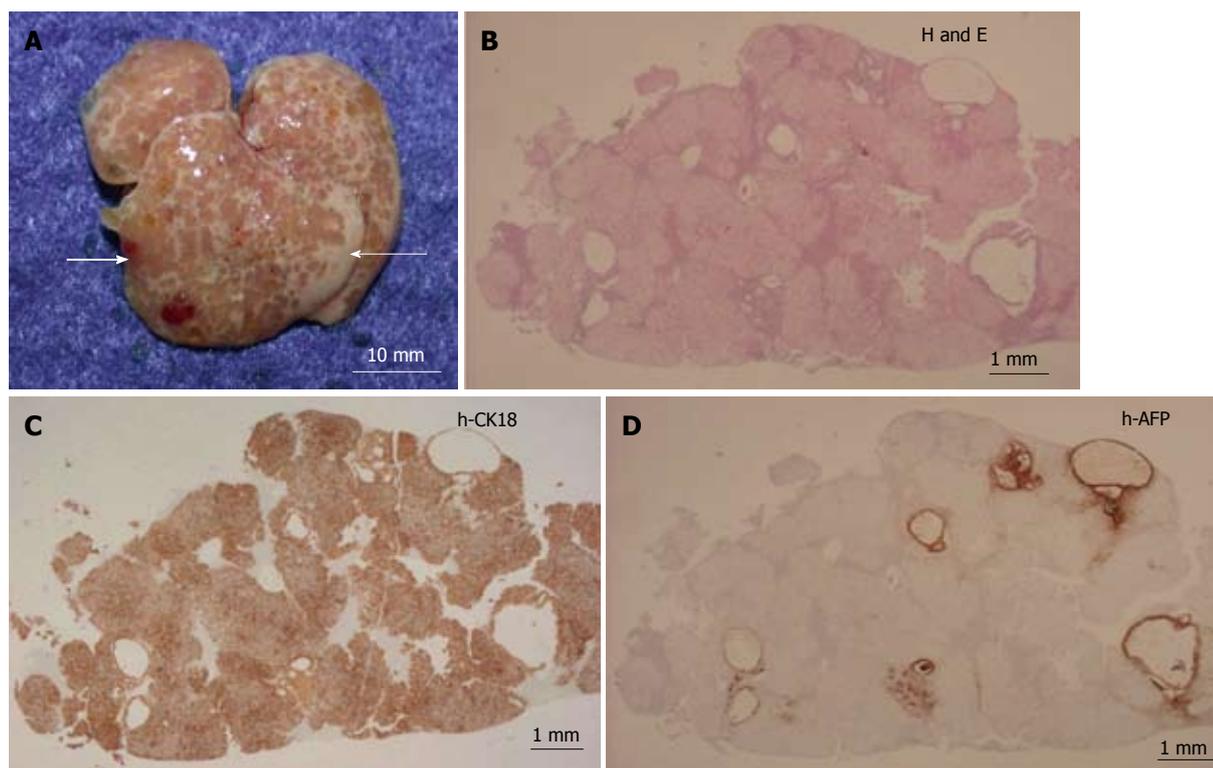


Figure 3 Macroscopic image of the liver of mouse No. 1 from Figure 2 at 56 d post-transplantation. A: The thick and thin white arrows point to h-cells [human hepatocytes (h-hepatocytes) and human gastric cancer cells (h-GCCs)] and m-liver cell regions, respectively; B: The liver was sectioned and stained with hematoxylin and eosin (H and E); C: The liver was sectioned and stained with anti-h-CK18; D: The liver was sectioned and stained with anti-human alpha-fetoprotein (h-AFP) antibodies. The h-AFP + (h-GCC) colonies were surrounded by less eosinophilic h-hepatocytes.

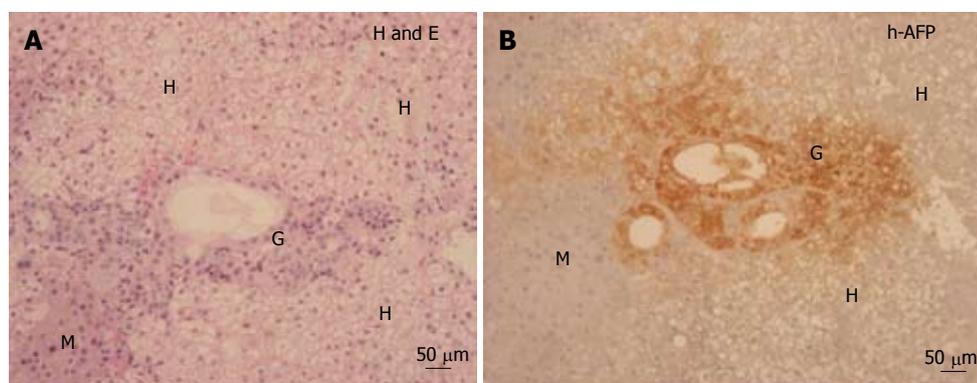


Figure 4 Magnified images of hepatic histology from group B mice. A: A serial section of the liver in Figure 3 was subjected to hematoxylin and eosin (H and E); B: A serial section of the liver in Figure 3 was subjected to human alpha-fetoprotein (h-AFP) staining. H, G and M represent the areas occupied by human-hepatocytes, human gastric cancer cells (h-GCCs), and host m-liver cells, respectively. h-GCCs composed moderately differentiated adenocarcinoma with disrupted glandular structures.

group B survived for the entire 56 d study. Extrahepatic sites and organs, such as the peritoneal cavity and kidney, were also examined for the presence of metastatic h-GCC lesions. The metastatic h-GCCs were not found in the extrahepatic regions during the observational period, indicating that the cells did not metastasize to any other regions.

DISCUSSION

An ideal animal model for liver metastasis of h-cancer

cells should possess at least two key features. First, the transplanted cancer cells need to invade and colonize in the host liver. Second, the liver of the host model has to provide the human cells with appropriate pathophysiological microenvironments that recapitulate the h-liver *in vivo*. Most of the conventional models to date manifest the first feature, but none of them have been able to sufficiently recapitulate the microenvironment of the h-liver^[4-6]. In the present study, we established a unique and novel that possessed both of these features.

In our study, we successfully engrafted the liver with

h-GCCs in the group A mice, and the cells formed relatively large colonies, with the MI as high as 25% at 56 d post-transplantation. However, such a considerably high MI could be a result of effects from either the donor or host side of the model. We chose h-AFP⁺ h-GCCs as a metastatic cancer cell line, since previous studies reported that patients with AFP⁺ gastric cancer showed a higher liver MI than those with AFP⁻ cells; more than 70% of the patients developed liver metastasis^[18,19]. These AFP⁺ cancer cells express c-Met^[19], which is the receptor for human hepatocyte growth factor (HGF), and therefore it is plausible that the cells have a high affinity for liver tissues under conditions where the levels of activated HGF in these tissues become high^[20]. In the present study, we utilized the uPA/SCID mice as hosts, which possessed a uPA transgene product that continuously damages the hepatocytes. In this model, the host hepatocytes generate pro-inflammatory environments in the liver, which stimulates the mobilization and expression of HGF in the liver tissues, including hepatocytes.

The role of uPA is an important aspect in this model. The host m-hepatocytes express unusually high levels of uPA, which is thought to induce severe damage in the replicative ability of m-hepatocytes through the activation of plasminogen, fibrinogen, and other proteins within the rough endoplasmic reticulum (RER) involved in proteolysis that lead to functional defects of the RER^[21]. In addition, uPA is secreted from m-hepatocytes into the plasma^[10], indicating that it circulates to liver tissues through sinusoidal capillaries and activates the conversion of blood plasminogen to plasmin. Therefore, the host liver tissue may provide h-GCCs with a pro-metastatic-like microenvironment. In fact, previous studies have indicated that uPA and its receptor (uPAR) play critical roles in the extravasation of tumors^[22-24]. Therefore, the injected h-GCCs are prone to extravasate liver tissues through the portal vein and sinusoid because of the uPA-induced fragility of vascular and sinusoidal endothelia and subsequently engraft liver tissues through an affinity for c-Met. Once the h-GCCs invade liver tissues, they can relatively easily propagate due to c-Met signaling in the host parenchyma, and can consequently replace m-hepatocytes as a result of the uPA-mediated damage. These conditions are also convenient for engraftment and proliferation of normal, healthy h-hepatocytes, as shown in this study when co-transplanted with h-GCCs.

The co-transplantation of h-hepatocytes with h-GCCs also resulted in the development of metastatic colonies in the mice similar to the transplantation of h-GCCs alone. In this type of transplantation experiment, large variances in serum concentrations of replacement marker proteins (h-Alb and h-AFP) were observed. The h-AFP kinetic curves were different from those of h-Alb and exhibited an increase of the serum level through "three steps": initial increase, followed by a plateau or decline, and then a sharp increase. This complex h-AFP kinetic pattern suggests the presence

of interactions between the invading cancer cells and the accepting host cells. There seemed to be two groups of animals within the experimental groups, one that more easily accepted xenogeneic cells and another that demonstrated resistance. However, we have consistently observed similar variances in h-Alb levels among individual mice when we generated h-hepatocyte chimeric mice^[12], though inbred mice were used as hosts. These variances are accidental in nature and might originate from some differences in manipulation procedures for transplantation as well as uncontrollable differences in the phenotypes of the uPA Tg mice^[10]. Despite these variances at the individual level, experimental group B of this study clearly demonstrated that we were able to reproducibly create mice whose livers were co-repopulated with healthy, normal h-hepatocytes and h-GCCs. Both h-hepatocytes and h-GCCs have high affinities for liver tissue, which drives engraftment of the liver and results in the generation of a humanized liver with metastatic cancer cells. We also found that the RI_{h-hepatocyte} (66.0% ± 12.3%) was significantly higher than MI_{h-GCC} (12.0% ± 6.8%), which may be a reflection of the difference in the inherent replication rates of the cells and adaptability to the host liver tissues. Our results indicate that h-hepatocytes are, as a whole, superior to h-GCCs in colony growth.

Relevant and reproducible animal models are indispensable tools for deducing the mechanisms of liver metastasis and pharmacokinetics of anti-cancer drugs, and several models have been developed to meet these practical needs, though they are quite limited^[2,25-30]. Preclinical tests of anti-cancer drugs for their effectiveness and toxicity in relevant animal models are required prior to application in humans^[31]. Toxicity data from non-primate species have been quite poor at predicting outcomes in subsequent human clinical trials, since there are significant differences in the metabolic activities of the hepatocytes between humans and rodent^[32-34]. Therefore, animal models with a humanized liver are more physiologic and will provide better tools for analyzing the pharmacokinetics of anti-cancer drugs as well as studying cancer metastasis^[35-37]. To our knowledge, no intrahepatic metastatic cancer model with a humanized liver has been available to date^[25,30,35-37]. The m-liver in the present study was chimeric and was composed of normal h-hepatocytes and m-hepatocytes. Previous studies have reported that the h-hepatocytes in these chimeric livers are functional and secreted a variety of hepatic proteins, such as Alb, -1 antitrypsin, apolipoprotein A, apolipoprotein E, several clotting factors, and complement proteins present in h-plasma^[38]. Transplanted h-hepatocytes also retain normal pharmacological responses, which makes the chimeric mouse model useful for studying the metabolism of compounds that cannot be easily administered to healthy volunteers^[14,15]. *In vivo* studies using these mice showed their utility in evaluating the metabolism of drugs catalyzed by both phase I and phase II enzymes^[13-15,39,40]. Since the liver functions of

the chimeric mice described in this study have not yet been characterized, future studies are needed to assess the model for anti-cancer drug testing. Taking together, the h-hepatocyte-chimeric mice may provide a useful bridge for studying human liver-related diseases because of the similarities with humans in physiological function and drug kinetics.

In conclusion, we have established a unique and novel animal model for studying liver cancer metastasis. The chimeric liver of the uPA/SCID mouse containing both human cancer cells and hepatocytes could be utilized as an appropriate model for *in vivo* testing of the efficacy and human-type metabolisms of candidate drugs for anti-cancer treatment as well as studying the mechanisms of liver cancer metastasis.

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COMMENTS

Background

One of the major target organs for cancer metastasis is the liver, and therefore, there has been increasing needs for animal models that can sufficiently mimic the pathophysiological situation in human liver and that are suitable for investigating the mechanisms of hepatic cancer metastasis.

Research frontiers

An ideal animal model for liver metastasis of human cancer cells should possess at least two key features. First, the transplanted cancer cells need to invade and colonize the liver of the host. Second, the liver of the host model has to provide the human cells with appropriate pathophysiological microenvironments that recapitulate the human liver *in vivo*. In the present study, the authors established a unique and novel animal model with both of these features.

Innovations and breakthroughs

A liver-humanized mouse was generated by transplanting healthy and normal h-hepatocytes into urokinase type plasminogen activator/severe combined immunodeficient (uPA/SCID) mice (immuno- and liver- compromised mice), and the liver was stably and reproducibly replaced with human hepatocytes. This is the first report of a novel experimental model that sufficiently mimics the pathophysiological situation of human liver.

Applications

The chimeric liver of the uPA/SCID mouse containing both human cancer cells and hepatocytes could be utilized as an appropriate model for the *in vivo* testing of anti-cancer drugs as well as studying the mechanisms of liver cancer metastasis.

Terminology

The uPA/SCID mouse is a transgenic mouse line that expressed uPA under the control of the albumin enhancer/promoter which constitutively damages the hepatocytes due to constant exposure to uPA. A liver-humanized mouse (chimeric mouse) was generated by transplanting healthy and normal human hepatocytes into mouse liver of the uPA/SCID mouse (immuno- and liver-compromised mouse), which had been generated by mating the uPA-Tg mouse with the SCID mouse. This mouse model sufficiently mimics the pathophysiological situation in human liver.

Peer review

This study tries to establish an animal model with h-hepatocyte-repopulated liver for *in vivo* study of liver cancer using uPA/SCID mouse, which could be useful for studying liver cancer metastasis. The authors transfected uPA/SCID mouse either with human gastric cancer cells (h-GCCs) or h-GCCs with h-hepatocytes and observed that both colonies can repopulate mouse liver. The study is well conducted, the manuscript is well-written and the figures are of good quality.

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Endoscopic ultrasound-guided fine needle aspiration in the differentiation of type 1 and type 2 autoimmune pancreatitis

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pancreatitis (LPSP) and idiopathic duct-centric pancreatitis (IDCP) mentioned in the International Consensus Diagnostic Criteria and examined if these findings make a contribution to the differential diagnosis of type 1 and type 2 AIP. A disposable 22-gauge needle was used for EUS-FNA.

RESULTS: Adequate specimens including pancreatic tissue for differentiating AIP from cancer were obtained from 43 of 47 patients who underwent EUS-FNA. EUS-FNA was performed from the pancreatic head in 21 cases, which is known to be technically difficult when performed by core biopsy; there was no significant difference in the results compared with pancreatic body-tail. Nine of 47 patients met level 1 findings of LPSP and 5 patients met level 2 findings of LPSP. No one met level 1 findings of IDCP, but 3 patients met level 2 findings of IDCP. Of 10 seronegative cases, 2 cases were diagnosed with "definitive type 1 AIP", and 3 cases were diagnosed with "probable type 2 AIP" when considering both the level 2 histological findings and response to steroids.

CONCLUSION: EUS-FNA is useful in the differentiation of type 1 and type 2 AIP, particularly in seronegative cases.

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Abstract

AIM: To investigate the usefulness of endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) in the differentiation of autoimmune pancreatitis (AIP).

METHODS: We retrospectively reviewed 47 of 56 AIP patients who underwent EUS-FNA and met the Asian diagnostic criteria. On 47 EUS-FNA specimens, we evaluated the presence of adequate material and characteristic features of lymphoplasmacytic sclerosing

Key words: Autoimmune pancreatitis; Endoscopic ultrasound-guided fine needle aspiration; Idiopathic duct centric pancreatitis; Lymphoplasmacytic sclerosing pancreatitis; Pancreatic cancer

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INTRODUCTION

Recently, the International Consensus Diagnostic Criteria (ICDC) for autoimmune pancreatitis (AIP) was proposed by Shimosegawa *et al.*^[1]. According to these criteria, AIP is classified into 2 types^[2]. The histological substance of type 1 AIP is known as lymphoplasmacytic sclerosing pancreatitis (LPSP)^[3-6], and type 2 AIP is characterized by a distinct histology called idiopathic duct centric pancreatitis (IDCP)^[7-10]. Type 2 AIP patients are generally seronegative and lack other organ involvement (OOI) in contrast to type 1 AIP. However, the absence of serological abnormalities or lack of OOI in patients with AIP does not necessarily imply the diagnosis of type 2, as type 1 also can be seronegative and without OOI. Taking these findings into consideration, ICDC made separate diagnostic criteria for type 1 and type 2 AIP, and histological differentiation is becoming more important for diagnosing AIP.

Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) is now widely accepted as a safe and effective modality for obtaining pancreatic tissue samples^[11-14]. There are reports on the usefulness of EUS-FNA in the diagnosis of AIP^[15,16] but only negative reports on the differentiation between LPSP and IDCP using specimens obtained by EUS-FNA^[17,18]. The findings of expert panel deliberations at the AIP International 2009 Honolulu Meeting reached a uniform consensus that essential histological features of LPSP can only be obtained or evaluated in tissues with preserved architecture, i.e., either a surgical resection specimen or a core biopsy but not FNA^[19]. However, a surgically resected specimen can only be obtained from a patient misdiagnosed with pancreatic cancer^[20-22], and a core biopsy device may not function properly when used in the duodenum. We thus investigated the usefulness of EUS-FNA in the differentiation of type 1 and type 2 AIP using EUS-FNA with a 22-gauge needle.

MATERIALS AND METHODS

Patients

We retrospectively reviewed 47 patients who underwent EUS-FNA of 56 AIP patients who met the Asian Diagnostic Criteria^[23] at our institute between July 2003 and July 2011. Forty-two men and 5 women with a mean age of 62.1 ± 13.6 years (range, 28-86 years) and a mean follow-up period of 839.8 ± 722.7 d (range, 19-2506 d) were included. The mean serum immunoglobulin G4 (IgG4) levels were 626.1 ± 1004.6 mg/dL (range of 4-5850 mg/dL), and 10 patients were seronegative. On

47 EUS-FNA specimens, we evaluated the presence of adequate material and characteristic features of LPSP [lymphoplasmacytic infiltration, storiform fibrosis, obliterative phlebitis, and abundant (> 10 cells per high-power field) IgG4-positive cells] and IDCP [granulocytic infiltration of the duct wall (GEL) or granulocytic acinar infiltrate] mentioned in the histological criteria for ICDC (Table 1). Adequate material indicated an adequate specimen including pancreatic tissue for differentiating AIP from cancer. EUS-FNA was performed from the pancreatic head in 21 cases, an approach that is known to be technically difficult when performed by core biopsy. The histological findings according to the locations of EUS-FNA were also evaluated. Using the results of EUS-FNA, we examined whether these findings make a contribution to the differential diagnosis of type 1 and type 2 AIP. Patients with jaundice or abdominal pain underwent steroid therapy with oral prednisolone (PSL). The initial dose of PSL was 30-40 mg/d, and it was tapered down to the maintenance dose (2.5-5 mg/d) within 12 wk. Relapse was defined as exacerbation of the pancreatic lesion or OOI morphology or emergence of new OOI. OOI include cholangitis^[24] [proximal (hilar/intrahepatic) or proximal and distal bile stricture], sialadenitis, nephritis^[25], inflammatory bowel disease (IBD), and retroperitoneal fibrosis.

EUS-FNA

After receiving written informed consent, the patients were submitted to conscious sedation with intravenous diazepam under appropriate cardiorespiratory monitoring. EUS-FNA was performed by expert endosonographers with experience of more than five thousand EUS cases. The apparatus used was a convex-type EUS, GF-UCT 240 (OLYMPUS Co., Ltd., Tokyo, Japan) and Pro-sound $\alpha 10$ (ALOKA Co., Ltd., Tokyo, Japan) with a frequency of 7.5 MHz. The needle used for EUS-FNA was a disposable 22-gauge needle (EZ shot; OLYMPUS Co., Ltd., Tokyo, Japan). After detailed evaluation of the pancreas with the B-mode and confirmation that no vessels were present in the puncture route in the color Doppler mode, EUS-FNA was performed from the stomach to puncture the pancreatic body or tail and from the duodenum to puncture the pancreatic head.

Statistical analysis

Statistical analyses were performed using SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, United States). The χ^2 test and Fisher's exact test were used to compare categorical parameters between the groups. Continuous parameters were presented as the mean \pm SD and/or median (range), and Student's *t* test was used. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

EUS-FNA

The number of FNA passes ranged from 1 to 4 with a

Table 1 Histological criteria for International Consensus Diagnostic Criteria		
	Level 1	Level 2
Type 1 AIP		
Histology of the pancreas	LPSP (core biopsy/resection) At least 3 of the following: (1) Periductal lymphoplasmacytic infiltrate without granulocytic infiltration (2) Obliterative phlebitis (3) Storiform fibrosis (4) Abundant (> 10 cells/HPF) IgG4-positive cells	LPSP (core biopsy) Any 2 of the following: (1) Periductal lymphoplasmacytic infiltrate without granulocytic infiltration (2) Obliterative phlebitis (3) Storiform fibrosis (4) Abundant (> 10 cells/HPF) IgG4-positive cells
Type 2 AIP		
Histology of the pancreas (core biopsy/resection)	IDCP Both of the following: (1) GEL with or without granulocytic acinar inflammation (2) Absent or scant (0-10 cells/HPF) IgG4-positive cells	Both of the following: (1) Granulocytic and lymphoplasmacytic acinar infiltrate (2) Absent or scant (0-10 cells/HPF) IgG4-positive cells

AIP: Autoimmune pancreatitis; LPSP: Lymphoplasmacytic sclerosign pancreatitis; IDCP: Idiopathic duct-centric pancreatitis; GEL: Granulocytic infiltration of the duct wall; IgG4: Immunoglobulin G4; HPF: High power field.

Table 2 Results of endoscopic ultrasound-guided fine needle aspiration specimen <i>n</i> (%)				
	Pancreatic head (<i>n</i> = 21)	Pancreatic body-tail (<i>n</i> = 26)	Total (<i>n</i> = 47)	<i>P</i> value
Average number of FNA passes	2.00 ± 0.43 (1-3)	2.04 ± 0.514 (1-4)	2.02 ± 0.48 (1-4)	0.78
Adequate sample material	17 (80.9)	26 (100)	43 (91.4)	0.07
Lymphoplasmacytic infiltration	6 (28.6)	10 (38.4)	16 (34.0)	0.68
Storiform fibrosis	12 (57.1)	22 (84.6)	34 (72.3)	0.07
Obliterative phlebitis	0 (0)	0 (0)	0 (0)	1
Abundant IgG4-positive plasmacyte infiltration	3/10 (30)	7/18 (38.8)	10/28 (35.7)	1
Granulocytic infiltration of duct wall	0 (0)	0 (0)	0 (0)	1
Granulocytic acinar infiltrate	1 (4)	2 (7.7)	3 (6.3)	1
Complications	0 (0)	0 (0)	0 (0)	1

IgG4: Immunoglobulin G4; FNA: Fine needle aspiration.

mean of 2.00 ± 0.48. One pass included approximately 15 to 20 back-and-forth movements in the target lesions. Adequate sample material was obtained from 43 of 47 patients who underwent EUS-FNA as well as 17 of 21 cases from the pancreatic head and all 26 cases from the body and tail. Sixteen of 47 EUS-FNA specimens showed lymphoplasmacytic infiltration, and 34 showed storiform fibrosis, but obliterative phlebitis could not be detected in any of the cases. Abundant IgG4-positive plasmacyte infiltration was shown in 10 of 28 patients who underwent immunostaining. Although GEL was not detected in any of the cases, three cases showed granulocytic acinar infiltrate. No significance was seen in the results of EUS-FNA between those performed at the pancreatic head and those obtained at the body-tail. There were no complications from EUS-FNA (Table 2).

On comparing the histological results of EUS-FNA against ICDC (Figure 1), 9 of 47 patients met level 1 findings of LPSP (Figure 2), and 5 patients met level

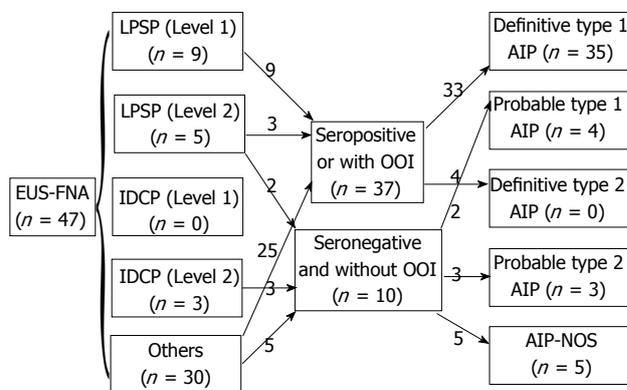


Figure 1 Comparison of endoscopic ultrasound-guided fine needle aspiration with International Consensus Diagnostic Criteria. EUS-FNA: Endoscopic ultra-sound-guided fine needle aspiration; LPSP: Lymphoplasmacytic sclerosing pancreatitis; IDCP: Idiopathic duct-centric pancreatitis; OOI: Other organ involvement; AIP: Autoimmune pancreatitis; NOS: Not otherwise specified.

2 findings of LPSP. Two of 5 patients who met level 2 findings of LPSP were seronegative and without OOI and were finally diagnosed with “definitive type 1 AIP” after considering both the level 2 histological findings and response to steroids (Table 3). No one met level 1 findings of IDCP (GEL), but 3 patients met level 2 findings of IDCP. All 3 patients were relatively young, seronegative, and had no OOI, including IBD. They were diagnosed with “probable type 2 AIP” (Figure 3) after considering the level 2 histological findings and response to steroids. They have shown improvement without relapse on radiological findings following steroid therapy thus far (Table 4).

DISCUSSION

EUS-FNA is an established and widely used technique to evaluate pancreatic masses. The diagnostic accuracy of EUS-FNA for pancreatic cancer is reported to be between 60% and 90%^[26-28], but conclusive diagnosis of

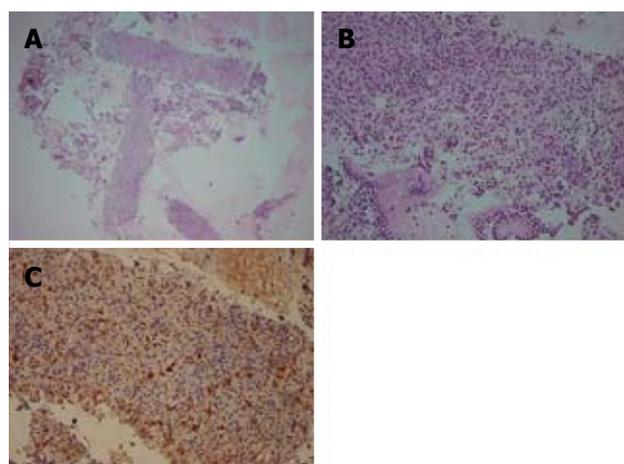


Figure 2 Endoscopic ultrasound-guided fine needle aspiration specimen of "definitive type 1 autoimmune pancreatitis". A, B: Hematoxylin and eosin staining of a resected pancreas specimen obtained by endoscopic ultrasound-guided fine needle aspiration shows replacement of the acinar structure by lymphoplasmacytic infiltration and fibrosis; C: Numerous plasma cells show positive immunoreactivity for immunoglobulin G4.

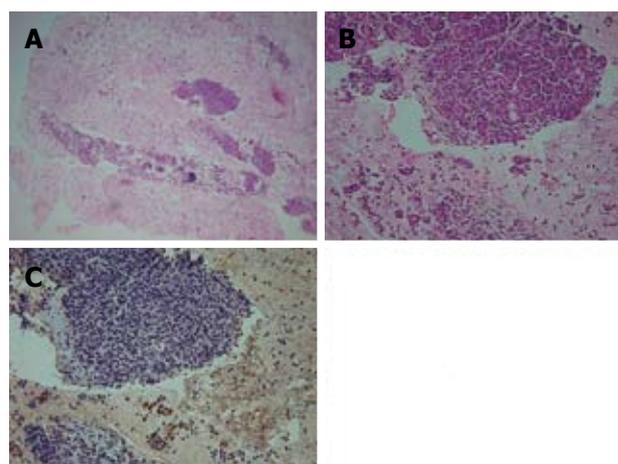


Figure 3 Endoscopic ultrasound-guided fine needle aspiration specimen of "probable type 2 autoimmune pancreatitis". A, B: Hematoxylin and eosin staining of a resected pancreas specimen obtained by endoscopic ultrasound-guided fine needle aspiration shows the infiltration of neutrophils in addition to lymphocyte infiltration and fibrosis; C: Immunostaining for immunoglobulin G4 is negative.

Table 3 Patients with level 2 histological findings of lymphoplasmacytic sclerosing pancreatitis

Case	Sex	Age, yr	IgG4 (mg/dL)	Location	Response to steroid	OOI	Diagnosis
1	Male	74	263	Diffuse	(+)	Nephritis	Definitive type 1 AIP
2	Female	71	364	Diffuse	(+)	Cholangitis	Definitive type 1 AIP
3	Male	54	230	Focal	(+)	Sialadenitis	Definitive type 1 AIP
4	Male	47	104	Focal	(+)	None	Definitive type 1 AIP
5	Male	57	46	Focal	(+)	None	Definitive type 1 AIP

IgG4: Immunoglobulin G4; OOI: Other organ involvement; AIP: Autoimmune pancreatitis.

AIP is often difficult due to the small size of specimens obtained by FNA. Recently, there have been several reports on the usefulness of EUS-guided tru-cut biopsy (EUS-TCB) for the diagnosis of AIP^[29-31]. Tru-cut biopsy needles have been developed to acquire samples while preserving tissue architecture, thus allowing histological examination^[32,33]. Previous reports describe the safety and the technical feasibility of performing EUS-TCB from a transgastric approach. However, the TCB device may not function properly when used in the second portion of the duodenum, and there is also some difficulty when using the TCB device from the duodenal bulb and along the greater curvature of the antrum^[29,34]. Moreover, because a 19-gauge needle is used for EUS-TCB, the risk of bleeding is higher compared with EUS-FNA using a 22-gauge needle, indicating that reexamination of safety is required. We previously reported^[35,36] the feasibility of EUS-FNA using a 22-gauge needle for the histological evaluation of gastrointestinal submucosal

Table 4 Patients with level 2 histological findings of idiopathic duct-centric pancreatitis

Case	Sex	Age, yr	IgG4 (mg/dL)	Location	Response to steroid	OOI	Follow-up, d	Relapse	Diagnosis
1	Male	28	69	Diffuse	(+)	(-)	973	(-)	Probable type 2 AIP
2	Female	31	43	Diffuse	(+)	(-)	425	(-)	Probable type 2 AIP
3	Male	30	23	Focal	(+)	(-)	120	(-)	Probable type 2 AIP

IgG4: Immunoglobulin G4; OOI: Other organ involvement; AIP: Autoimmune pancreatitis.

tumors, and we believe that this method can also be applied to pancreatic lesions. In our study, adequate material for differentiating cancer from AIP was obtained in 43 of 47 cases (91.4%), and no significant difference in EUS-FNA results was seen between those obtained from the pancreatic head and body-tail. Nine of 47 patients (19.1%) met 3 of 4 characteristic features of LPSP and were diagnosed with "definitive type 1 AIP" based on histological findings alone. Detailed analysis of 8 patients who showed level 2 histological findings of type 1 or type 2 AIP revealed that 3 patients with level 2 findings of type 1 were seropositive and/or with OOI and could be diagnosed with "definitive type 1 AIP" without histological findings, but the other 5 patients were seronegative and without OOI and diagnosed with "definitive type 1 AIP" or "probable type 2 AIP" based on combination of the level 2 histological findings and the response to steroid treatment. Therefore, out of 10 seronegative cases, 2 cases were diagnosed with "definitive type 1 AIP", and 3 cases were diagnosed with "probable type 2 AIP" using the histological findings of EUS-FNA. As mentioned earlier, type 1 AIP often can be di-

agnosed without histology, but it is difficult to differentiate type 1 and type 2 AIP when results are seronegative and without OOI. We believe histological evaluation of EUS-FNA is rather important in such cases.

In conclusion, EUS-FNA is useful in diagnosing AIP even when performed from the pancreatic head and may also provide complementary histological information to distinguish type 1 and type 2 AIP, particularly in seronegative cases.

COMMENTS

Background

Recently, the International Consensus Diagnostic Criteria (ICDC) for autoimmune pancreatitis (AIP) was proposed. ICDC made separate diagnostic criteria for type 1 and type 2 AIP, and histological differentiation is becoming more important for diagnosing AIP. There have been reports on the usefulness of endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) in the diagnosis of AIP but only negative reports on the differentiation between type 1 and type 2 AIP using specimens obtained by EUS-FNA.

Research frontiers

In the area of AIP, the research hotspot is how to obtain sufficient materials from AIP patients and differentiate type 1 and type 2 AIP correctly.

Innovations and breakthroughs

Adequate specimens including pancreatic tissue for differentiating AIP from cancer were obtained from 43 of 47 patients who underwent EUS-FNA. EUS-FNA was performed from the pancreatic head in 21 cases, which is known to be technically difficult when performed by core biopsy; there was no significant difference in the results compared with pancreatic body-tail. Of 10 seronegative cases, 2 cases were diagnosed with "definitive type 1 AIP," and 3 cases were diagnosed with "probable type 2 AIP" when considering both the level 2 histological findings and response to steroids.

Applications

The study results suggested that EUS-FNA (instead of core biopsy) was useful in diagnosing AIP even when performed from the pancreatic head and may also provide complementary histological information to distinguish type 1 and type 2 AIP, particularly in seronegative cases.

Terminology

Type 1 and type 2 AIP: The histological substance of type 1 AIP is known as lymphoplasmacytic sclerosing pancreatitis, and type 2 AIP is characterized by a distinct histology called idiopathic duct centric pancreatitis. Type 2 AIP patients are generally seronegative and lack other organ involvement (OOI) in contrast to type 1 AIP. However, the absence of serological abnormalities or lack of OOI in patients with AIP does not necessarily imply the diagnosis of type 2, as type 1 also can be seronegative and without OOI.

Peer review

The authors reported the usefulness of EUS-FNA in the diagnosis of type 1 and type 2 AIP and also stressed the importance of this method for the differential diagnosis between AIP and pancreatic cancer especially in the cases with negative results of serology and absence of other organ involvement. The content is clear and the discussion is straightforward. This paper is useful for understanding the ICDC and the classification of type 1 and 2 AIP.

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Non-invasive determination of hepatic steatosis by acoustic structure quantification from ultrasound echo amplitude

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Abstract

AIM: To use leptin-deficient (*ob/ob*) mice with demonstrated differences in steatosis levels to test a new diagnostic method using the acoustical structure quantification (ASQ) mode and the associated analytical parameter, "focal disturbance ratio" (FD-ratio).

METHODS: Nine *ob/ob* mice, at 5, 8, and 12 wk of age ($n = 3$ in each age group), were used as models for hepatic steatosis. Echo signals obtained from ultrasonography in the mice were analyzed by ASQ, which uses a statistical analysis of echo amplitude to estimate inhomogeneity in the diagnostic region. FD-ratio, as calculated from this analysis, was the focus of the present study. FD-ratio and fat droplet areas and sizes were compared between age groups.

RESULTS: No fibrosis or inflammation was observed in any of the groups. The fat droplet area significantly ($P < 0.01$) increased with age from $1.25\% \pm 0.28\%$ at 5 wk to $31.07\% \pm 0.48\%$ at 8 wk to $51.69\% \pm 3.19\%$ at 12 wk. The median fat droplet size also significantly ($P < 0.01$) increased with age, from 1.33 (0.55 - 10.52) μm at 5 wk, 2.82 (0.61 - 44.13) μm at 8 wk and 6.34 (0.66 - 81.83) μm at 12 wk. The mean FD-ratio was 0.42 ± 0.11 at 5 wk, 0.11 ± 0.05 at 8 wk, and 0.03 ± 0.02 at 12 wk. The FD-ratio was significantly lower at 12 wk than at 5 wk and 8 wk ($P < 0.01$). A significant negative correlation was observed between the FD-ratio and either the fat droplet area ($r = -0.7211$, $P = 0.0017$) or fat droplet size ($r = -0.9811$, $P = 0.0052$).

CONCLUSION: This tool for statistical analysis of signals from ultrasonography using the FD-ratio can be used to accurately quantify fat *in vivo* in an animal model of hepatic steatosis, and may serve as a quantitative biomarker of hepatic steatosis.

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Key words: Non-alcoholic fatty liver disease; Quantitation of hepatic steatosis; Animal model; Focal disturbance ratio; Acoustic structure quantification; Ultrasonography

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a clinically important disease that occurs in subjects with underlying conditions such as obesity or insulin resistance, and is frequently accompanied by metabolic syndrome, including diabetes, hyperlipidemia and/or hypertension^[1-5]. Non-alcoholic steatohepatitis (NASH) is the most extreme form of NAFLD, and is regarded as a major cause of cirrhosis of the liver of unknown cause^[6-10]. Methods for early detection and assessment of NAFLD through quantitative measurement of steatosis are needed to achieve earlier intervention and avoid the progression to cirrhosis.

The gold standard for quantitative assessment of steatosis has been considered to be liver histology^[11]. However, liver biopsy shows various limitations, such as potential sampling error, difficulties repeating the procedure because of ethical concerns, and complications including bleeding^[12]. Non-invasive alternatives to liver biopsy thus need to be established.

Several methods have recently been established for non-invasively quantifying steatosis using imaging techniques. Non-invasive modalities such as ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI) have been employed for the assessment of hepatic steatosis^[13-15]. However, using these modalities for the repeated evaluation of NAFLD is difficult, because CT involves radiation exposure and MRI is expensive to perform. From these perspectives, ultrasonography represents an excellent examination modality that is minimally invasive, inexpensive, and can be performed repeatedly with no risk to the patient. Furthermore, ultrasonography is known to be highly sensitive for detecting fat accumulation in the liver^[16]. However, the diagnostic performance of ultrasonography depends on the empirical and qualitative reading skills of the examiner, and quantitative methods to evaluate liver lipogenesis have yet to be established.

In recent years, Yamaguchi *et al.*^[17,18] have reported that diffuse pathological changes in the liver tissue can be quantitatively evaluated based on the statistical deviation of ultrasound signals compared to normal liver. Generally, ultrasonographic images of parenchymal organs such as the liver are designated as having a “speckle pattern” consisting of numerous fine echo spots. The speckle pattern is constructed by ultrasonic interference of scattered ultrasound waves generated by innumerable reflexive objects that are distributed closer than the ultrasonic wavelength^[17-19]. Image analysis of speckle patterns has been used to identify tissue characteristics associated with chronic liver diseases, because the pattern changes according to the structural characteristics of the medium. One such analytical method, the probability density function (PDF) of the echo amplitude of a speckle pattern, has been reported to be approximated by a function called the Rayleigh distribution. Moreover, Toyoda *et al.*^[20] proposed the acoustical structure quantification (ASQ) method and reported the possibility of quantifying diffuse liver disease or monitoring regression/progression in cases of liver fibrosis and during treatment. However,

no reports have yet described assessments of hepatic steatosis in NAFLD patients using this tool for the statistical analysis of ultrasonic signals.

The present study aimed to validate a quantitative imaging technique used to detect and measure steatosis with statistical information from ultrasound echo signals with the focal disturbance ratio (FD-ratio) as a parameter in leptin-deficient (*ob/ob*) mice, a pure NAFLD model.

MATERIALS AND METHODS

This study was a collaborative effort between Iwate Medical University and Toshiba Medical Systems. However, no direct financial support was received from Toshiba Medical Systems for this study.

Animals

The animal research protocols for this prospective study were approved by our institutional research animal resource center. Nine 5 wk old (at the start of the study) male *ob/ob* mice were purchased from Charles River Laboratories (Yokohama, Japan) and maintained on conventional food and water throughout the experiment. These mice were divided into 3 groups ($n = 3$ each) that underwent the experiment described below at 5, 8 and 12 wk old, respectively.

General anesthesia was induced in mice by intraperitoneal administration of 40-50 mg/kg pentobarbital sodium (Ovation Pharmaceutical, Deerfield, IL), and underwent laparotomic ultrasonography, prior to having the liver extracted for histological examination.

Ultrasonographic imaging

An AplioXG ultrasound scanner (Toshiba Medical Systems, Otawara, Japan) was combined with a 12 MHz linear transducer (PLT-1204BT) for ultrasonographic investigations in this study. The scan mode was harmonic B-mode imaging (T: 6.0/R: 12.0 MHz). Display depth and transmit focus were fixed at 15 mm and 7.5 mm, respectively, and cross-sectional images of the hepatic parenchyma were recorded digitally with raw data, consisting substantially of the linear amplitudes without any cosmetic image processing. Raw data were uploaded to a personal computer in the DICOM format. FD-ratio was determined using ASQ software (details described in the next section). A region of interest (ROI) was set to a fixed depth of 2.5 mm to the liver surface (Figure 1). FD-ratio was measured 10 times in succession, and the mean value (after excluding outliers) was used as the final result. In parallel, mean echo intensity of the hepatic parenchyma was measured using Image-J image analysis software (NIH, United States).

Analytical method

The principles of the ASQ method^[20] are as follows. When echo signals are generated from very small, dense scatters located beyond the limit of spatial resolution, the pattern of the ultrasound image is constructed based on the interference of the sound waves (speckle noise). In that

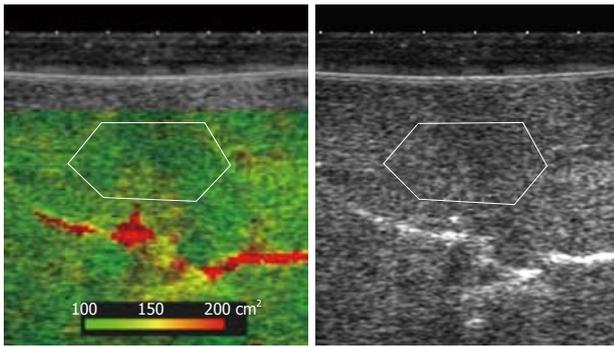


Figure 1 Acoustic structure quantification imaging. In parametric imaging, the intensity distribution can be visualized and displayed on a split screen by color coding. The large-region of interest was set at a fixed depth of 2.5 mm from the liver surface.

case, the PDF of the echo amplitude can be approximated using the Rayleigh distribution function^[21]. In normal liver parenchyma, such statistical results are not described by the Rayleigh distribution due to the existence of structures such as vessel walls. Results for livers with either nodules or fibrosis, i.e., liver cirrhosis, are even less similar to the Rayleigh distribution. We hypothesized that in the case of progression of fat drops, these scatters would generate wave interference or mask the original small structures, which would change the PDF to more closely resemble a Rayleigh distribution.

Once the examiner sets a comprehensive ROI (hereinafter referred to as a large-ROI) on the image, several hundred small ROIs (small-ROIs hereinafter) are automatically set therein to calculate the PDF (Figure 2A). The essential parameter, C_m^2 , in the analysis is defined by the equation:

$$C_m^2 = \frac{\sigma_m^2}{\sigma_R^2(\mu_m)} = \left(\frac{\pi}{4 - \pi} \right) \frac{\sigma_m^2}{\mu_m^2}$$

where μ and σ^2 are the average and variance of the echo amplitude in a small-ROI, respectively. The $\sigma_R^2(\mu)$ is a variant if the Rayleigh distribution is estimated from the measured average. Multiple results for small-ROIs in a large-ROI are displayed as an occurrence histogram of C^2 (real line in Figure 2B). If samples consist of speckle noise, the C^2 histogram will gather to 100 with narrow variance, while structural information will make the average value larger and variance wider.

FD-ratio is calculated in the following manner. First, C_m^2 is defined as:

$$C_m^2 = \frac{\sigma_m^2}{\sigma_R^2(\mu)} = \left(\frac{\pi}{4 - \pi} \right) \frac{\sigma_m^2}{\mu^2}$$

where σ_m is the variance calculated from limited samples less than $\mu + 4\sigma$. If the ratio C^2/C_m^2 is larger than the threshold α , the result of C_m^2 is eliminated from the histogram (real line), but added to the alternative histogram (dotted line). The FD-ratio is the ratio of the area under the curve (AUC) for these two histograms: $R_{FD} = [\text{AUC (real)}]/[\text{AUC (dotted)}]$.

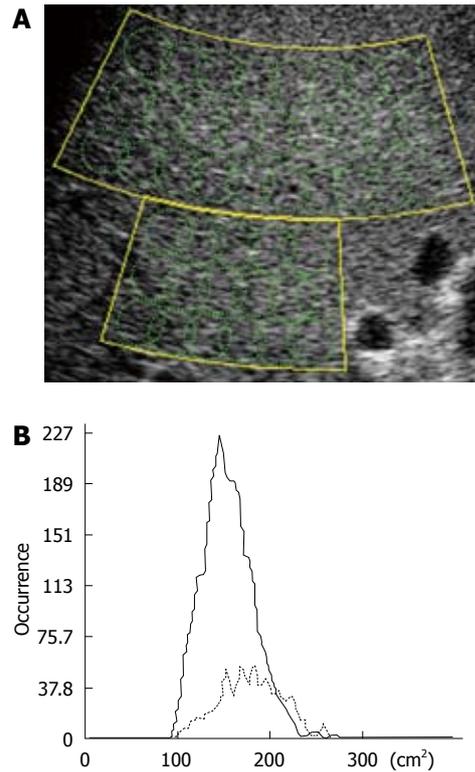


Figure 2 Schematic of region of interests used for statistical analysis of the radio frequency signal with the acoustic structure quantification method, and the C_m^2 -histogram. A: The set large-region of interest (ROI) actually consists of several hundred small ROIs used to calculate multiple C_m^2 (intensity or amplitude) values; B: Results are shown as the occurrence in the C_m^2 histogram.

Here, the threshold α was set to 1.2, so $R_{FD} = 0$ when the samples show a Rayleigh distribution for the PDF, and has a positive value in the presence of tiny structural changes.

The ASQ software also has an imaging function that reconstructs the 2-dimensional color map of the C^2 measured for each position (parametric image).

Histopathological analysis

Histopathological examinations were performed by an experienced pathologist certified by the Japanese Society of Pathology. Images from liver biopsy samples were recorded using the JPEG format. Using Image-J software, the percentage fat area was calculated from the ratio between total fat tissue area and total specimen area, and the mean value was used to denote the fat droplet area. In addition, again using Image-J software, the maximum diameter of fat droplets in 8 and 12 wk old mice was measured, and median values were used to denote the size of fat droplets. The fat droplet area and size obtained in this manner were compared with the FD-ratio.

Statistical analysis

Values are shown as mean \pm SD, or median (range) according to the distribution of values. Stat View software (version 5.0; SAS Institute, Cary, NC, United States) was used for all statistical analysis. The Spearman rank-order

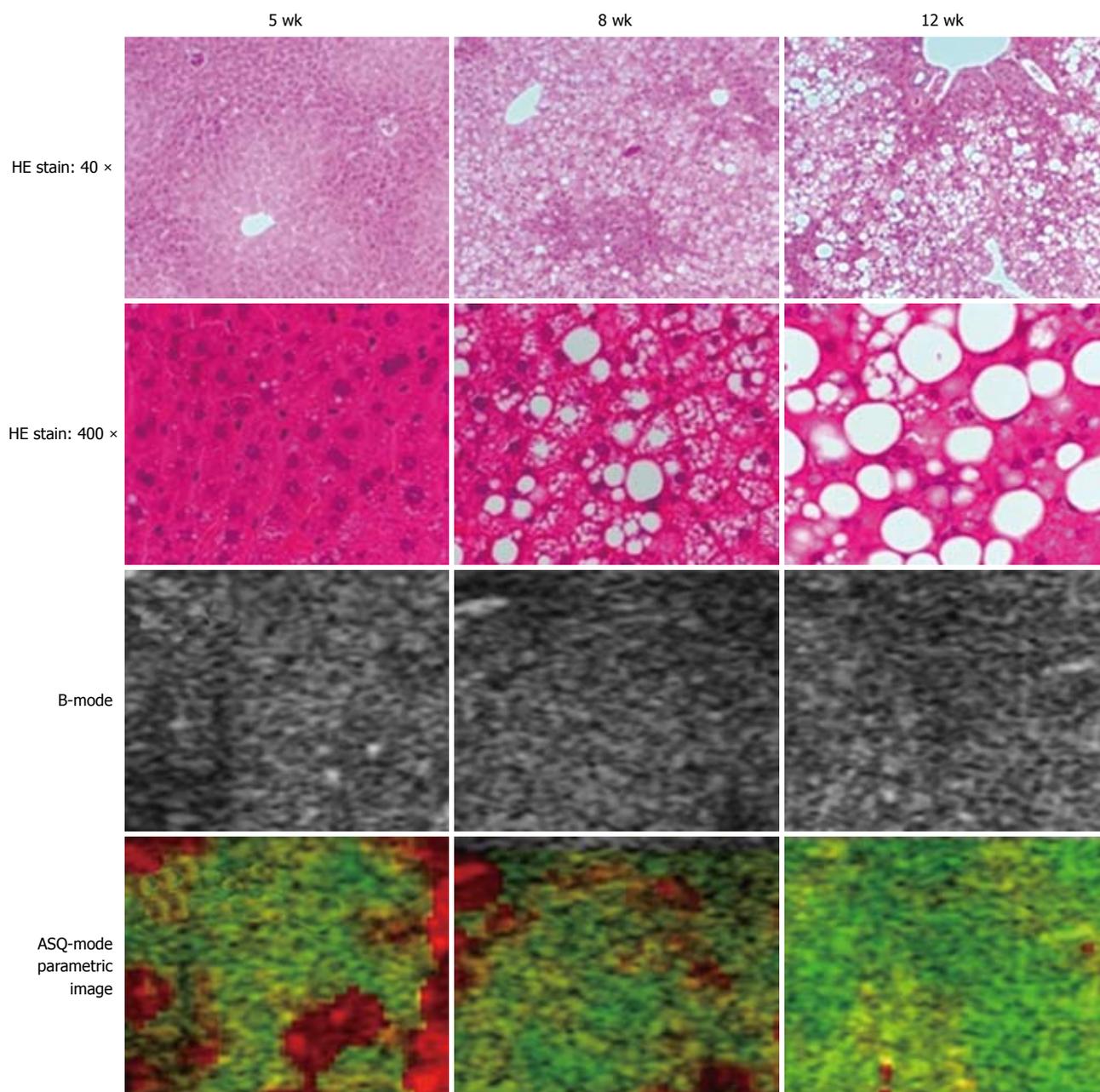


Figure 3 Representative histological findings, B-mode images and acoustic structure quantification-mode images for each group. No fibrosis or inflammation was observed in any groups. Large fat droplets were mainly observed at 12 wk old. In parametric imaging, red and green existed together at 5 wk, and red decreased and green was more abundant at 12 wk. ASQ: Acoustical structure quantification.

correlation test was used to study correlations between two variables, with a significant correlation considered to exist for values of $P < 0.05$, and for correlation coefficient $r \geq 0.40$. The Tukey-Kramer method was used for multiple comparison tests, and values of $P < 0.05$ were considered to indicate a significant difference.

RESULTS

Comparison of histological findings and liver echogenicity

No fibrosis or inflammation was observed in any groups. Large fat droplets were mainly observed at 12 wk

(Figure 3). Fat droplet area increased significantly ($P < 0.01$ each) with age from $1.25\% \pm 0.28\%$ at 5 wk to $31.07\% \pm 0.48\%$ at 8 wk, and to $51.69\% \pm 3.19\%$ at 12 wk. Median fat droplet size also increased significantly ($P < 0.01$ each) with age, from $1.33 \mu\text{m}$ (range: $0.55\text{-}10.52 \mu\text{m}$) at 5 wk to $2.82 \mu\text{m}$ (range: $0.61\text{-}44.13 \mu\text{m}$) at 8 wk and $6.34 \mu\text{m}$ (range: $0.66\text{-}81.83 \mu\text{m}$) at 12 wk (Figure 4). Mean Gray values in each group were 65.31 ± 22.52 at 5 wk, 65.95 ± 19.41 at 8 wk and 91.32 ± 21.83 at 12 wk (Figure 5). Although no differences were observed between the 5 and 8 wk old groups, mean gray value was significantly elevated in the 12 wk old group, with an increase observed in the brightness of the hepatic parenchyma.

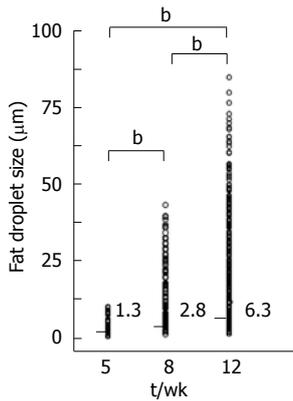


Figure 4 Comparison of fat droplet size. Median fat droplet size increased significantly ($^bP < 0.01$) with age, from 1.33 μm (range: 0.55-10.52 μm) at 5 wk, to 2.82 μm (range: 0.61-44.13 μm) at 8 wk and 6.34 μm (range: 0.66-81.83 μm) at 12 wk.

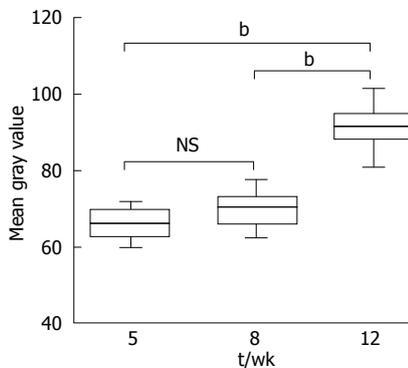


Figure 5 Comparison of liver echogenicity. Mean gray values in each group were 65.31 ± 22.52 at 5 wk, 65.95 ± 19.41 at 8 wk, and 91.32 ± 21.83 at 12 wk. Although no difference was apparent between the 5 and 8 wk old groups, mean gray value was significantly elevated in the 12 wk old group, with increased brightness of the hepatic parenchyma. $^bP < 0.01$. NS: Not significant.

Relationship between FD-ratio and fat droplet area or size

Mean FD-ratio was 0.42 ± 0.11 at 5 wk, 0.11 ± 0.05 at 8 wk, and 0.03 ± 0.02 at 12 wk. The FD-ratio was significantly lower at 12 wk than at 5 or 8 wk ($P < 0.01$ each). In parametric imaging, red and green existed together at 5 wk, and the red had decreased and green was more abundant at 12 wk (Figure 3). A significant negative correlation was observed between FD-ratio and both fat droplet area ($r = -0.7211$, $P = 0.0017$) (Figure 6A) and fat droplet size ($r = -0.9811$, $P = 0.0052$) (Figure 6B).

DISCUSSION

The results of this study demonstrated a close correlation between FD-ratio and the degree of histologically evaluated fat accumulation in the liver. These data suggest that ASQ analysis of liver ultrasonography and its representative parameter, FD-ratio, may offer a reliable new clinical modality for the assessment of liver steatosis.

With regard to the ultrasonographic diagnosis of fatty liver disease, Joseph *et al*^[16] advocated the bright liver pat-

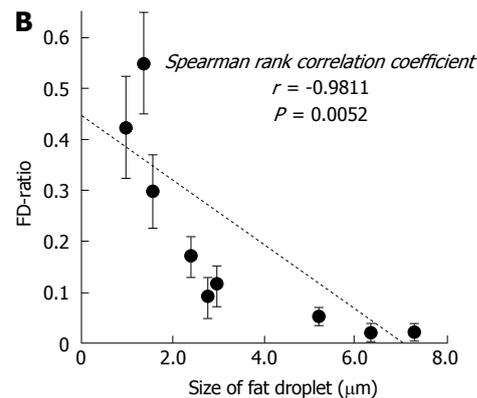
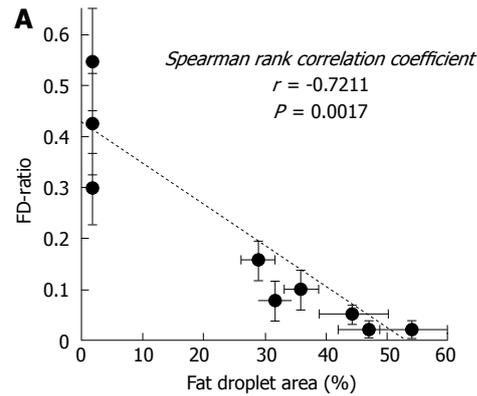


Figure 6 Relationship between “focal disturbance ratio” and fat droplet area or size of fat droplets. A: Significant negative correlations were observed between focal disturbance ratio (FD-ratio) and either fat droplet area ($r = -0.7211$, $P = 0.0017$); B: Fat droplet size ($r = -0.9811$, $P = 0.0052$).

tern, and attempts to quantify the hepatosplenic contrast or hepatorenal contrast were subsequently proposed^[22-24]. Although findings of a bright liver pattern and hepatorenal contrast are widely accepted as sensitive, reliable findings for the presence of fatty liver disease, no quantitative method to evaluate the severity of hepatic fat accumulation has previously been established. The difficulties in ultrasonographic diagnosis may originate from the fact that image information obtained by conventional ultrasonography lacks an objective or quantitative nature, unlike the X-ray absorbance in CT. In contrast, ASQ analysis generate objective data and provide a quantitative assessment of liver histology with respect to fat accumulation. Taking into account the non-invasive and inexpensive nature of ultrasonography, ASQ analysis could become a mainstay in the diagnosis of fatty liver disease and evaluations of disease severity and response to treatment. In addition, these studies of tissue characterization using ASQ analysis could lead to the development of methods for the quantitative diagnosis of other diffuse liver diseases, including fibrosis or inflammation, thus decreasing the need for liver biopsies.

With regard to the ASQ method, procedures are undertaken to analyze the RF signals for each of a large number of small-ROIs set up within a large ROI, for the purpose of improving analytical precision^[17-19]. The assumption is that in small-ROIs with a high degree of de-

viation from the Rayleigh distribution, the strength of the signals contained therein would be non-homogeneous. We also assumed the presence of two kinds of inhomogeneous samples, i.e., diffuse inhomogeneity and focal inhomogeneity, and by placing our focus on the small-ROIs with a high degree of deviation from the Rayleigh distribution resulting from a focally inhomogeneous structure, we established FD-ratio as a parameter.

Conversely, a bright liver pattern and vascular blurring are observed in fatty livers, due to reflection and scattering of the ultrasound waves and physical pressure on small blood vessels by ballooning hepatocytes induced by the fat droplets. We predicted that, due to the large number of fat droplets assembled densely (and thereby enveloping structures such as small blood vessels and bile ducts), the brightness of the hepatic parenchyma would be increased, and hepatic vein walls would become blurred, thus resulting in homogenization of the signal strength in each small-ROI and a decrease in the number of focally inhomogeneous small-ROIs. As a result, we believed that, as the area and diameter of fat droplets increased, the FD-ratio would decrease. Interestingly, no significant difference was seen in the brightness of hepatic parenchyma between 5 and 8 wk old mice, while FD-ratios were lowest in 8 wk old mice. We can therefore infer that a tool for the statistical analysis of ultrasonic signals may detect, beyond a qualitatively oriented reading ability, small changes in the speckle pattern caused by steatosis.

In conclusion, a novel tool for the statistical analysis of ultrasonic signals using FD-ratio as a parameter would be useful for the quantitative evaluation of liver steatosis. FD-ratio can therefore be used as a non-invasive biological marker for the early detection and quantitative evaluation of hepatic steatosis.

ACKNOWLEDGMENTS

We wish to thank Ms. Yuriko Mikami for her invaluable technical assistance with ultrasonography.

COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease. The gold standard for quantitative assessment of steatosis has been considered to be liver histology. However, liver biopsy shows various limitations and repeating the procedure is difficult because of ethical concerns. Non-invasive alternatives to liver biopsy are thus needed.

Research frontiers

Non-invasive modalities such as ultrasonography, computed tomography, and magnetic resonance imaging have been employed for the assessment of hepatic steatosis. Researchers have recently reported that diffuse pathological changes in liver tissue can be quantitatively evaluated as the statistical deviation of ultrasound signals compared to normal liver. However, no reports have described the assessment of hepatic steatosis in NAFLD patients using this tool for the statistical analysis of ultrasonic signals.

Innovations and breakthroughs

This study validated a quantitative imaging technique used to detect and measure hepatic steatosis with statistical information from ultrasound echo signals using focal disturbance ratio (FD-ratio) as a parameter in an animal model.

Applications

FD-ratio can be used as a non-invasive biological marker for the early detection and quantitative evaluation of hepatic steatosis.

Peer review

The authors present some pilot data from their research on the diagnostic utility of a new ultrasound technique in an animal model of fatty liver.

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Differential roles of EPS8 in carcinogenesis: Loss of protein expression in a subset of colorectal carcinoma and adenoma

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Abstract

AIM: To analyze the epidermal growth factor receptor pathway substrate 8 (EPS8) expression status and role in colorectal carcinogenesis given that EPS8 has a conserved actin barbed-end capping function that is required for proper maturation in intestinal cells.

METHODS: We studied 8 colon cancer cell lines and 58 colorectal tumors (19 adenomas and 39 carcinomas). We performed expression microarray analysis of colon cancer cell lines followed by loss of heterozygosity (LOH)

analysis and immunohistochemistry for EPS8 expression in colon tumors. Subsequently, we performed mutation analysis by direct sequencing and methylation analysis by bisulfite sequencing and methylation-specific polymerase chain reaction assays.

RESULTS: Expression microarray analysis of colon cancer cell lines showed overexpression of EPS8 transcript in all lines but RKO. Genome wide loss of heterozygosity (LOH) analysis of colon tumors, showed considerable LOH at the *EPS8* gene locus. Immunohistochemically, EPS8 was constitutively expressed in normal colonic mucosa with a dot-like supranuclear localization with accentuation at the luminal surface supporting its proposed role in epithelial maturation. Nineteen colon tumors (4 adenoma, 15 carcinoma) out of 51 (37%) showed strikingly tumor specific EPS8 protein loss. Of the remaining tumors, 5/51 (2 adenoma, and 3 carcinoma, 10%) showed marked overexpression, while 27/51 tumors (53%) showed retained expression. Mutation analysis revealed a missense mutation (c.794C>T, p.R265C) in exon 8 in RKO. The *EPS8* promoter was also methylated in RKO, but there was no significant methylation in other cell lines or carcinoma specimens.

CONCLUSION: The loss of EPS8 expression in colorectal adenomas and carcinomas suggests that down regulation of this gene contributes to the development of a subset of colorectal cancers, a finding which could have applications in diagnosis and treatment.

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Key words: Actin capping; Colon cancer; Epidermal growth factor receptor pathway substrate 8; Hypermethylation; Immunohistochemistry; RKO

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INTRODUCTION

Epidermal growth factor receptor pathway substrate 8 (EPS8) is a 97-kDa protein that is tyrosine phosphorylated following stimulation of receptor tyrosine kinases (RTK)^[1]. EPS8 plays a role in signal transduction from RTK and PI3K^[2,3] leading to Rac-mediated actin remodeling, ruffle formation and cell motility^[4]. In *Caenorhabditis elegans* (*C. elegans*), *eps-8* knockdown animals were zygotic lethal due to major defects in the gut, and the isoform EPS-8A was shown to be required for proper apical morphogenesis in the intestinal cells. This phenotype was correlated with an actin barbed-end capping activity, which is present in the C terminus of the EPS-8A isoform and is required for coordinately terminated elongation of the microvillar actin bundle core^[5]. This function of EPS8 protein is conserved throughout evolution^[6].

EPS8 was recently shown to be overexpressed in advanced stage human cancers including colon cancer cell lines and specimens^[7]. Our expression microarray analysis of colon cancer cell lines confirmed this overexpression. Interestingly, there was a strikingly low level of *EPS8* in RKO, a colon cancer cell line with a marked lack of constitutive β -catenin regulated transcription^[8], which prompted us to conduct a comprehensive immunohistochemical, genetic, and epigenetic analysis of *EPS8* alterations in colorectal cell lines and patient specimens.

MATERIALS AND METHODS

Patients and samples

We studied 8 colon cancer cell lines (RKO, HCA7, KM12, LoVo, DLD1, HCT116, SW48, LIM1215) and 58 colorectal tumors (19 adenomas and 39 carcinomas) of which 21 tumors (4 adenomas and 17 carcinomas) belong to a well characterized series of familial colon cancer type X (FCC-X). 15 adenomas and 22 carcinomas were sporadic. Clinicopathological characteristics of these cohorts are available in our previous publications^[9-11]. The FCC-X originated from 19 cancer families clinically indistinguishable from Lynch syndrome (hereditary non-polyposis colon cancer), but screening negative for the known predisposing genes by multiple techniques^[12]. We identified distinct molecular features in these tumors including high frequency of genomically stable carcinomas with membranous β -catenin, however, the predisposing defects in these families remain elusive^[9]. The sporadic colorectal tumors were selected from a larger cohort with the aim to include equal numbers of tumors with membranous *vs* nuclear β -catenin.

Fresh frozen and/or paraffin derived specimens of tumor and matching normal tissues were collected from pathology departments of different hospitals and used for immunohistochemical analysis and DNA extraction according to standard protocols. All human specimens were obtained after informed consent and approvals from the appropriate institutional review boards of the Helsinki University Central Hospital.

mRNA expression analysis by microarrays

Analyses were performed using HG-U133 Plus 2.0 array (Affymetrix, Santa Clara, CA, United States). The protocols for HG-U133 Plus 2.0 arrays were as described by the manufacturer (Affymetrix, Santa Clara, CA, United States). Briefly, total RNA was extracted from cell lines by RNeasy (Qiagen, Valencia, CA, United States). An aliquot of each RNA sample was run on a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States) to visualize and quantify the degree of RNA integrity. Double-stranded cDNA was synthesized from 5 μ g of total RNA using the GeneChip One-Cycle cDNA synthesis kit, followed by cleanup with the GeneChip Sample Cleanup Module, *in vitro* transcription (IVT) and Biotin labeling reaction using the GeneChip IVT Labeling kit, and clean-up and quantification of the biotin-labeled cRNA yield by spectrophotometric analysis. All kits were from Affymetrix. Fragmentation of the 8 μ g cRNA and hybridizations to test chips and the HG-U133 Plus 2.0 array were carried out according to Affymetrix protocols, and microarrays were processed by the Affymetrix Fluidics Station 450 and scanned with an Affymetrix GeneChip Scanner 7G. Captured images were analyzed using Microarray Suite version 5.0 algorithm (Affymetrix). All quality control criteria recommended by Affymetrix were observed in the "Test" chips and sample chips.

The hybridization data were pre-processed using Robust Multi-array Average (RMA^[13]), designed to enhance the comparability of expression measures between separate arrays. RMA pre-processing produces a single expression measure for each probe set in the Affymetrix array which can be readily used in subsequent analyses. As duplicate arrays were available for each cell line, the median of the two RMA values was used as the expression value. Gene assignments of the probes were extracted from the Affymetrix annotation files and genes with ambiguous information about the physical location were excluded from the analysis.

EPS8 loss of heterozygosity analysis

For the *EPS8* loss of heterozygosity (LOH) analysis we chose two microsatellite markers spanning the *EPS8* gene locus at Ensembl cytogenetic band 12p12.3 and surrounding the gene from both directions (<http://www.ensembl.org>). The physical distances between loci in mega-bases according to Ensembl are given in parentheses: pter D12S1580 - (2.4 Mb) - EPS8 - (0.4 Mb) - D12S1728 qter. The polymerase chain reaction (PCR) amplification primers were from Généthon Microsatellite Maps at <http://>

www.genlink.wustl.edu/genethon_frame. The forward primers were fluorescently labeled with carboxyfluorescein and PCR fragments were run on the ABI3730 sequencer/genotyper and results analyzed using GeneMapper v3 software (Applied Biosystems, Forster City, CA, United States) as described previously^[9]. A sample was scored as showing LOH, if one of the alleles had decreased 40% or more, and borderline LOH or allelic imbalance, if the decrease was 25%-39% for one allele.

Microsatellite instability analysis

Microsatellite instability status was determined using the Bethesda panel of 5 microsatellite markers and additional markers as described^[9,14,15]. Tumors with two or more unstable markers were considered to have high-degree microsatellite instability (MSI-H), while those with one unstable marker had low-degree microsatellite instability (MSI-L) and those with no unstable markers were microsatellite stable (MSS). MSI-H cancers were mostly excluded from this study cohort to enable the LOH study.

Immunohistochemical analysis

Four-micrometer sections from formalin-fixed paraffin-embedded tissues were mounted on silanized slides (Dako, Glostrup, Denmark) and air-dried overnight at 37 °C. After de-waxing and re-hydration in distilled water, sections were subject to heat-induced target retrieval in 1 mmol/L ethylenediaminetetraacetic acid buffer pH 8.0 for 5 min at 750 W followed by 5 min at 450 W in a microwave oven. After cooling, the slides were washed in Tris-buffered saline pH 7.2 and subsequent staining steps were performed manually with the Dako EnVision+ System, Peroxidase (DAB), according to the manufacturer's instructions (Dako, Glostrup, Denmark). In addition, after blocking endogenous peroxidase activity, and prior to incubation with the primary antibody, the sections were incubated with 10% normal (non-immune) goat serum (Dako, Glostrup, Denmark) for 30 min. The primary antibodies were purified rabbit polyclonal anti EPS8 Antibody (C-terminal, clone RB4006, Abgent, San Diego, CA, United States) and purified mouse monoclonal anti- β -catenin antibody (clone 14, BD Transduction Laboratories, Ermbodegem, Belgium). Paired tumor and normal mucosa were in the same section and the normal tissues were used as an internal reference for evaluation of staining results. β -catenin immunohistochemical staining for identification of its sub-cellular localization and the interpretation of results were performed as described^[9]. β -catenin expression was considered aberrant if there was nuclear staining of more than 10% or cytoplasmic staining of more than 50% of tumor cells (not observed in the matching normal tissue). For approximately half of the tumors, β -catenin data were available from our earlier studies^[9], while for the rest, these results were generated in the present investigation.

EPS8 mutation analysis

All coding exons of the *EPS8* gene were examined

by direct sequencing. The primer sequences and PCR conditions are given in Table 1. *EPS8* sequences were compared to that of GenBank accession number RefSeq NC_000012.10, and exon information was from Ensembl ENST00000389337. DNA mutation numbering is based on cDNA sequence where +1 corresponds to the A of the ATG translation initiation codon and the initiation codon is codon 1. Sequence changes reported here were present in sequence tracing from both the forward and reverse direction and were reproducibly found in 2 independent PCR products from cases of interest.

EPS8 methylation analysis

To search for CpG islands in the *EPS8* promoter, the EMBOSS CpG Plot program was used with default definitions (<http://www.ebi.ac.uk/emboss>). Two adjacent CpG islands were identified, together spanning 750 bp within and upstream of the untranslated exon number 1. This area was divided into two overlapping segments to screen cell lines and normal lymphocytes for methylation by bisulfite sequencing. The primers for the distal region were, forward, 5'-gggagatttttagggatttgatgg-3' and reverse, 5'-ccaaattatcaaaaccacaatcaaaatc-3', and for the proximal region (closest to and in part including the untranslated exon 1), forward, 5'-ttagttagttttgtagggatttttgg-3' and reverse, 5'-ctaactactacataaaatctaaacc-3'. Only the distal region showed any evidence of methylation, which is why we focused on this region when designing methylation-specific PCR (MSP) assays for the studies of patient specimens.

MSP^[11] was performed to separately amplify either methylated or unmethylated alleles from the distal region of the *EPS8* promoter (see above). Two alternative pairs of primers (MF1 + MR1, or MF3 + MR3) were used for the methylated reaction, and primers UF1 + UR1 for the unmethylated reaction. The primer sequences were: MF1, 5'-tggtattatagatgcggttttggttgc-3', MR1, 5'-gtataaaaacttcgccccgcagc-3'; MF3, 5'-ggtgttgaattgagcgttttttc-3', MR3, 5'-aacgtataaaaacttcgccccgc-3'; and UF1, 5'-ttggttagatgtgttttggttt-3', UR1 5'-ccaacaaaataaacacccccaaaca-3'. DNA (1 μ g) was modified with sodium bisulfite treatment (CpGenome DNA Modification kit, Chemicon) and subjected to MSP. MSP was performed in a volume of 25 μ L containing 24 ng of bisulfite-modified template per reaction with HotStarTaq DNA polymerase (Qiagen). Cycling conditions were according to the manufacturers' standard cycling protocol for HotStarTaq DNA polymerase, with 35 cycles. Annealing temperatures were 58 °C for the methylated reaction MF1 + MR1, 64 °C for the methylated reaction MF3 + MR3, and 58 °C for the unmethylated reaction. MSP products were run through 2%-3% agarose gel, stained with ethidium bromide, and visualized with ultraviolet transillumination. All sodium bisulfite modifications and MSP runs were repeated at least twice. A negative control without template was included in each MSP run.

RESULTS

RKO is a special colon cancer cell line as it lacks constitu-

Table 1 Epidermal growth factor receptor pathway substrate 8 genomic primers (ENST00000389337)

Primer	Sequence	Primer for sequence	Tm	PCR fragment (bp)
EPS8ex1F	tctggcagcaacacatatt	F	59	227
EPS8ex1R	ccaaatcaaatcccccaaa		62	
EPS8ex2F	aaccaacacaatgacctttt		60	251
EPS8ex2R	tcactgctcattccaaaca	R	60	
EPS8ex3F	gagatagccacatgataccaaca		59	195
EPS8ex3R	tgttctcaagggtcactctaaa	F	60	
EPS8ex4F	tcttttcttttgccaat		56	280
EPS8ex4R	ttcatccattttcaacaatc	R	58	
EPS8ex5F	gattgttgaaaatggatggaa	F	59	261
EPS8ex5R	aaagctcccagacaactgc		59	
EPS8ex6F	tcagacaaggaacaatccctt	F	60	251
EPS8ex6R	tttttctaacttttgggaaaaa		60	
EPS8ex7F	agtaccacaagttagtaattgat	F	55	264
EPS8ex7R	tccaacccaaagtaagtgtc		60	
EPS8ex8F	ggcaaatggctcctctttt	F	60	206
EPS8ex8R	ccagtgatctaaaggcgactc		59	
EPS8ex9F	tgggctgctcttttctaa	F	60	280
EPS8ex9R	ctggagatctaacaggcatt		58	
EPS8ex10F	cctcctctcgcttattca	F	58	234
EPS8ex10R	cacacccccaaaaatctat		57	
EPS8ex11F	gaccgtcccctctgtgcta	F	60	229
EPS8ex11R	ccagacagacactggggta		60	
EPS8ex12F	ctgttttgccatgggtt	F	60	265
EPS8ex12R	aaggcattatagggtgtaaatgct		59	
EPS8ex13F	tatgcttcattccctcctg	F	60	297
EPS8ex13R	tgaataaaaatgagaactgcaatca		60	
EPS8ex14F	tgacctgagtctgattcaaa	F	59	274
EPS8ex14R	gacctgtcacctctgtagcac		59	
EPS8ex15F	cttaggaagagctagcagaat		54	250
EPS8ex15R	aatacttgaaggaaagttagttat	R	54	
EPS8ex16F	gggaactcttctagaaatgg	F	59	267
EPS8ex16R	aagagtataactctgtaaatgtgt		56	
EPS8ex17F	aaagtataattgtttctagacc	F	55	315
EPS8ex17R	tgccctcctgggaaacttac		61	
EPS8ex18F	ggggttctagagggtgatgt	F	61	292
EPS8ex18R	tgtgtacacacagaattgcaaag		60	
EPS8ex19F	tttctcttggtttaggcaat		59	256
EPS8ex19R	aatagttgttccagactttcaa	R	59	
EPS8ex20F	gcagcctgcacaagttagta	F	60	203
EPS8ex20R	aatgccaaaaacaatggagtt		59	

EPS8: Epidermal growth factor receptor pathway substrate 8; PCR: Polymerase chain reaction; F: Forward; R: Reverse.

tive β -catenin regulated transcription compared to other colon cancer cell lines^[8]. To detect genes that show the most remarkable differential expression in the RKO cell line compared to all the other cell lines (HCA7, KM12, LoVo, DLD1, HCT116, SW48, and LIM1215, each being mismatch repair-deficient like RKO), maximum deviance in signal between RKO and the remaining lines was calculated for each Affymetrix probe. When identifying putative over-expressed genes, the deviance was defined as the difference between the signal in RKO and the maximum signal in the other cell lines. In the case of under-expressed genes, the deviance was defined as the difference between the signal in RKO and the minimum signal in the other cell lines. The Affymetrix probe “202609_at” corresponding to the *EPS8* gene showed remarkable reduction in the RKO cell line when compared to the other cell lines. The signal detected in RKO was 118, whereas the other cell lines showed signals of 1454, 1361, 3792, 429, 683, 758 and 1804 (Log2 ratio-3.53).

Most patient samples were MSS apart from 5/43 (12%) which showed the MSI phenotype. By immunohistochemical analysis of clinical specimens, normal colonic mucosa showed dot-like supranuclear cytoplasmic expression pattern of EPS8 protein (Figure 1A). In some cases we noticed a gradient of expression with more intense staining at the luminal surface that faded away towards the intestinal crypts (Figure 1B). Colorectal adenomas and carcinomas showed three patterns of expression compared to their matching normal mucosae. 19 (4 adenoma, 15 carcinoma) out of 51 tumors (37%) showed tumor specific EPS8 protein loss, 5 (2 adenoma, and 3 carcinoma) out of 51 (10%) showed marked overexpression, while 27/51 tumors (53%) showed retained expression comparable to what was observed in the matching normal mucosae (Table 2, Figure 1C-F). However, there was no significant correlation between EPS8 expression pattern and β -catenin subcellular localization (in contrast to the finding in the RKO cell line), or tumor stage and location within the colon.

Table 2 Analysis of epidermal growth factor receptor pathway substrate 8 gene status and protein expression in uncultured tumor specimens *n* (%)

	Total number	Membranous β -catenin	LOH ¹	EPS8 protein loss	EPS8 mutation ²	Methylation ³
Sporadic carcinomas	22	10/22 (45)	7/19 (37)	11/22 (50)	0/11	0/7
Sporadic adenomas	15	4/10 (40)	6/15 (40)	4/14 (29)	0/4	ND
FCC-X	21	11/18 (61)	7/17 (41)	4/15 (27)	ND	ND
Total	58		20/51 (39)	19/51 (37)	0/15	0/7

¹Based on informative (i.e., constitutionally heterozygous) cases; ²Cases with protein loss by immunohistochemistry and/or presence of loss of heterozygosity (LOH) were selected for this analysis; ³Cases were selected on the basis of immunohistochemical protein loss without LOH. ND: Not done; FCC-X: Familial colon cancer type X; EPS8: Epidermal growth factor receptor pathway substrate 8.

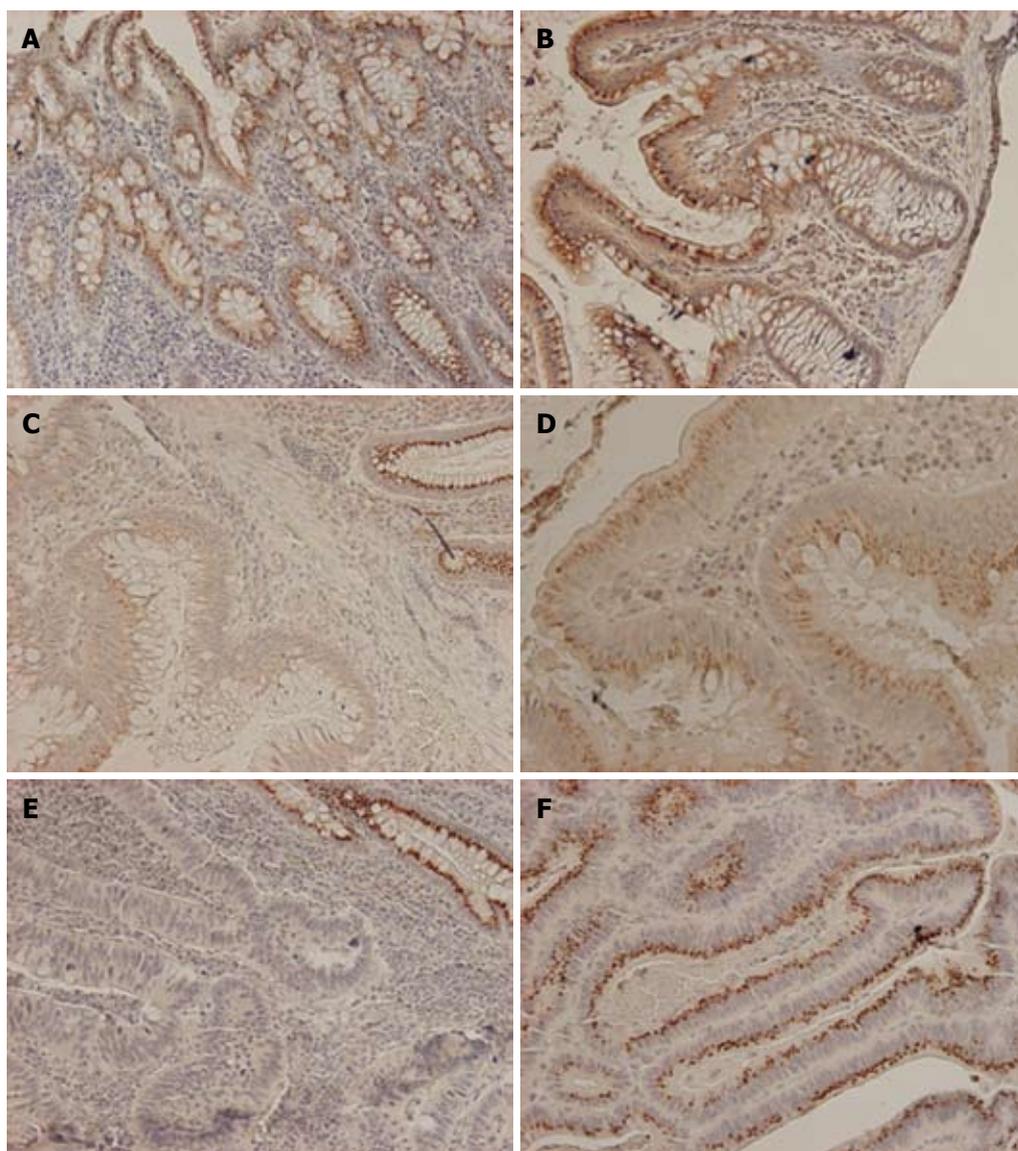


Figure 1 Epidermal growth factor receptor pathway substrate 8 immunohistochemistry. A: Normal colon mucosa with dot-like, supranuclear expression; B: Normal colon mucosa with clear gradient of expression stronger at the luminal aspect compared to the crypt bases; C: Marked reduction to complete loss of epidermal growth factor receptor pathway substrate 8 (EPS8) expression in adenoma (lower left) compared to normal mucosa (upper right); D: EPS8 positive adenoma; E: Marked reduction to complete loss of EPS8 expression in carcinoma (lower left) compared to normal mucosa (upper right); F: EPS8 positive carcinoma.

As possible mechanisms underlying expression changes, LOH, mutation, and promoter methylation were evaluated. We report LOH at EPS8 locus if, at least, one of the two markers D12S1580 and D12S1728 showed a clear cut

LOH (40% or more reduction) while borderline-LOH (ratio reduction ranging from (25%-39%) at one marker only was ignored. Overall, 20/51 (39%) tumors showed *EPS8* locus LOH with similar frequencies in adenomas (40%)

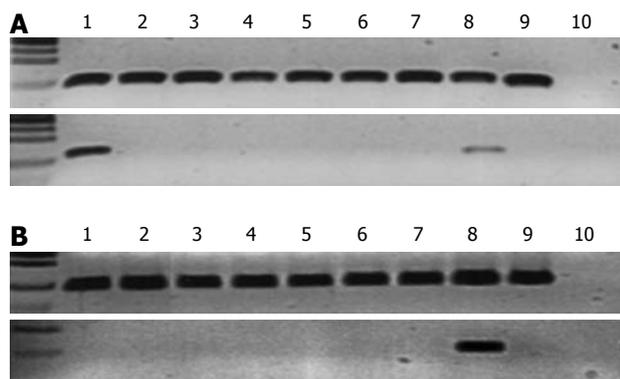


Figure 2 Methylation-specific polymerase chain reaction assays for epidermal growth factor receptor pathway substrate 8 gene. A: Cell lines analysis; upper panel is the unmethylated (UF1 + UR1) reaction and lower panel is the methylated (MF1 + MR1) reaction. Sample order in both panels from left to right is 1, RKO; 2, LoVo; 3, LIM1215; 4, HCA7; 5, HCT116; 6, KM12; 7, HCT15; 8, TK6 (a lymphoblastoid cell line); 9, unmethylated (negative) control; 10, water; B: Primary uncultured colon cancer analysis; upper panel is the unmethylated reaction and lower panel is the methylated reaction (MF3 + MR3). Sample order in both panels from left to right is 1-7, uncultured colon cancer specimens; 8, RKO (used as methylated control); 9, unmethylated (negative) control; 10, water.

and carcinomas (37%-41%) (Table 2). LOH was observed in 6/16 (38%) of informative tumors with absent *EPS8* protein *vs* 9/39 (23%) of cases with retained or elevated protein expression ($P = 0.53$).

All coding exons and flanking intronic regions of the *EPS8* gene were examined by direct sequencing. The focus of this analysis was the cell line RKO and tumors with loss of *EPS8* expression and/or LOH. We also included one of the control cell lines (HCA7 because of other special features^[16]). We identified only one tumor specific missense mutation c.794C>T (p.R265C) in exon 8 in the RKO cell line. Since the matching normal tissue for the RKO cell line was not available, we further analyzed more than 100 normal DNA samples and none of them showed this change. To our knowledge, this nucleotide change is also not reported in any sequence or single nucleotide polymorphism (SNP) database. The nature of the amino acid change suggests that this is not likely to be a SNP since Arginine (R) is a positively charged, large polar amino acid that mostly prefers to substitute for the other positively charged amino acid Lysine, although in some circumstances it will also tolerate a change to other polar amino acids, but substitution with the small amino acid cysteine (C) is not tolerated in any cellular location^[17]. We tested this particular substitution using the SIFT program (<http://blocks.fhcrc.org/sift/SIFT.html>) that sorts intolerant from tolerant amino acid substitutions based on evolutionary conservation, and cysteine substitution was regarded as intolerant.

Regarding *EPS8* methylation analysis, our very first observation was that the well-established human lymphoblastoid cell line TK6 was methylated in the distal region of the *EPS8* promoter, which indicated that the promoter was sensitive to methylation in general (Figure 2A). *EPS8* promoter methylation was examined by bisulfite sequencing in all cancer cell lines. These included

cell lines in which the *MLH1* promoter was known to be methylated (RKO, KM12, HCA7) as well as cell lines with unmethylated *MLH1* promoter (HCT15, HCT116, LoVo, LIM1215)^[11]. Only RKO was methylated (Figure 2A). Encouraged by *EPS8* methylation in RKO, we designed MSP reactions to investigate *EPS8* methylation status in patient specimens of colorectal cancer. We focused on those cases that had no LOH at chromosome 12 markers (including cases that were uninformative for LOH), yet *EPS8* protein was reduced or lost by immunohistochemistry, suggesting that there had to be alternative mechanisms for inactivation. There was no methylation in any of the seven tumor specimens analyzed, including five MSS tumors and two with MSI (Figure 2B). Given the lack of methylation in these samples of perhaps the highest interest, we did not extend these analyses to additional specimens.

DISCUSSION

Our data shed light on the role of *EPS8* in tumorigenesis in several important respects. *EPS8* is involved in actin dynamics through its actin barbed-end capping activity and its ability to modulate Rac activity. Accordingly, *EPS8* is crucial for the formation of actin networks that support cellular structures such as lamellipodia, filopodia, stress fibers and focal adhesions^[18]. It appears that this is the most significant function of *EPS8* in carcinogenesis also, since it did not colocalize with epidermal growth factor receptors but colocalized with F-actin in circular ruffles and at the leading edge of pancreatic cancer cells^[19]. The data presented here support an important role of *EPS8* in maturation and differentiation of the normal human colonic mucosa since normal colonic mucosa showed strong constitutive supranuclear cytoplasmic expression of *EPS8* with increasing intensity towards the luminal surface away from the crypt base. These data are consistent with the well established role of *EPS8* in the maturation of intestinal epithelium in *C. elegans*^[5] and the previously described expression pattern in pancreatic ductal cells^[19]. Potential roles of *EPS8* in normal colonic epithelium might include the migration of proliferating cells from the bases of the crypt to the colonic luminal surface and/or stabilization of cell-cell junctions, as *EPS8* was shown to be involved in cell-cell junction stability in fibroblasts^[20], and *EPS8* knockdown impaired actin cell-cell junction in confluent pancreatic cancer cells^[19].

Regarding colon carcinoma, we noticed high levels of *EPS8* mRNA in all cell lines except RKO. However, immunohistochemical analysis of *EPS8* protein in uncultured tumor biopsies showed that only around 10% of uncultured patient biopsies showed protein overexpression. This discrepancy between the cell line mRNA approach and patient biopsies' protein expression was consistent with the observation in pancreatic ductal adenocarcinomas^[19] and may be explained by the apparent need of the cell lines to over-express motility and invasion markers. We are currently undertaking studies to ex-

plore the role of miRNA in posttranscriptional regulation of EPS8 protein expression. However, studies showed good correlation between mRNA and protein levels within the same tumor model^[21].

The remarkable finding in this work was loss of EPS8 protein expression in subsets of colon adenoma and carcinoma. This finding is intriguing given the large number of published reports on EPS8 upregulation in different types of cancers, including those of the colon^[7,19,22-24]. We also noted this upregulation at the levels of mRNA and protein expression in some tumors as discussed above. A careful analysis of the published reports shows, however, that most cases of EPS8 upregulation were characteristic of advanced stage and metastatic cancers^[7,19,23,24]. The published literature suggests that EPS8 is most likely to be upregulated at the stage of metastasis. This hypothesis is best highlighted by the finding of EPS8 upregulation in the metastatic cell line SW620 as compared to its primary colon cancer cell line SW480^[7]. These two cell lines are a well established model that have been used to study the markers associated with metastasis in colon carcinomas^[25,26]. Similarly, the metastatic HN12 cells expressed high levels of EPS8 compared to its primary squamous cell carcinoma-derived cell line HN4^[22]. In pancreatic cancer, cell lines from primary tumors had low levels of *EPS8* mRNA expression; cell lines from pancreatic cancer metastases had medium levels of *EPS8* mRNA expression; and a cell line derived from malignant ascites (AsPC-1) had high levels of *EPS8* mRNA expression^[19]. These data could explain the apparent lack of mutation in our study, particularly in those tumors with loss of EPS8 protein which should leave a space for upregulation and overexpression at later stages; since reversion mutations are known to be extremely rare. In this regard, epigenetic and other regulatory mechanisms that could be easily reversed would be a preferable mode for controlling this gene expression status. Consistent with this, we noted the susceptibility of the *EPS8* promoter to methylation in the lymphoblastoid TK6 cells (Figure 2A) and its methylation in the RKO cell line associated with EPS8 mRNA underexpression. This is in agreement with RKO being a prototype of CpG island methylator phenotype that is usually observed in combination with MSI tumors^[27]. We, however, did not observe promoter methylation in the primary tumors considered to have the highest a priori likelihood for methylation, suggesting that *EPS8* inactivation in these tumors occurred by other, as yet unknown mechanisms.

In conclusion, we report EPS8 loss of expression in colorectal adenomas and carcinomas and propose that EPS8 downregulation plays a role in the development of these tumors.

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COMMENTS

Background

Epidermal growth factor receptor pathway substrate 8 (EPS8) is a 97-kDa protein that is required for intestinal cell maturation. EPS8 was recently shown to be overexpressed in advanced stage human cancers including colon cancer cells. In this study, the authors analyzed EPS8 status in colorectal cancers.

Research frontiers

This work applies multiple approaches to gain insight into the expression status of EPS8 in colorectal cancer cell lines and primary tumors. Furthermore, it sheds light on the possible mechanisms of the observed expression alterations.

Innovations and breakthroughs

The remarkable finding in this work was loss of EPS8 protein expression in colorectal adenoma and carcinoma. This finding is intriguing given the previously published reports on EPS8 upregulation in different types of cancers, including those of the colon. Thus, the results show, for the first time, that EPS8 downregulation plays a role in the development of subsets of colorectal tumors.

Applications

The current findings could have applications in diagnosis and treatment of a subset of colon tumors. The observed expression differences of EPS8 here raise a note of caution about generalization of the previously reported findings of EPS8 overexpression in some tumors and re-emphasize the significance of personalized medicine in the treatment of cancer patients.

Peer review

It is an interesting study worth to be considered.

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Choice of approach for hepatectomy for hepatocellular carcinoma located in the caudate lobe: Isolated or combined lobectomy?

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Abstract

AIM: To investigate the significance of the surgical approaches in the prognosis of hepatocellular carcinoma (HCC) located in the caudate lobe with a multivariate regression analysis using a Cox proportional hazard model.

METHODS: Thirty-six patients with HCC underwent caudate lobectomy at a single tertiary referral center between January 1995 and June 2010. In this series, left-sided, right-sided and bilateral approaches were used. The outcomes of patients who underwent isolated caudate lobectomy or caudate lobectomy combined with an additional partial hepatectomy were compared. The survival curves of the isolated and combined resection groups were generated by the Kaplan-Meier method and compared by a log-rank test.

RESULTS: Sixteen (44.4%) of 36 patients underwent isolated total or partial caudate lobectomy whereas 20

(55.6%) received a total or partial caudate lobectomy combined with an additional partial hepatectomy. The median diameter of the tumor was 6.7 cm (range, 2.1-15.8 cm). Patients who underwent an isolated caudate lobectomy had significantly longer operative time (240 min *vs* 170 min), longer length of hospital stay (18 d *vs* 13 d) and more blood loss (780 mL *vs* 270 mL) than patients who underwent a combined caudate lobectomy ($P < 0.05$). There were no perioperative deaths in both groups of patients. The complication rate was higher in the patients who underwent an isolated caudate lobectomy than in those who underwent combined caudate lobectomy (31.3% *vs* 10.0%, $P < 0.05$). The 1-, 3- and 5-year disease-free survival rates for the isolated caudate lobectomy and the combined caudate lobectomy groups were 54.5%, 6.5% and 0% and 85.8%, 37.6% and 0%, respectively ($P < 0.05$). The corresponding overall survival rates were 73.8%, 18.5% and 0% and 93.1%, 43.6% and 6.7% ($P < 0.05$).

CONCLUSION: The caudate lobectomy combined with an additional partial hepatectomy is preferred because this approach is technically less demanding and offers an adequate surgical margin.

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Key words: Hepatocellular carcinoma; Hepatectomy; Caudate lobectomy; Caudate lobe; Combined resection

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INTRODUCTION

The caudate lobe is a segment of the liver that is surgically difficult to approach because of its deep location in the hepatic parenchyma^[1-3]. The anatomic relationship of the caudate lobe to the hepatic vasculature was initially described by Couinaud. The caudate lobe is located anterior to the inferior vena cava (IVC), which may envelop this structure circumferentially. It extends to the hilum of the liver just posterior to the bifurcation of the portal vein. Cephalad, the caudate lobe lies posterior to the confluence of the left and middle veins as they enter the IVC on the left^[4].

The caudate lobe is generally divided into three regions: the left Spiegelian lobe, the process portion and the paracaval portion. As these regions are supplied by different vasculobiliary branches of the portal triad and they are drained separately by branches of the hepatic veins, each region can be resected independently, thus making partial caudate lobectomy possible^[5].

Caudate lobectomy is commonly indicated for hepatocellular carcinoma (HCC). It has been performed infrequently in the past, partly because of technical difficulties and the inadequate understanding of the anatomy^[5-7]. Precise anatomic knowledge of the caudate lobe, and improvement in perioperative care and the surgical techniques have resulted in more performance of caudate lobectomies. However, caudate lobectomy remains a technical challenge, even for experienced hepatic surgeons. Caudate lobectomy is classified as total or partial lobectomy, and as isolated caudate lobectomy or caudate lobectomy combined with an additional partial hepatectomy. This study aimed to evaluate the surgical outcomes of caudate lobectomy and the optimal surgical approach for HCC in the caudate lobe.

MATERIALS AND METHODS

Study subjects

Thirty-six patients with HCC underwent caudate lobectomy at the Department of Hepatobiliary Surgery and Liver Transplantation Surgery, Navy General Hospital between January 1995 and June 2010. Informed consent was obtained from each patient. Surgical outcomes for patients who underwent isolated caudate lobectomy or caudate lobectomy combined with an additional partial hepatectomy were compared. The data were collected prospectively and analyzed retrospectively.

Procedure

The surgeries of this series were completed over the past

15 years. The choice of approach mainly depended on the prevailing conditions and surgeon's experience. Surgery was performed through a bilateral subcostal incision in eight patients, while a Mercedes-Benz incision was used in 28 patients. After an exploratory laparotomy, the liver was fully mobilized from all its peritoneal attachments. The liver was then assessed with intraoperative ultrasound. We carefully searched the abdominal cavity for the extent of local disease, extrahepatic metastases and peritoneal seedings. In this series, three approaches were used^[8,9]: (1) a left-sided approach for tumors situated mainly in the Spiegelian lobe, or when a caudate lobectomy was combined with a left hepatectomy; (2) a right-sided approach for tumors situated mainly in the caudate process or paracaval portion, or when a caudate lobectomy was combined with a right hepatectomy; and (3) a bilateral approach for tumors situated in the whole caudate lobe. Although we started with one particular surgical approach in most patients, we had to combine different approaches to facilitate the caudate lobectomy. The suprahepatic and infrahepatic IVC was slung with vascular loops. Resection began with a pringle maneuver in cycles of 15/5 min of clamp/unclamp times. Total vascular exclusion was used only when patients had excessive bleeding from a lacerated IVC or hepatic vein. Liver resection was carried out by a clamp crushing method.

Statistical analysis

The survival curves of the isolated and combined resection groups were generated by the Kaplan-Meier method and compared with a log-rank test. To investigate the prognostic significance of the operative procedure, we performed a multivariate regression analysis with a Cox proportional hazard model, using a variable-selection method by a backward-elimination procedure. $P < 0.15$ was set as the cutoff for elimination. In the multivariate analysis, we chose 12 factors as potential confounders, considering their clinical significance and the results of previous reports^[10,11]. Because any factors that are of potential importance can be incorporated into a multivariate analysis, whether or not they are statistically significant^[12], we entered some nonsignificant factors in the univariate analysis into the model of the multivariate analysis in the present study. The 12 factors included: age (older *vs* younger than 65 years), sex, preoperative serum total bilirubin level (more *vs* less than 1 mg/dL), Child-Pugh class (A *vs* B), background liver status (cirrhosis *vs* noncirrhosis) as assessed histologically, tumor size (larger *vs* smaller than 30 mm), cancer spread (present or absent), tumor cell differentiation (well *vs* moderate or poor), serum-fetoprotein level (more *vs* less than 100 ng/mL), history of red blood cell transfusion (yes *vs* no), surgical margin (greater *vs* smaller than 5 mm) and tumor exposure (yes *vs* no). The Mann-Whitney U test and χ^2 test were used for the continuous and categorical data, respectively. All statistical analysis were performed using statistical software (SPSS 11.5 for Windows, SPSS, Inc., Chicago, IL). $P < 0.05$ was considered to be statistically significant.

Table 1 Patient characteristics

	Isolated caudate lobectomy group (<i>n</i> = 16)	Combined caudate lobectomy group (<i>n</i> = 20)	<i>P</i> value
Age (yr)	51 ± 14	48 ± 17	NS
Gender			NS
Male	12	15	
Female	4	5	
Liver cirrhosis			NS
Present	10	14	
Absent	6	6	
Child-Pugh class			NS
A	14	17	
B	2	3	
Liver function			
Albumin (g/dL) ¹	2.9	3.5	0.03
ALT (IU/L) ¹	54	32	0.04
Total bilirubin (mg/dL) ¹	1.2	0.8	0.04
Prothrombin time (%) ¹	75	79	NS
Location of the tumor			NS
Spiegel	2	3	
Paracaval portion	2	3	
Caudate process	1	2	
Spiegel + paracaval portion	3	4	
Paracaval portion + caudate process	3	2	
Complete caudate lobe	5	6	
Surgical margin (mm)			0.04
< 5 mm	5	1	
≥ 5 mm	11	19	
α-Fetoprotein (ng/mL) ¹	23	25	
Cancer spread ²			NS
Positive	3	5	
Negative	13	15	
Differentiation of tumor			NS
Edmondson I	1	1	
Edmondson II	5	4	
Edmondson III	10	14	
Edmondson IV	0	1	
Tumor size, median (range), cm	6.1 (2.1-13.4)	7.5 (2.3-15.8)	NS

¹Median; ²Cancer spread was defined by presence of microscopic vascular invasion and/or intrahepatic metastasis. ALT: Alanine aminotransferase; NS: Not significant.

RESULTS

During the study period, 36 patients (28 males and 8 females) underwent caudate lobectomy for HCC. The median age was 49 years (range 31-74 years), and 66.7% of the patients had liver cirrhosis. The median diameter of the tumor was 6.7 cm (range 2.1-15.8 cm). Tumors were present in all three parts of the caudate lobe in 11 patients, in the Spiegel lobe in five patients, in the paracaval portion in five patients, in the caudate process in three patients, in the paracaval portion and caudate process in five patients, and in the Spiegel and paracaval portion in 7 patients. The comparative data are shown in Table 1.

Surgical procedures

The operative procedures are listed in Table 2. Sixteen patients (44.4%) received an isolated complete or partial caudate lobectomy, whereas 20 (55.6%) underwent a

Table 2 Operative procedures

Operations	<i>n</i> (%)
Isolated caudate lobectomy	16 (44.4)
Complete caudate lobectomy	8
Partial caudate lobectomy	8
Concomitant procedures	
Partial IVC resection + repair	2
Approaches	
Left-side	2
Right-side	2
Bilateral	12
Combined caudate lobectomy	20 (55.6)
Complete caudate lobectomy + left hepatectomy	2
Complete caudate lobectomy + left lateral sectionectomy	2
Complete caudate lobectomy + right hepatectomy	1
Complete caudate lobectomy + right posterior hepatectomy	1
Partial caudate lobectomy + left hepatectomy	7
Partial caudate lobectomy + left lateral sectionectomy	3
Partial caudate lobectomy + right hepatectomy	2
Partial caudate lobectomy + right posterior hepatectomy	2
Concomitant procedures	
Partial IVC resection + repair	4
Approaches	
Left-side	4
Right-side	2
Bilateral	14

IVC: Inferior vena cava.

complete or partial caudate lobectomy combined with an additional partial hepatectomy. Five patients required a partial resection and repair of the IVC because of tumor invasion into the anterior wall of the IVC. The left-sided, right-sided and bilateral approaches were used in 6, 4 and 26 patients, respectively.

Surgical outcomes

The surgical outcomes were compared between isolated caudate lobectomy and caudate lobectomy combined with an additional partial hepatectomy. The median operating time was 198 min (range 150-310 min) and the median blood loss was 620 mL (range 150-1470 mL). Patients that underwent an isolated caudate lobectomy had significantly longer operative time, length of hospital stay and blood loss than patients who underwent caudate lobectomy combined with an additional partial hepatectomy ($P < 0.05$). There were no perioperative deaths in both groups of patients. Patients that underwent an isolated caudate lobectomy had a higher complication rate than those who underwent caudate lobectomy combined with an additional partial hepatectomy (31.3% *vs* 10.1%, $P < 0.05$, Table 3).

The 1-, 3- and 5-year disease-free survival rates for the isolated caudate lobectomy and the combined caudate lobectomy groups were 54.5%, 6.5% and 0% and 85.8%, 37.6% and 0%, respectively ($P < 0.05$, Figure 1A). The corresponding overall survival rates were 73.8%, 18.5% and 0% and 93.1%, 43.6% and 6.7% ($P < 0.05$, Figure 1B). Multivariate analysis identified combined resection as significantly influencing the overall survival rate and the disease-free survival rate (Table 4).

Table 3 Surgical outcomes			
	Isolated caudate lobectomy group (n = 16)	Combined caudate lobectomy group (n = 20)	P value
Time of vascular control, median (range), min	52 (32-68)	33 (25-39)	0.04
Blood loss, median (range), mL	780 (250-1470)	460 (150-980)	0.03
No. of patients with blood transfusion	14	11	0.02
Operative time, median (range), min	240 (170-310)	170 (150-225)	0.04
Hospital stay, median (range), d	18 (11-22)	13 (9-17)	0.04
Mortality	0	0	-
Complications			0.03
Liver failure	0	0	
Post-operative hemorrhage	2	0	
Bile leak	2	0	
Intra-abdominal collection	2	1	
Pleural effusion	1	1	

DISCUSSION

Although studies on caudate lobectomies have been increasingly reported, most of them were single case reports or small series reports^[13-15]. Some series contained cases of caudate lobectomy carried out for microscopic involvement of hilar cholangiocarcinoma^[16]. Comparative studies are very rare.

Caudate lobectomy is classified as a total or partial resection, and is also classified as an isolated or combined resection^[17]. Several approaches have been described for caudate lobectomy, including the left-sided approach, right-sided approach, combined left- and right-sided approach and the anterior transhepatic approach. Peng *et al.*^[18] also described the retrograde approach for resecting tumors in the caudate lobe that had invaded the IVC. The selection of an appropriate surgical approach is essential for a safe caudate lobectomy. When the tumor is large or the IVC and/or major hepatic vein is compressed by the tumor, caudate lobectomy is technically very difficult and the resection has to be carried out using a combination of different approaches^[19].

In an isolated caudate lobectomy, especially for a bulky tumor, it is important to recognize the danger of tearing the middle hepatic vein posteriorly when the caudate lobe is dissected away from this vein. To prevent major hemorrhage from a torn middle hepatic vein, the common trunk of the middle and left hepatic veins should be isolated and slung with a vascular loop before any attempt is made to dissect the caudate lobe within the tunnel formed by the IVC and the hepatic veins^[20]. In a caudate lobectomy that is combined with either a right or left hepatectomy, a caudate lobectomy can be performed with little danger of bleeding from the middle hepatic vein since this vessel is usually controlled extrahepatically, or it can be sacrificed and resected together with the

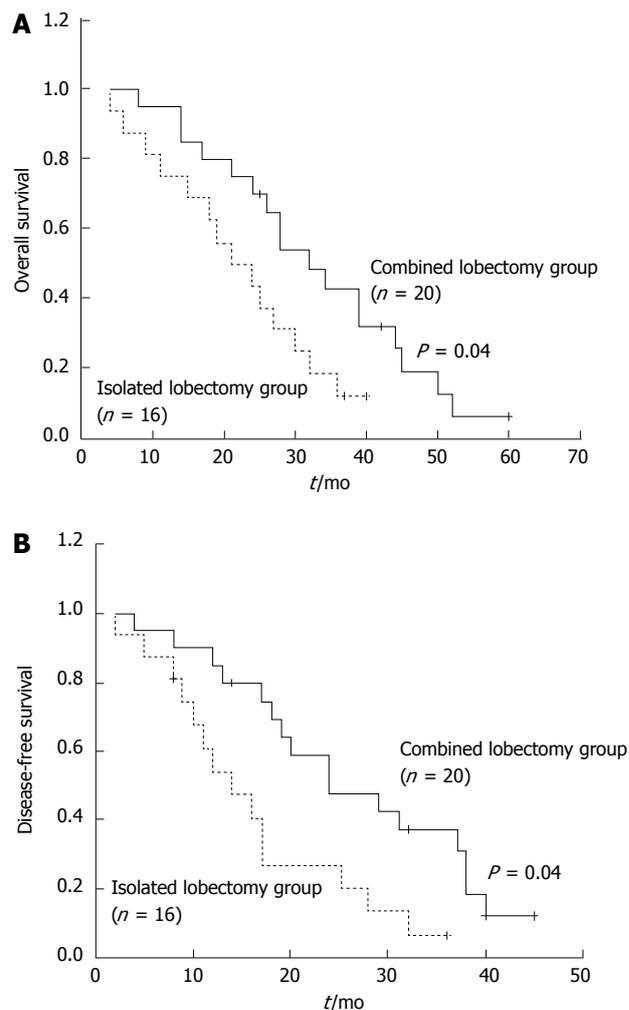


Figure 1 Overall survival rate curves and disease-free survival rate curves after isolated and combined resections for hepatocellular carcinoma originating from caudate lobe. A: Overall survival rate curves after isolated and combined resections for hepatocellular carcinoma originating from caudate lobe; B: Disease-free survival rate curves after isolated and combined resections for hepatocellular carcinoma originating from the caudate lobe.

specimen. Thus, an isolated caudate lobectomy is technically more difficult than a caudate lobectomy combined with either a right or a left hepatectomy.

The choice of isolated or combined resection is based primarily on the extent of HCC invasion and liver function reserve. The group that had an isolated resection of the tumor was characterized by well-differentiated, capsule intact, and poor liver function reserve, which could be easily removed. On the contrary, the group that had a combined resection of the tumor was characterized by poorly-differentiated, capsule incomplete, and better liver function reserve, which could be ablated with an extended resection to achieve the purpose of a complete resection.

We found that the isolated resection group had a worse long-term prognosis than the combined resection group. The main reasons were related to the following factors. First, the caudate lobe HCC was very close to the other lobe with limited growth space. Especially when the tumor was located in the paracaval part, it often infil-

Table 4 Multivariate analysis

Variables	Hazard ratio	95% CI	P value
Overall survival			
Absence of cancer spread ¹	0.44	0.24-0.69	0.007
Child-Pugh class A	0.86	0.66-1.33	0.17
Combined resection	0.57	0.32-0.92	0.04
Tumor size < 30 mm	0.61	0.32-1.05	0.08
Total bilirubin < 1 mg/dL	0.52	0.28-1.06	0.07
α -Fetoprotein < 100 ng/mL	0.61	0.33-1.19	0.07
Disease-free survival			
Absence of cancer spread ¹	0.61	0.37-0.82	0.001
Combined resection	0.66	0.42-0.94	0.03
Negative tumor exposure	0.39	0.20-0.77	0.04
Total bilirubin < 1 mg/dL	0.56	0.37-0.89	0.02

¹Cancer spread was defined by presence of microscopic vascular invasion and/or intrahepatic metastasis.

trated the other lobe, such as segment IV, V, VI, VII or VIII. Due to the unclear boundary, an isolated resection of the tumor could not achieve a complete resection. Second, the caudate HCC was often close to the main branch of the main portal and hepatic veins, which increased the likelihood of vascular invasion leading to an inadequate surgical margin. A caudate lobe resection combined with the other lobe could obtain a clear exposure and acquire a more adequate surgical margin. Third, from the no-touch point of view, repeated over-turning and pulling on the caudate lobe can cause HCC cells to transfer to other locations along the portal vein and hepatic vein, increasing the possibility of metastasis in the isolated resection. Although the anterior approach can avoid this problem, its application is limited by varying degrees of liver cirrhosis. The anterior approach required segment IV resection, which prolonged the operation time and increased the amount of bleeding^[21]. Obtaining a negative margin may not be easy particularly in large and very large HCC, especially for those located in the caudate lobe^[22-24]. Therefore, the style of the combined resection can solve the above problem, which is an optimal method. We advocate that the caudate lobe should be ablated from the combined adjacent lobe to get an adequate margin and reduce the stretching and compression of the tumor, thereby achieving a good long-term prognosis.

If confounders in a multivariate analysis are limited only to the significant factors in a univariate analysis, some factors, which are not significant despite their potential importance, may be excluded. Therefore, according to Tralhão *et al.*^[25], we chose 12 factors as confounders, after weighing their clinical importance, whether or not they were significant in the univariate analysis. Indeed, this method was also adopted in a previous study^[26,27]. The present study indicated that anatomic resection would be a suitable option of choice for HCC. Our multivariate analysis showed that liver function was an important prognostic factor for the overall survival, though the Child-Pugh class between the two groups showed no difference. In the other study, we found that segmentectomy or lobectomy might be recommended as an initial treatment for patients with good hepatic func-

tion and a solitary hepatic nodule because such patients have a chance of achieving long-term survival and wider surgical resections could minimize the chance of microscopic residual tumors or occult metastases^[28-32].

Approaches to a caudate lobectomy thus depend largely on the size and location of the lesion and liver functional reserve. For patients with sufficient liver functional reserve, partial or complete caudate lobectomy combined with other partial hepatic resections is preferred because such an operation is technically less demanding. For patients with a poor liver function, we are left with no choice but to carry out an isolated caudate lobectomy. HCC originating from the caudate lobe is relatively rare. As the study sample is small, a more accurate conclusion requires a multi-center randomized controlled study to confirm our results.

COMMENTS

Background

The caudate lobe is a segment of the liver that is surgically difficult to approach because of its location deep in the hepatic parenchyma, which is surrounded by branches of the porta hepatis, the hepatic veins and the inferior vena cava (IVC). Caudate lobectomy is commonly indicated for hepatocellular carcinoma (HCC). Currently, caudate lobectomy remains a technical challenge, even for experienced hepatic surgeons. This study gives some instructions for hepatectomy for HCC located in the caudate lobe, with the choice of isolated or combined lobectomy.

Research frontiers

Caudate lobectomy is classified as total or partial lobectomy; it is also classified as isolated caudate lobectomy or caudate lobectomy combined with an additional partial hepatectomy. The selection of an appropriate surgical approach is essential for a safe caudate lobectomy.

Innovations and breakthroughs

Although increasing numbers of studies on caudate lobectomy have been reported in the medical literature, most are single case reports or small series studies. Some series contained cases of caudate lobectomy carried out for microscopic involvement of hilar cholangiocarcinoma. Comparative studies are very rare. In this paper, 36 patients with HCC underwent caudate lobectomy at a single tertiary referral center between January 1995 and June 2010. The surgical outcomes of patients who underwent isolated caudate lobectomy or caudate lobectomy combined with an additional partial hepatectomy were compared. For patients with sufficient liver functional reserve, caudate lobectomy combined with an additional partial hepatectomy is preferred because such an approach is technically less demanding and offers an adequate surgical margin. For patients with a marginal liver functional reserve, the viable surgical option is an isolated caudate lobectomy.

Applications

This study showed that, in patients with sufficient liver functional reserve, a caudate lobectomy combined with an additional partial hepatectomy is preferred because such an approach is technically less demanding and achieves adequate surgical margin. However, for patients with marginal liver functional reserve, the viable surgical option is an isolated caudate lobectomy.

Terminology

Caudate lobectomy is classified as total or partial resection, and is also classified as an isolated or combined resection. Several approaches have been described for caudate lobectomy, such as the left-sided approach, right-sided approach, combined left- and right-sided approach and the anterior transhepatic approach. Recently, a retrograde approach for resecting tumors in the caudate lobe that have invaded the IVC has also been described.

Peer review

This manuscript emphasizes the optimal surgical approach for HCC in the caudate lobe. The manuscript sections are very clearly described and the conclusion is an opened door for further investigation. They observed that the 16 patients who underwent isolated lobectomy had longer operative times, greater blood loss, a higher complication rate, longer hospital stays and higher mortality. They concluded that, in patients with adequate functional reserve, combined hepatectomy is the preferred choice.

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Normal carcinoembryonic antigen indicates benefit from perioperative chemotherapy to gastric carcinoma patients

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Abstract

AIM: To evaluate pretreatment serum carcinoembryonic antigen (CEA) as a predictor of survival for patients with locally advanced gastric cancer receiving perioperative chemotherapy.

METHODS: We retrospectively studied a cohort of 228 gastric cancer patients who underwent D2 gastrectomy combined with chemotherapy at the Sun Yat-sen University Cancer Center between January 2005 and December 2009. Among them, 168 patients received 6-12 cycles of oxaliplatin-based adjuvant (post-operative) chemotherapy, while 60 received perioperative chemotherapy (2 cycles of FOLFOX6 or XELOX before surgery and 4-10 cycles after surgery). Serum CEA was measured using an enzyme immunoassay. The follow-up lasted until December 2010.

RESULTS: In the group that had elevated serum CEA, the difference in survival time between patients receiving perioperative chemotherapy and those receiving adjuvant chemotherapy had no statistical significance ($P > 0.05$). However, in the group that had normal serum CEA, patients receiving perioperative chemotherapy had a longer survival time. In multivariate analysis, T staging and lymph node metastatic rate were independent prognostic factors for the patients. Perioperative chemotherapy improved the overall survival of patients who had a normal pretreatment CEA level ($P = 0.070$).

CONCLUSION: Normal pretreatment serum CEA is a predictor of survival for patients receiving perioperative chemotherapy.

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Key words: Carcinoembryonic antigen; Perioperative chemotherapy; Prognosis; Gastric adenocarcinoma; Survival

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INTRODUCTION

Gastric cancer is one of the most common cancers worldwide. It is the second leading cause of cancer deaths in the world^[1-3], and most of those patients are diagnosed at an advanced stage of disease^[4,5]. Surgery is the main

treatment for gastric cancer. Many meta-analyses have demonstrated that adjuvant (post-operative) chemotherapy can improve the prognosis for gastric cancer patients^[6-8], and in some prospective clinical trials, adjuvant chemotherapy has improved the prognosis of patients with locally advanced gastric cancer^[9-11]. The Cunningham trial showed for the first time that perioperative chemotherapy (treatment both before and after surgery) is superior to surgery alone in treating gastric cancers. Further studies showed that preoperative chemotherapy combined with chemoradiotherapy provided substantial responses that improved the prognosis^[12-14].

Carcinoembryonic antigen (CEA) was first identified in 1965 by Gold and Freedman in human colon cancer tissue extracts^[15]. In the last two decades, CEA has been widely used as a tumor marker in the diagnosis and monitoring of some malignancies^[16]. Since the 1990s, tumor markers including CEA, carbohydrate antigen 19-9, and others have been widely used to monitor gastric cancer progression and even to assess the prognosis of gastric cancer patients, although their specificities have not been satisfactory^[17-20]. The controversial conclusions resulting from the use of these biomarkers are therefore understandable^[21]. In the present study, we retrospectively evaluated the predictive value of pretreatment serum CEA in patients with late-stage gastric cancer in China.

MATERIALS AND METHODS

Patient inclusion and exclusion criteria

Inclusion criteria: (1) age: 20 to 75 years; World Health Organization performance status 0 to 1; (2) histologically proven adenocarcinoma of the stomach; T3 or T4 tumor based on endoscopic ultrasound; no evidence of distant metastases or of disease considered nonresectable by endoscopic ultrasonography, computed tomography (CT), or extended diagnostic laparoscopy; (3) no prior gastric surgery; (4) no previous radiotherapy or other treatments, including immunotherapy or Chinese traditional medicine; (5) no uncontrolled infectious or cardiac disease; adequate hepatic and renal functions; and (6) no synchronous or metachronous cancers.

Exclusion criteria: (1) age: older than 75 years or younger than 20 years; (2) hepatic, renal, pulmonary, or cardiac dysfunction; and (3) severe postoperative complications, such as anastomosis leakage or anastomosis stenosis, that may cause malnutrition or make the patients intolerant to postoperative chemotherapy.

Patient characteristics

We included 228 patients who underwent D2 gastrectomy at the Sun Yat-sen University Cancer Center between January 2005 and December 2009. Among them, 173 patients had a normal pretreatment serum CEA (≤ 5 ng/mL) and 55 patients had elevated pretreatment serum CEA (> 5 ng/mL). Sixty patients among both CEA groups (43 with normal serum CEA and 17 with

Table 1 Clinicopathologic characterization of patients with gastric adenocarcinoma treated by surgery in combination with chemotherapy

Data	Normal serum CEA (median 52 yr, range: 22-74 yr)	Elevated serum CEA (median 54 yr, range: 32-73 yr)	P
	n (%)	n (%)	
Sex			0.090
Male	116 (67.1)	44 (80.0)	
Female	57 (32.9)	11 (20.0)	
Tumor location			0.115
Upper	52 (30.0)	26 (47.3)	
Middle	42 (24.3)	12 (21.8)	
Lower	68 (39.3)	15 (27.3)	
Total	11 (6.4)	2 (3.6)	
Histological grade			0.128
G1	1 (0.6)	0 (0)	
G2	31 (17.9)	18 (32.7)	
G3	112 (64.7)	29 (52.7)	
G4	29 (16.8)	8 (14.6)	
Tumor size			0.053
≤ 2 cm	23 (13.3)	1 (1.8)	
2 cm < diameter ≤ 5 cm	98 (56.6)	36 (65.5)	
> 5 cm	52 (30.1)	18 (32.7)	
Boarrman type			0.093
I	3 (1.7)	0 (0)	
II	90 (52.0)	20 (36.4)	
III	69 (39.9)	28 (50.9)	
IV	11 (6.4)	7 (12.7)	
Pathological T staging ¹			0.664
T0	6 (3.5)	1 (1.8)	
T3	156 (90.2)	49 (89.1)	
T4	11 (6.4)	5 (9.1)	
Lymph node metastasis rate			0.951
0	12 (6.9)	3 (5.5)	
$0 < r \leq 0.1$	29 (16.8)	8 (14.5)	
$0.1 < r \leq 0.3$	50 (28.9)	17 (30.9)	
$r > 0.3$	82 (47.4)	27 (49.1)	
Surgery			0.313
Radical	165 (95.4)	50 (90.9)	
Palliative	8 (4.6)	5 (9.1)	
Chemotherapy			0.384
Adjuvant	130 (75.1)	38 (69.1)	
Perioperative	43 (24.9)	17 (30.9)	

¹Pathological T staging was based on the 6th Union for International Cancer Control's staging systems for gastric cancer. CEA: Carcinoembryonic antigen.

elevated serum CEA) received oxaliplatin-based perioperative chemotherapy, with 2 cycles before surgery and 4-10 cycles of the same regimen after surgery. The exception was 6 patients suffering from progressive disease who received second-line chemotherapy for 4-6 cycles (see Treatment section). Among both CEA groups, 168 patients received only adjuvant chemotherapy (Figure 1). The clinicopathological characteristics of all patients are presented in Table 1.

Treatment

The two cycles of preoperative chemotherapy included the XELOX and FOLFOX regimens. All the chemotherapy regimens were used under standard protocols. The XELOX regimen consisted of oxaliplatin at 130 mg/m² (i.v. drip, day 1) and capecitabine at 1000 mg/m² (oral,

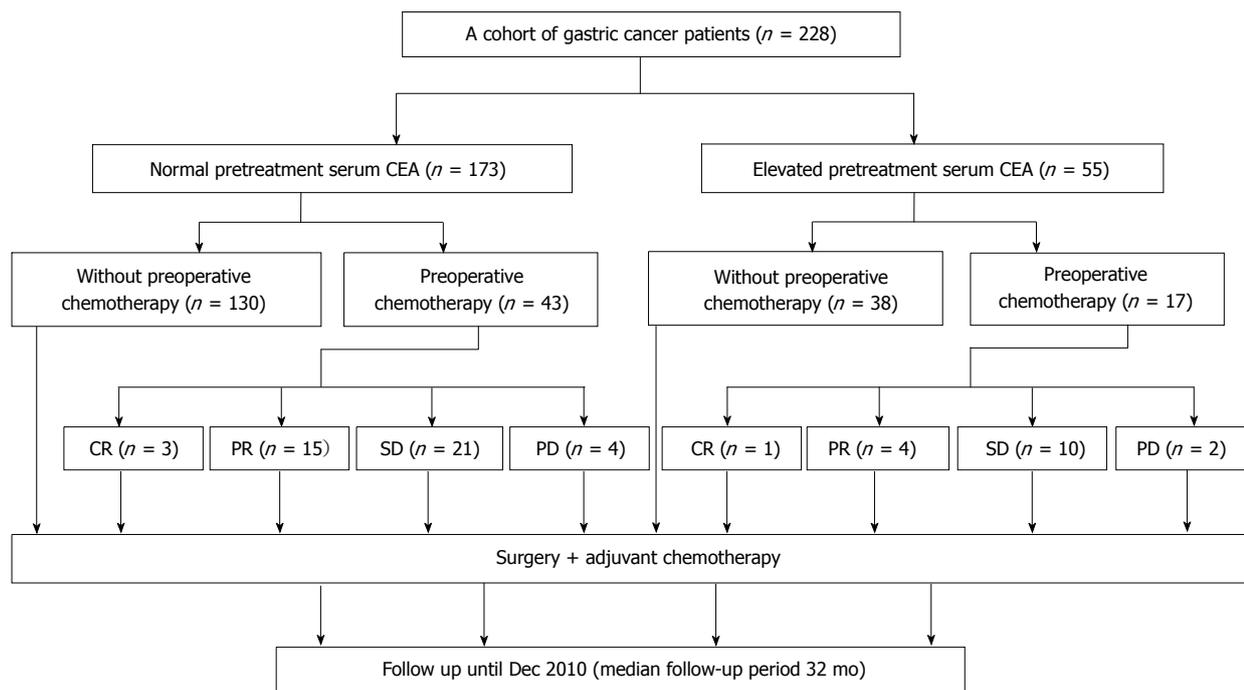


Figure 1 The treatment subgroups of the cohort of 228 patients in this retrospective study. CEA: Carcinoembryonic antigen; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

day 1-14), followed by one week of no treatment. Starting on day 22, the cycle was repeated, and surgery took place between day 43 and 47.

The FOLFOX6 regimen started on day 1 with oxaliplatin at 100 mg/m² (i.v. drip) with folic acid at 400 mg/m² (racemic) or 200 mg/m² (L-form), plus 5-fluorouracil (5-FU) as a 400 mg/m² bolus, followed by 2400 mg/m² of 5-FU as a continuous 46 h infusion. When the infusion was completed, there was no further treatment through day 14. On day 15, the cycle was repeated, with surgery taking place between day 30 and day 33.

After surgery, all of the patients received adjuvant chemotherapy starting within 2-4 wk. The median number of cycles for each regimen was 9 for FOLFOX6 (range: 7-12) and 7 for XELOX (range: 6-8). The six progressive disease patients received paclitaxel plus 5-FU (1 patient), docetaxel plus 5-FU (2 patients), or S-1 oral administration (3 patients) chemotherapy; these patients received a median of 5 cycles (range: 4-6).

Chemotherapy response evaluation

Assessment of the response to preoperative chemotherapy was based on the reduction of primary tumor size (as measured by endoscopic ultrasonography and CT scan) and the Response Evaluation Criteria in Solid Tumors criteria.

Complete response: Disappearance of all target lesions. Any pathological lymph nodes (whether target or non-target) must have a reduction of the short axis to less than 10 mm.

Partial response: At least a 30% decrease in the sum of diameters of target lesions, taking as a reference the baseline sum of diameters.

Stable disease: Neither sufficient shrinkage to qualify as a partial response nor sufficient increase to qualify as progressive disease.

Progressive disease: At least a 20% increase in the sum of diameters of target lesions, taking as reference the smallest sum in the study (which may include the baseline sum). The sum must also show an absolute increase of at least 5 mm.

Patient follow-up

After treatment, the patients were monitored every 3 mo for the first 2 years, then every 6 mo thereafter. Telephone calls and letters were used to assess patients who could not be physically present. Complete data were collected from all 228 patients until December 2010. The follow-up period ranged from 8 mo to 59 mo (median, 32 mo). The total follow-up times are shown in Figure 2A.

Statistical analysis

The χ^2 test was used to compare categorical variables between the normal and elevated serum CEA groups. Univariate survival analysis was performed using the Kaplan-Meier method. Survival curves were compared with the log-rank test. Multivariate statistical survival analysis was performed using Cox regression. Analysis were performed with SPSS software version 16.0 for Windows

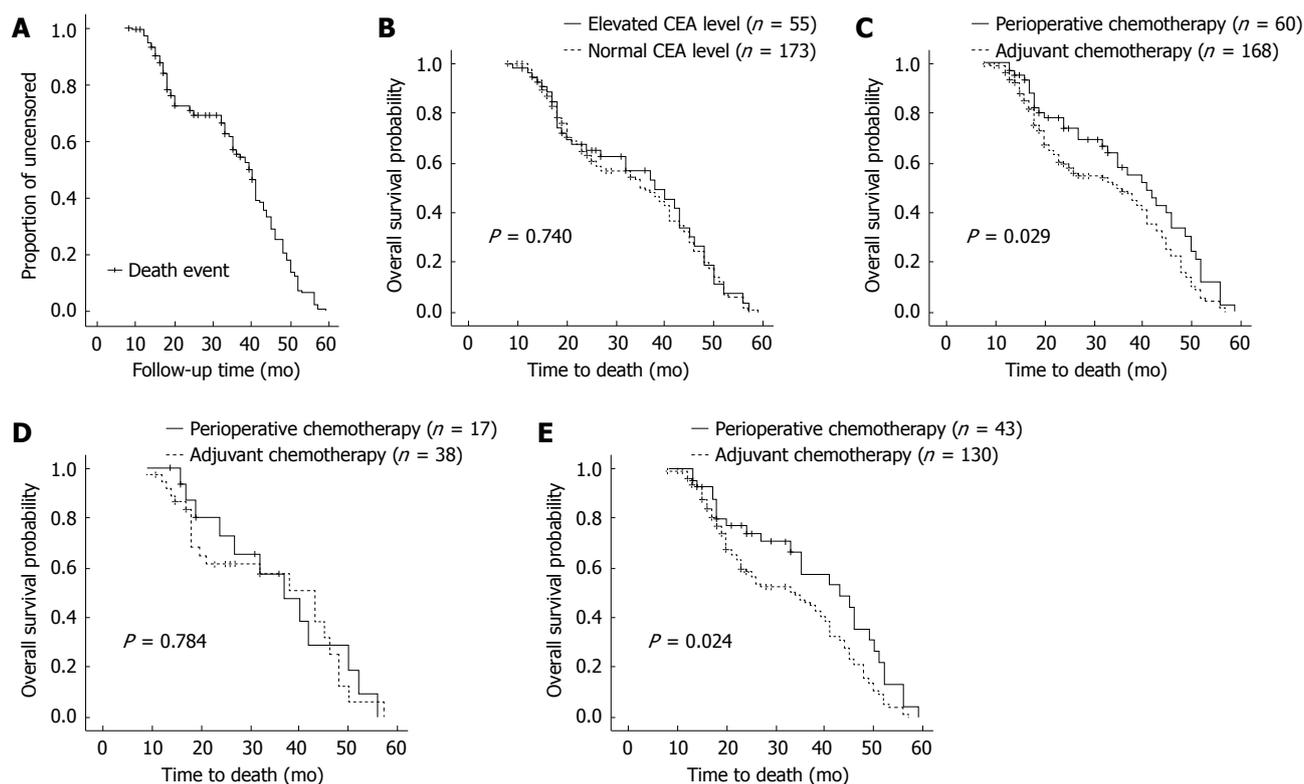


Figure 2 Survival curves for carcinoembryonic antigen patients with different treatments. A: The 228 patients in follow-up over this study; follow-up times ranged from 8 to 59 mo (median, 32 mo); B: Survival curves for the elevated carcinoembryonic antigen (CEA) patients and the normal CEA patients, with two-year survival rates of 57% and 51%, respectively, and no statistically significant difference between the groups ($P = 0.740$); C: Survival curves for patients receiving perioperative chemotherapy or adjuvant chemotherapy, with two-year survival rates of 58% and 50%, respectively. The difference is statistically significant ($P = 0.029$); D: Survival curves for the elevated CEA patients who received perioperative or adjuvant chemotherapy, with two-year survival rates of 58% and 58%, respectively, with no statistical significance ($P = 0.784$); E: Survival curves for normal CEA patients who received perioperative or adjuvant chemotherapy, with a two-year survival rate of 59% and 48%, respectively. The difference is statistically significant ($P = 0.024$).

(SPSS, Inc., Chicago, IL, United States). Statistical significance was defined as $P < 0.05$.

RESULTS

There was no statistically significant difference in overall survival between the normal CEA group ($n = 173$) and the elevated CEA group ($n = 55$). The survival curves are shown in Figure 2B.

The efficacy of preoperative chemotherapy was evaluated. Among these 60 patients, 4 (6.67%) had complete clinical response and 19 (31.7%) had partial clinical response, yielding an overall response rate of 40.0%. The response rates of the patients who had normal pretreatment serum CEA *vs* those who had elevated CEA were not significantly different, although patients with normal CEA had a higher response rate (complete response + partial response) (41.9% *vs* 29.4%). These results are shown in Table 2.

The 60 patients who received perioperative chemotherapy (i.e., preoperative plus adjuvant) had a significantly better overall survival rate than the 168 who received only adjuvant chemotherapy, with median survival time of 41 mo for the perioperative group *vs* 35 mo for the adjuvant group ($P = 0.029$). The survival curves are shown in Figure 2C. For the patients with elevated se-

rum CEA, there was no significant difference in overall survival rate between perioperative chemotherapy ($n = 17$) and adjuvant chemotherapy ($n = 38$, $P = 0.784$, Figure 2D). For the patients with normal serum CEA, the overall survival rate was significantly better in the perioperative group ($n = 43$) and the median survival time was 43 mo *vs* 34 mo for the adjuvant group ($n = 130$, $P = 0.024$). The survival curves are shown in Figure 2E.

In univariate analyses, perioperative chemotherapy, T staging, and the lymph node metastasis rate significantly correlated with overall survival (Table 3). In multivariate analysis, T staging and the lymph node metastatic rate were independent prognostic factors. Perioperative chemotherapy improved the overall survival of patients who had normal pretreatment serum CEA (Table 4).

DISCUSSION

Perioperative chemotherapy, although it is a large physical and psychological burden, has been proven to be effective for some gastric cancer patients. The European Organization for Research and Treatment of Cancer Randomized Trial 40 954 showed no survival benefit from preoperative chemotherapy compared with surgery alone for locally advanced cancer^[22]. However, this study had low statistical power; a high number of proximal

Table 2 The efficacy of preoperative chemotherapy on locally advanced gastric cancer patients *n* (%)

Pretreatment serum CEA	Response to preoperative chemotherapy			
	Complete response	Partial response	Stable disease	Progressive disease
Normal	3 (7.0)	15 (34.9)	21 (48.8)	4 (9.3)
Elevated	1 (5.9)	4 (23.5)	10 (58.8)	2 (11.8)

CEA: Carcinoembryonic antigen.

Table 3 Univariate analysis of overall survival in all patients and in patients with normal pretreatment serum carcinoembryonic antigen

Variable	No. of patients	2-yr survival rate (%)	Median survival (mo)	<i>P</i> value
All patients				
Pathological T staging				0.001
T0	7	86	52	
T3	205	52	37	
T4	16	36	27	
Lymph node metastasis				0.001
0	15	79	48	
0 < <i>r</i> ≤ 0.1	37	68	31	
0.1 < <i>r</i> ≤ 0.3	67	42	32	
<i>r</i> > 0.3	109	50	33	
Chemotherapy				0.029
Adjuvant	168	50	35	
Perioperative	60	58	41	
Normal-CEA patients				
Pathological T staging				0.001
T0	6	83	50	
T3	156	50	35	
T4	11	39	17	
Lymph node metastasis				0.002
0	12	74	44	
0 < <i>r</i> ≤ 0.1	29	70	45	
0.1 < <i>r</i> ≤ 0.3	50	38	26	
<i>r</i> > 0.3	82	49	33	
Chemotherapy				0.024
Adjuvant	130	48	34	
Perioperative	43	59	43	

CEA: Carcinoembryonic antigen.

gastric cancers (which involved the gastroesophageal junction and were different from most of the cases in endemic areas); and an increased R0 resection rate, indicating a better outcome in those patients suffering from early-stage gastric cancer. In China, most gastric cancer patients are diagnosed as having locally advanced disease, suggesting that different treatments should be considered for increasing the survival of the patients. However, there is no reliable marker to determine which patients with advanced gastric cancer can benefit from perioperative chemotherapy. The goal of the present study was to determine whether the pretreatment serum CEA level could be used as a marker to select patients for this aggressive treatment.

Our study revealed that perioperative chemotherapy can improve overall survival in patients with advanced gastric cancer. Dividing the patients in two groups based

Table 4 Multivariate analyses (Cox regression model) of overall survival of all patients and of patients having a normal pretreatment serum carcinoembryonic antigen

Variable	Hazard ratio	95% CI	<i>P</i> value
All patients			
Perioperative chemotherapy	0.723	0.501–1.044	0.084
T staging	1.422	1.067–1.896	0.016
Lymph node metastasis rate	1.302	1.101–1.539	0.002
Normal-CEA patients			
Perioperative chemotherapy	0.670	0.434–1.033	0.070
T staging	1.443	1.041–2.000	0.028
Lymph node metastasis rate	1.274	1.053–1.542	0.013

CEA: Carcinoembryonic antigen.

on their pretreatment serum CEA, we found that perioperative chemotherapy improved the survival rate only for patients with a normal level of pretreatment serum CEA.

Although the biological functions of CEA are not fully known, the close correlation of CEA with cancer aggressiveness has been known for decades. Higher preoperative CEA correlates with more aggressive gastric cancer and a lower patient survival rate^[23]. Our findings imply that patients with elevated CEA might have gastric cancers more resistant to chemotherapy, resulting in no survival benefit even from aggressive chemotherapy. CEA has been reported to have roles in homotypic adhesion and cellular aggregation^[24], and it cooperates with Myc and Bcl-2 in cellular transformation^[25]. In colon cancer, CEA is up-regulated in the microadenoma stage in the colon of patients with APC mutations^[26], and CEA plays antiapoptotic and prometastatic roles in colon cancer cells^[27]. Overexpression of CEA can protect tumor cells from apoptosis induced by loss of cell contact with the extracellular matrix (anoikis)^[28].

Interestingly, those gastric cancer cells expressing alpha-fetoprotein (which is another oncofetal antigen) show P-glycoprotein overexpression and drug resistance in both animal models and human cancer^[29,30]. In some case reports, drug-resistant patients always had elevated serum CEA^[31,32], and CEA overexpression was observed in multidrug-resistant breast carcinoma cell lines^[33]. The CEA promoter (AdCEAIacZ) can increase the IC50 of ganciclovir against gastric cancer cell lines by improving CEA production^[34]. All of these pieces of evidence suggest that CEA may induce or promote drug resistance in cancer cells.

This study is retrospective, with its own weaknesses such as confounding factors and low persuasiveness. The cycles of adjuvant chemotherapy were different among the patients, and there were two main chemotherapy regimens: XELOX and FOLFOX6. We believe that the study would be more convincing if there had been a standard regimen, although most investigators report the efficacy of these two regimens as having no statistically significant difference in gastric cancer patients. Thus, more randomized controlled trials are needed to confirm

that pretreatment serum CEA can be used as a marker to select patients for the aggressive perioperative treatment or to find other markers for assigning patients to an appropriate treatment.

To our knowledge, there is no strong evidence that CEA is a marker of drug resistance in gastric cancer. More experiments and clinical trials are needed to validate whether CEA levels can predict such drug resistance. In summary, our study showed that only patients with normal pretreatment serum CEA obtained a survival benefit from cytotoxic perioperative chemotherapy. The role of CEA in the drug resistance of gastric cancers warrants further exploration.

ACKNOWLEDGMENTS

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COMMENTS

Background

Perioperative chemotherapy has been proven to be effective for some gastric cancer patients. However, there is no reliable marker for determining which patients with advanced gastric cancer can benefit from perioperative chemotherapy.

Research frontiers

In the last two decades, carcinoembryonic antigen (CEA) has been widely used as a tumor marker in the diagnosis and monitoring of some malignancies. The research hotspot is to determine whether pretreatment serum CEA can be used as a marker to select patients for this aggressive perioperative treatment.

Innovations and breakthroughs

The study revealed that perioperative chemotherapy can improve overall survival in patients with advanced gastric cancer. After dividing the patients in two groups based on their pretreatment serum CEA, the authors found that perioperative chemotherapy improved the survival rate only for patients with a normal level of pretreatment serum CEA.

Applications

The study results suggest that normal pretreatment serum CEA is a predictor of survival benefit for the patients receiving perioperative chemotherapy.

Peer review

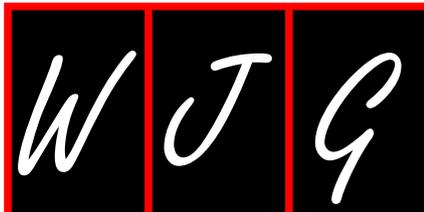
This is an interesting paper aimed to evaluate the role of pretreatment serum CEA level as a predictor of survival for patients with locally advanced gastric cancer receiving neoadjuvant chemotherapy. This retrospective study is well conducted and well written.

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Thrombosis of celiacomesenteric trunk: Report of a case

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Abstract

Here we present the case of a 79-year-old woman who complained of acute abdominal pain, vomiting and diarrhoea. Laboratory exams demonstrated a severe metabolic imbalance. Abdominal X-rays showed bowel overdistension and pneumatosis of the stomach wall. Abdominal tomography revealed infarction of the stomach, duodenum and small bowel due to thrombosis of the celiacomesenteric trunk. Exploratory laparotomy revealed ischemia of the liver, spleen infarction and necrosis of the gastro-intestinal tube (from the stomach up to the first third of the transverse colon). No further surgical procedures were performed. The patient died the following day. To our knowledge, this is the first reported case about severe gastro-intestinal ischemia due to thrombosis of the celiacomesenteric trunk, a rare anatomic variation of the gastrointestinal vascularisation.

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Key words: Celiacomesenteric trunk; Celiac trunk; Thrombosis; Anomalies; Gastrointestinal vascularisation

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INTRODUCTION

The majority of the blood supply of the gastrointestinal tract is provided by the anterior branches of the abdominal aorta: celiac trunk and superior mesenteric artery. Usually, the aforementioned branches arise independently from the abdominal aorta, the first one at the level of the twelfth thoracic vertebra, the second one at the level of the first lumbar vertebra. Anomalies of vascularisation of the gastro-intestinal tract are frequent, but the presence of the celiacomesenteric trunk (derived by common origin of celiac trunk and superior mesenteric artery) is rare. The injury of the trunk can have lethal effects on the organism. Here we describe the first reported case of severe ischemia of the gastrointestinal tract due to thrombosis of the celiacomesenteric trunk.

CASE REPORT

In September 2011, a 79-year-old woman arrived at the emergency department of San Biagio hospital with severe and diffuse abdominal pain and tenderness, more marked in the lower abdominal quadrants, with signs of peritoneal irritation. The area of hepatic dullness was



Figure 1 Abdominal X-rays. Gaseous overdistension of the small bowel. The arrow points the pneumatosis of the stomach wall.

present at percussion and intestinal peristalsis was diminished. The patient was afebrile and eupneic at rest. Laboratory analyses demonstrated increased inflammatory markers (neutrophilic leukocytes, polymerase chain reaction), acute renal failure (creatinine: 5.85 mg/dL) and severe metabolic acidosis. No pathological signs were found at chest X-rays, whereas abdominal X-rays revealed gaseous overdistension of the small bowel and pneumatosis of the stomach wall (Figure 1).

The radiological findings were subsequently confirmed by computed tomography of the abdomen performed without iodinated contrast due to renal failure of the patient. Tomography demonstrated pneumatosis of the wall of the stomach, duodenum and small bowel. Air was also present within the superior mesenteric and portal veins with intraparenchymal distribution to ventral portions of the left liver and fourth segment (Figure 2A), because of the advanced stage of arterial infarction. The axial scan evidenced the thrombosis of the celiacomesenteric trunk, which justified the radiological findings (Figure 2B); no further arterial vessel directed to the gastro-intestinal tract was identified by tomography.

Exploratory laparotomy showed ischemia of the liver, spleen infarction and necrosis of the stomach, duodenum, small bowel and large intestine (from the caecum to the first third of transverse colon). No further surgical procedures were performed. The patient died the following day.

DISCUSSION

The celiac trunk and superior mesenteric artery supply the majority of the blood to the gastrointestinal tract. Usually, the celiac trunk is a short artery that arises from the anterior wall of the abdominal aorta at the level of the twelfth thoracic vertebra; it divides almost immediately into three branches: left gastric, splenic and common hepatic artery.

The left gastric artery courses upwards to the left toward the cardias, where it turns downward and, following the lesser gastric curvature, descends to the right toward the pylorus. The left gastric artery forms an ar-

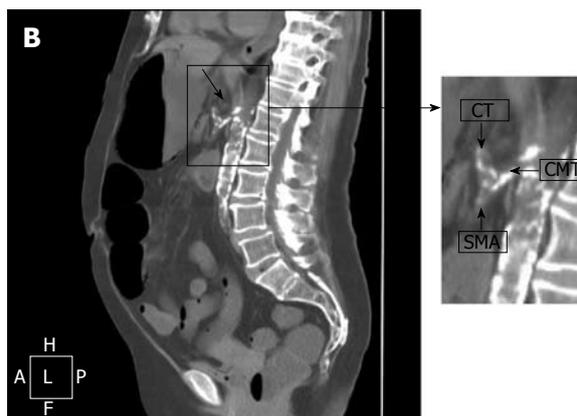


Figure 2 Computed tomography of the abdomen. A: Pneumatosis of the wall of stomach and small bowel (arrow in the right). Intraparenchymal air in ventral portions of the left liver and fourth segment (arrow in the left); air within the branches of the mesenteric vein (arrow in the centre); B: Thrombosis of the celiacomesenteric trunk (CMT) (arrow). In the detail: common origin of celiac trunk (CT) and superior mesenteric artery (SMA) from the CMT.

cing anastomotic loop with the right gastric artery, a branch of the common hepatic artery and, less frequently, of the gastroduodenal artery.

The splenic artery from its origin takes a short loop to the right and then runs along the cephalic border of the pancreas to supply the spleen. The splenic artery gives rise to several branches directed to the pancreas (dorsal pancreatic artery, arteria pancreatica magna, caudal pancreatic arteries) and to the stomach (left gastro-epiploic artery, short gastric arteries).

The common hepatic artery arises on the right side of the celiac trunk and runs to the right reaching the first part of duodenum, where it gives rise to a branch called the gastroduodenal artery and continues into the hepatic artery proper (some authors don't utilise this definition, but they call this branch of celiac trunk the common hepatic artery "before" or "after" the origin of the gastroduodenal artery). At the porta hepatis it divides into right and left hepatic arteries. The common hepatic artery emerges partially or entirely from the superior mesenteric artery in approximately 18% of the population.

The celiac trunk supplies the liver, stomach, pancreas and superior part of the duodenum^[1,2].

The superior mesenteric artery arises from the ante-

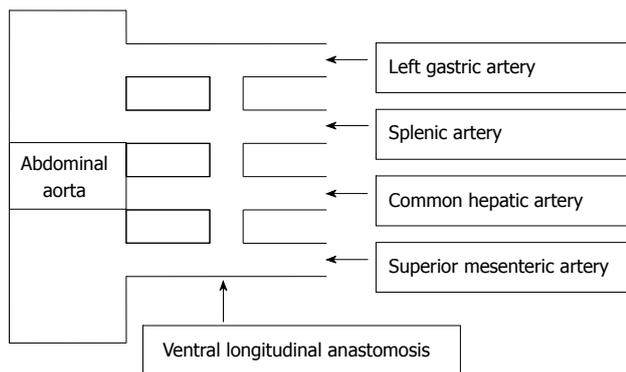


Figure 3 Primitive splanchnic vascularisation. Ventral longitudinal anastomosis (Lang's anastomosis) between four primitive splanchnic roots arising from the abdominal aorta.

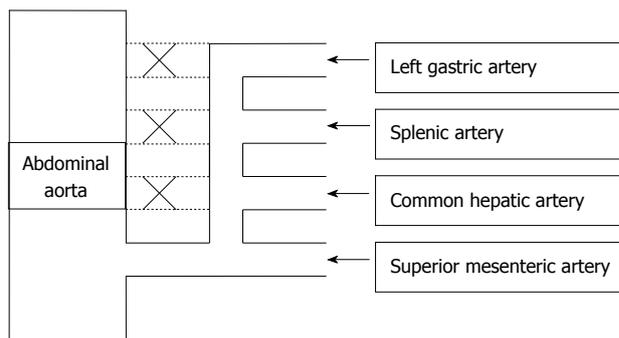


Figure 4 Celiacomesenteric trunk. Retention of the ventral longitudinal anastomosis higher than the fourth root keeps one or more celiac trunk branches with the superior mesenteric artery, disappearance of the first or fourth root causes a common celiacomesenteric trunk.

rior wall of the abdominal aorta at the level of the first lumbar vertebra. It runs down behind the head of the pancreas and ahead of the uncinat process of the pancreas and the third part of the duodenum. It descends anteriorly into the mesentery of the small intestine. Its right branches are inferior pancreatico-duodenal (this vessel forms an anastomosis with the superior pancreatico-duodenal artery, branch of the gastroduodenal artery), right colic, middle colic and ileo-colic; its left branches are 4-6 jejunal and 9-13 ileal arteries.

The superior mesenteric artery supplies the pancreas, duodenum (from the second to the fourth part), small bowel and colon from the caecum to the right half of the transverse colon^[1,2].

Variations of the normal vascularisation described above may be caused by the retention or disappearance of the roots of the primitive arterial plexus, as indicated by Tandler^[3] in 1908. The fetal digestive tube is supplied by four primitive splanchnic roots which arise from the abdominal aorta. There is a ventral longitudinal anastomosis (Lang's anastomosis) between these branches (Figure 3): the closure of the longitudinal anastomosis between the third and the fourth root and the disappearance of the central two roots lead to normal anatomy. Retention of the ventral longitudinal anastomosis higher than the fourth root keeps one or more celiac trunk branches with the superior mesenteric artery; disappearance of the first or fourth root causes a common celiacomesenteric trunk^[2,4,5]. Moreover, the simple arboriform scheme of the gastroduodenal and hepatobiliary vasculature is profoundly altered by the growth of the liver and pancreas, and by the assumption of a curved form in the stomach and duodenum. These factors operate to complicate the branching of the coeliac axis and the superior mesenteric artery^[6].

In our case, the celiacomesenteric trunk was formed by an anomalous separation of the ventral longitudinal anastomosis, with the common hepatic, left gastric and splenic arteries joining with the fourth root, as shown in Figure 4.

The celiacomesenteric trunk is one of the most striking among the different variations of the normal vascularisation of the gastro-intestinal tract^[1,2,4-7]: it is found in 1%-2% of all anomalies involving the coeliac axis^[1,8-10].

Full comprehension of the topics as knowledge of the different anatomical variations of the arterial supply of the gallbladder, liver, stomach and colon is crucial in cholecystectomy, hepatobiliary and gastro-intestinal surgical procedures^[6]. Without knowledge of the arterial architecture of the patient in this critical region, surgery may lead to a considerable risk of errors occasionally leading also to lethal complications^[11].

Different classifications of these anatomical variations are present in the literature; however the most widely used are those indicated by Morita^[12], Michels^[13] and Olry *et al*^[14]. The present case belongs to type P'b of Morita's classification and to type 6 of the Michels's classification.

The discovery of a celiacomesenteric trunk is often fortuitous during autoscopic dissections^[15-17] or can be accidentally detected by angiography or abdominal computed tomography scanning^[5,8,10]. It can accompany different clinical situations, such as aneurysm^[7,10,18], chronic occlusive disease^[9,18], compression by abdominal aorta aneurysm or aortic dissection^[18], celiac compression syndrome^[9,19], but the large gastrointestinal infarction caused by thrombosis of the celiacomesenteric trunk, to our knowledge, has never been previously reported.

In the current case, the celiacomesenteric trunk arose at the level of the first lumbar vertebra and then, after a stretch of about one centimetre, it divided in two branches, superior (hepato-gastro-splenic trunk or celiac trunk) and inferior (superior mesenteric artery). The thrombosis of the origin of the celiacomesenteric trunk had a lethal effect on the patient because it caused the full stoppage of the splanchnic arterial supply and consequent ischemia. In fact, in this rare situation a single artery is the sole source of vascularisation of the supramesocolic organs and collateral flow is only possible from the inferior mesenteric, phrenic, oesophageal and retroperitoneal arteries^[1]. Evidently, the condition of generalized atherosclerosis has prevented any collateral flow.

In conclusion, the present clinical report describes a

rare anomaly involving the celiac axis and, especially, the dramatic consequences related to the complete thrombosis of the celiacomesenteric trunk. This condition has important wide-ranging clinical implications because it compromises the blood supply of a large portion of the gastrointestinal tract and it may put at severe risk most of the abdominal viscera.

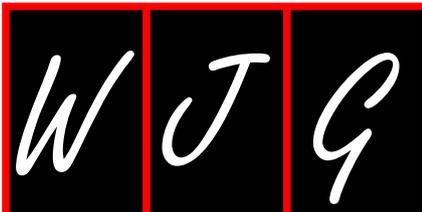
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Opioid/naloxone prolonged release combinations for opioid induced constipation

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Abstract

I read with great interest the recent article by Chen *et al* in a recent issue of your esteemed journal. The article is highly thought provoking. One emerging therapeutic alternative for opioid induced constipation is the emergence of opioid/naloxone prolonged release combinations. For instance, naloxone when administered in a 1:2 ratio with oxycodone reverses the inhibitory effect of oxycodone on the gastrointestinal tract. The advantage of oxycodone/naloxone prolonged release (OXN) is that while its anti-nociceptive efficacy is equivalent to that of oxycodone prolonged release (OXC), it significantly decreases the "Bowel Function Index" thereby ameliorating symptoms of opioid induced constipation to a large extent. Schutter *et al* in a recent study have reported a decrease in the bowel function index from 38.2 to 15.1. Similarly, Löwenstein *et al* in another recent study have reported that following a month of therapy, complete spontaneous bowel movements per week is increased from one in OXC therapy to three in OXN therapy.

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Key words: Opioid naloxone; Cancer; Morphine; Carcinogenesis

TO THE EDITOR

I read with great interest the article by Chen *et al*^[1] in a recent issue of your esteemed journal. The article is highly thought provoking. One emerging therapeutic alternative for opioid induced constipation is the emergence of opioid/naloxone prolonged release combinations.

For instance, naloxone when administered in a 1:2 ratio with oxycodone reverses the inhibitory effect of oxycodone on the gastrointestinal tract^[2]. The advantage of oxycodone/naloxone prolonged release (OXN) is that while its anti-nociceptive efficacy is equivalent to that of oxycodone prolonged release (OXC), it significantly decreases the "Bowel Function Index" thereby ameliorating symptoms of opioid induced constipation to a large extent. Schutter *et al*^[3] in a recent study have reported a decrease in the bowel function index from 38.2 to 15.1. Similarly, Löwenstein *et al*^[4] in another recent study have reported that following a month of therapy, complete spontaneous bowel movements per week is increased from one in OXC therapy to three in OXN therapy.

In fact, the colonic transit time is reduced by almost two hours with OXN 20/10 mg combination therapy^[5]. This is further affirmed by the fact that in patients receiving OXN therapy the mean laxative use is decreased by almost 20% while the stool consistency as measured by the "Bristol

Stool Form Scale” is improved from type 2 to type 5^[6,7].

In addition, in a recent study, the quality of life was accentuated by 47% following OXN therapy for management of chronic severe neuropathic pain^[8]. Similarly, a low mean Brief Pain Inventory Short Form “sleep interference” score is maintained with OXN therapy and is comparable to OXC therapy^[9].

Clearly, OXN therapy is highly effective in mitigating the symptoms of opioid induced constipation and provides a safe and efficacious alternative to methylnaltrexone.

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 Asian Pacific *Helicobacter pylori*
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 Kuala Lumpur, Malaysia

January 19-21, 2012
 American Society of Clinical
 Oncology 2012 Gastrointestinal
 Cancers Symposium
 San Francisco, CA 3000,
 United States

January 19-21, 2012
 2012 Gastrointestinal Cancers
 Symposium
 San Francisco, CA 94103,
 United States

January 20-21, 2012
 American Gastroenterological
 Association Clinical Congress of
 Gastroenterology and Hepatology
 Miami Beach, FL 33141,
 United States

February 3, 2012
 The Future of Obesity Treatment
 London, United Kingdom

February 16-17, 2012
 4th United Kingdom Swallowing
 Research Group Conference
 London, United Kingdom

February 23, 2012
 Management of Barretts
 Oesophagus: Everything you need
 to know
 Cambridge, United Kingdom

February 24-27, 2012
 Canadian Digestive Diseases Week
 2012
 Montreal, Canada

March 1-3, 2012
 International Conference on
 Nutrition and Growth 2012
 Paris, France

March 7-10, 2012
 Society of American Gastrointestinal
 and Endoscopic Surgeons Annual
 Meeting
 San Diego, CA 92121, United States

March 12-14, 2012
 World Congress on
 Gastroenterology and Urology
 Omaha, NE 68197, United States

March 17-20, 2012
 Mayo Clinic Gastroenterology and
 Hepatology
 Orlando, FL 32808, United States

March 26-27, 2012
 26th Annual New Treatments in
 Chronic Liver Disease
 San Diego, CA 92121, United States

March 30-April 2, 2012
 Mayo Clinic Gastroenterology and
 Hepatology
 San Antonio, TX 78249,
 United States

March 31-April 1, 2012
 27th Annual New Treatments in
 Chronic Liver Disease
 San Diego, CA 92121, United States

April 8-10, 2012
 9th International Symposium on
 Functional GI Disorders
 Milwaukee, WI 53202, United States

April 13-15, 2012
 Asian Oncology Summit 2012
 Singapore, Singapore

April 15-17, 2012
 European Multidisciplinary
 Colorectal Cancer Congress 2012
 Prague, Czech

April 18-20, 2012
 The International Liver Congress
 2012
 Barcelona, Spain

April 19-21, 2012
 Internal Medicine 2012
 New Orleans, LA 70166,
 United States

April 20-22, 2012
 Diffuse Small Bowel and Liver
 Diseases
 Melbourne, Australia

April 22-24, 2012
 EUROSON 2012 EFSUMB Annual

Meeting
 Madrid, Spain

April 28, 2012
 Issues in Pediatric Oncology
 Kiev, Ukraine

May 3-5, 2012
 9th Congress of The Jordanian
 Society of Gastroenterology
 Amman, Jordan

May 7-10, 2012
 Digestive Diseases Week
 Chicago, IL 60601, United States

May 17-21, 2012
 2012 ASCRS Annual Meeting-
 American Society of Colon and
 Rectal Surgeons
 Hollywood, FL 1300, United States

May 18-19, 2012
 Pancreas Club Meeting
 San Diego, CA 92101, United States

May 18-23, 2012
 SGNA: Society of Gastroenterology
 Nurses and Associates Annual
 Course
 Phoenix, AZ 85001, United States

May 19-22, 2012
 2012-Digestive Disease Week
 San Diego, CA 92121, United States

June 2-6, 2012
 American Society of Colon and
 Rectal Surgeons Annual Meeting
 San Antonio, TX 78249,
 United States

June 18-21, 2012
 Pancreatic Cancer: Progress and
 Challenges
 Lake Tahoe, NV 89101, United States

July 25-26, 2012
 PancreasFest 2012
 Pittsburgh, PA 15260, United States

September 1-4, 2012
 OESO 11th World Conference
 Como, Italy

September 6-8, 2012
 2012 Joint International

Neurogastroenterology and Motility
 Meeting
 Bologna, Italy

September 7-9, 2012
 The Viral Hepatitis Congress
 Frankfurt, Germany

September 8-9, 2012
 New Advances in Inflammatory
 Bowel Disease
 La Jolla, CA 92093, United States

September 8-9, 2012
 Florida Gastroenterologic Society
 2012 Annual Meeting
 Boca Raton, FL 33498, United States

September 15-16, 2012
 Current Problems of
 Gastroenterology and Abdominal
 Surgery
 Kiev, Ukraine

September 20-22, 2012
 1st World Congress on Controversies
 in the Management of Viral Hepatitis
 Prague, Czech

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

November 3-4, 2012
 Modern Technologies in
 Diagnosis and Treatment of
 Gastroenterological Patients
 Dnepropetrovsk, Ukraine

November 4-8, 2012
 The Liver Meeting
 San Francisco, CA 94101,
 United States

November 9-13, 2012
 American Association for the Study
 of Liver Diseases
 Boston, MA 02298, United States

December 1-4, 2012
 Advances in Inflammatory Bowel
 Diseases
 Hollywood, FL 33028, United States

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1352 experts in gastroenterology and hepatology from 64 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

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

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

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

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

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- 15 Morse SS. Factors in the emergence of infectious dis-

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- 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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Osteopontin as potential biomarker and therapeutic target in gastric and liver cancers

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Abstract

Gastric cancer and liver cancer are among the most common malignancies and the leading causes of death worldwide, due to late detection and high recurrence rates. Today, these cancers have a heavy socioeconomic burden, for which a full understanding of their pathophysiological features is warranted to search for promising biomarkers and therapeutic targets. Osteopontin (OPN) is overexpressed in most patients with gastric and liver cancers. Over the past decade, emerging evidence has revealed a correlation of OPN level and clinicopathological features and prognosis in gastric and liver cancers, indicating its potential as an independent prognostic indicator in such patients. Functional studies have verified the potential of OPN knockdown as a therapeutic approach *in vitro* and *in vivo*. Furthermore, OPN mediates multifaceted roles in the interaction be-

tween cancer cells and the tumor microenvironment, in which many details need further exploration. OPN signaling results in various functions, including prevention of apoptosis, modulation of angiogenesis, malfunction of tumor-associated macrophages, degradation of extracellular matrix, activation of phosphoinositide 3-kinase-Akt and nuclear factor- κ B pathways, which lead to tumor formation and progression, particularly in gastric and liver cancers. This editorial aims to review recent findings on alteration in OPN expression and its clinicopathological associations with tumor progression, its potential as a therapeutic target, and putative mechanisms in gastric and liver cancers. Better understanding of the implications of OPN in tumorigenesis might facilitate development of therapeutic regimens to benefit patients with these deadly malignancies.

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Key words: Osteopontin; Gastrointestinal cancer; Metastasis; Prognosis; Biomarker

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INTRODUCTION

Gastric and liver cancers are among the most common malignancies and leading causes of death worldwide,

which carries a heavy socioeconomic burden. Until now, surgical resection has remained the frontline treatment for patients with early stage gastric and liver cancers. Nevertheless, the majority of such patients have poor prognosis due to high rates of tumor recurrence as well as lymph node (LN) and systemic metastases. Therefore, a full understanding of gastric and liver cancers is crucial to develop useful prognostic markers and therapeutic targets. During the past decade, emerging evidence has refined the value of osteopontin (OPN) as a candidate biomarker and target for cancer therapy^[1]. OPN is a secretory extracellular matrix (ECM) protein that is involved in a series of physiological and pathophysiological processes including but not limited to cell attachment, migration, invasion, proliferation, tissue remodeling, bone formation and even inflammation^[1-4]. OPN is frequently overexpressed in human cancers and contributes to tumor formation and progression^[5,6]. OPN belongs to the small integrin binding ligand N-linked glycoprotein family, which consists of members serving as markers of early cancer progression, due to their capabilities in modulating the activity of matrix metalloproteinases (MMPs)^[7]. OPN participates in the interactions between cancer cells and tumor stroma, which plays a pivotal role in malignant cancer phenotype. A more thorough understanding of the functional role of OPN in the tumor microenvironment is warranted. There have been many reports on OPN and gastric and liver cancers, therefore, this review aims to summarize recent findings on clinical implications of OPN, its potential as a therapeutic target, and its related mechanisms in these two types of cancer. Further understanding on the role of OPN in gastric and liver cancers may facilitate development of therapeutic strategies in such patients.

OPN GENE AND PROTEIN STRUCTURE

OPN is a matrix glycoprotein secreted by a variety of cell types including osteoclasts, endothelial cells, epithelial cells, and activated immune cells such as macrophages and T cells^[8]. It is also known as bone sialoprotein I, early T lymphocyte activation 1 and secreted phosphoprotein 1^[9-11]. Human *OPN* gene is located on chromosome 4q21-q25, spans approximately 11 kb, and consists of seven exons encoding the OPN protein with 314 amino acid residues^[12]. It contains several highly conserved structural elements, including arginine-glycine-aspartate and Ser-Val-Val-Tyr-Gly-Leu-Arg domains for integrin binding, a calcium binding site and a heparin binding domain for CD44 receptor binding^[13] (Figure 1). Alternative splicing produces three OPN isoforms, OPN-a, OPN-b and OPN-c, which probably display different expression profiles and functional heterogeneity in a tumor-specific manner^[14,15]. Moreover, OPN protein is subjected to a series of post-translational modifications including serine/threonine phosphorylation, glycosylation and tyrosine sulfation, resulting in molecular variants ranging from 25 to 75 kDa^[16]. These modifications are cell type specific and depend on physiological and pathophysiological factors, which likely affect both OPN structure and functions^[17].

ological factors, which likely affect both OPN structure and functions^[17].

OPN OVEREXPRESSION AND CLINICAL VALUE IN PATIENTS WITH GASTRIC CANCER

OPN expression is significantly elevated in most gastric cancer patients at both transcriptional and translational levels^[18-24]. OPN protein is overexpressed in both primary gastric cancer and metastatic lesions, mildly expressed in the epithelial cells in chronic atrophic gastritis that is a precancerous lesion for gastric cancer, and negatively in normal gastric mucosa, which indicates that OPN may play a role and serve as a potential biomarker in the formation and progression of gastric cancer^[18-20]. Moreover, Wu *et al*^[19] have found higher OPN plasma level in gastric cancer patients as compared with healthy individuals, suggesting that OPN plasma level may also be a biomarker for gastric cancer, and is of particular clinical interest because plasma-derived biomarkers are more convenient in clinical application than biomarkers from tissues. In gastric cancer tissues, OPN protein is diffusely located in the cytoplasm of tumor cells as well as tumor-associated macrophages (TAMs), which is in line with its implications in the interactions between cancer cells and tumor stroma.

Until now, the diagnostic and prognostic values of OPN have been implicated in gastric cancer patients. Microarray studies have identified gene signatures including OPN in gastric cancer patients^[18]. OPN overexpression is significantly associated with clinicopathological parameters in gastric cancer such as low apoptotic index, high proliferative index, low grade, high stage, LN and vascular invasion, and distant metastasis^[20-24]. In addition, OPN overexpression is an independent predictor of poor prognosis and tumor recurrence in patients with gastric cancer^[21,22]. Dai *et al*^[22] have suggested that patients with OPN-positive gastric cancer have poorer outcome than OPN-negative cases. Multivariate analysis has revealed OPN expression as an independent prognostic indicator of poor disease-free and overall survival in patients with gastric cancer, particularly for survival in cases in tumor, node, metastasis (TNM) stage II and III. The prognostic value of the marker combinations of OPN with conventional biomarkers has also been explored in gastric cancer patients. Zhang *et al*^[24] have found the combination of OPN and caudal-related homeobox gene 2 (*CDX2*) as a survival predictor of advanced gastric cancer patients. OPN plasma level is commonly elevated in patients with gastric cancer, and is significantly associated with the clinicopathological features including late stage, serosal invasion, LN and vascular invasion, and liver metastasis^[19]. High OPN plasma level is inversely correlated with poor prognosis in gastric cancer patients, especially in those with invasive phenotypes. Thus, elevated OPN plasma level may serve as an independent risk factor for poor survival in gastric cancer patients.

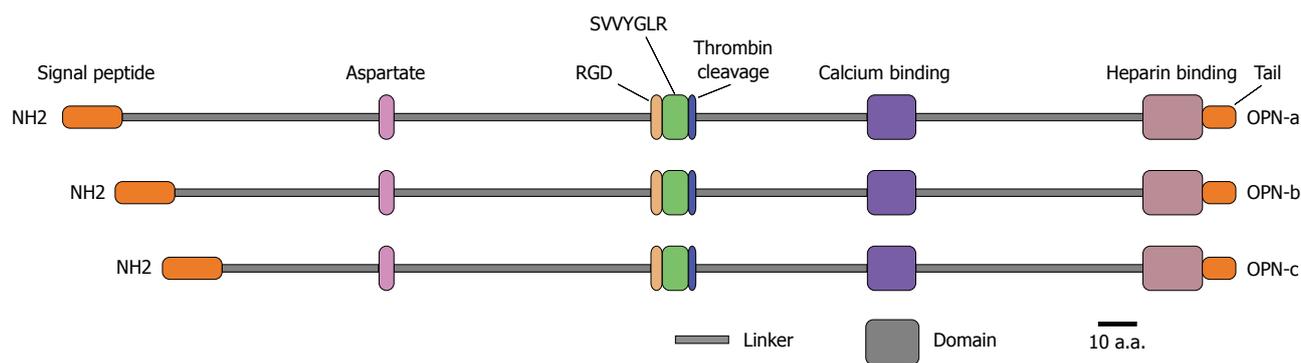


Figure 1 Structural features of osteopontin isoforms. Three isoforms of osteopontin (OPN), OPN-a, OPN-b and OPN-c, are known. All of them possess identical domains [aspartate domain, arginine-glycine-aspartate domain, Ser-Val-Val-Tyr-Gly-Leu-Arg (SVVYGLR) domain, thrombin cleavage domain, calcium binding domain and heparin binding domain] that are linked together with various linkers. These isoforms distinguish themselves by having a variable length of the linker between signal peptide and aspartate domain. RGD: Arginine-glycine-aspartate.

OPN OVEREXPRESSION AND CLINICAL VALUE IN PATIENTS WITH LIVER CANCER

OPN is positive in most hepatocellular carcinoma (HCC) patients at both transcriptional and translational levels^[25-35]. Yuan *et al.*^[28] demonstrated OPN mRNA overexpression in 79 (51%) of 156 primary HCC patients. Kim *et al.*^[35] disclosed that OPN protein was expressed in 92 (32.3%) of 285 tumors. The expressions of OPN mRNA and protein display a positive correlation^[29]. In HCC, OPN is secreted by both cancer cells and TAMs, and secreted by bile duct epithelium and stellate cells, but not by normal hepatocytes or Kupffer cells, in normal liver conditions^[34,35]. OPN⁺ cancer cells are often dispersed in the periphery of cancer nodules and are adjacent to stromal cells^[34,35]. In addition, OPN plasma level is also significantly elevated in HCC patients, especially in those with cirrhosis or in advanced stages^[35-39]. Kim *et al.*^[35] determined that OPN plasma level in HCC patients was significantly higher than in patients with chronic liver diseases or healthy controls (954 ng/mL *vs* 381 ng/mL; 954 ng/mL *vs* 155 ng/mL). Zhang *et al.*^[36] also found that OPN plasma level of HCC patients was significantly higher than that of healthy controls (176.90 ng/mL *vs* 63.74 ng/mL). These data propose that elevated OPN plasma level can serve as a potential biomarker for HCC.

Meanwhile, several microarray studies have identified OPN-containing gene signatures of HCC patients^[30-32]. Ye *et al.*^[31] have identified *OPN* as a leading gene in the gene signature that was relevant to tumor metastasis and patient survival. Luo *et al.*^[32] have found that overexpressed *OPN* gene belongs to a specific gene signature in HCC. In addition, many studies have established a significant correlation between OPN overexpression and clinicopathological features of HCC, including the severity of liver damage according to Child-Pugh class, high grade, late stage, LN/vascular/bile duct/capsular invasion, and intrahepatic or distant metastases^[26-30,40-44]. Until now, OPN overexpression has been revealed as an independent prognostic factor for poor overall and disease-free survival in HCC patients^[25-28,33,42-47]. In 2010, Weber

et al.^[42] performed a meta-analysis and found that OPN level correlated with poor overall and disease-/relapse-free survival, and as a biomarker for stage, grade, and early tumor progression in HCC. Chen *et al.*^[25] disclosed that OPN expression was a prognostic marker for HCC patients at TNM stage I. Furthermore, novel biomarker combinations are evaluated to predict patient outcome in HCC, since classical parameters cannot provide exact information. The biomarker combinations, OPN and α -fetoprotein (AFP), or OPN and CD44s, are revealed to have better prognostic value than the classical diagnostic biomarkers^[29,44]. Huang *et al.*^[48] have suggested that the combination of OPN and caspase-3 can be an effective indicator for HCC patients after curative resection. However, because the published data are conflicting in many cases, further large-scale studies are necessary to confirm their clinical value^[49].

Tumor recurrence is a persistent issue after surgical resection. A number of studies have suggested OPN as a useful marker for predicting early recurrence in HCC patients^[25-27,33,44-46,50]. OPN polymorphisms and the combination of OPN and CD44 are potential predictors of tumor recurrence in HCC^[45,46]. OPN overexpression is associated with early recurrence of hepatitis C virus (HCV)-related HCC^[50]. Chen *et al.*^[25] found that OPN expression was correlated with early postoperative recurrence in patients at stage I. Sieghart *et al.*^[33] have revealed that OPN is an independent predictor of tumor recurrence and survival in HCC patients beyond Milan criteria undergoing orthotopic liver transplantation. Thus, OPN may be able to help determine the patients who need adjuvant therapy to prevent early recurrence after surgical resection.

At present, many serum biomarkers are under evaluation for the detection of HCC, but none of them has sufficient sensitivity and specificity to be considered in the guidelines. OPN plasma level increases significantly with advanced Child-Pugh class, large tumor size, high grade, and late stage^[35,38]. OPN plasma level is suggested as an adverse prognostic factor for both overall survival, disease-free survival and relapse-free survival in hepatitis

Table 1 Osteopontin as a potential therapeutic target for gastric and liver cancers

Cell lines	Mouse model	Method of study	Resultant effects	Possible mechanisms
Gastric cancer SGC7901	Nude mice	siRNA knockdown	Reduced angiogenesis <i>in vitro</i> and <i>in vivo</i>	Decreasing microvessel density
BGC-823	Nude mice	Transient/stable siRNA knockdown	Inhibited cell growth, anchorage-independent growth, migration and invasion <i>in vitro</i> , and suppressed tumor growth and prolonged survival <i>in vivo</i>	Inhibition of MMP-2 and uPA expression, NF- κ B DNA binding activity, and Akt phosphorylation
SGC7901	Nude mice implanted with SGC-OPN-cells	Lentivirus-mediated stable depletion	Suppressed metastases and prolonged survival time <i>in vivo</i>	Reducing expression of VEGF
HCC MHCC97-L, MHCC97-H, HCC-LM3		siRNA knockdown	Decreased cell invasion and cell cloning number <i>in vitro</i>	
HuH1/4/7, MHCC97, SMMC7721, SK-Hep-1, Hep3B, CCL13, HCCLM3	Nude mice of lung metastasis	OPN-neutralizing antibody	Blocked invasion of SK-Hep-1 and Hep3B cells <i>in vitro</i> , inhibited pulmonary metastasis of HCC-LM3 cells <i>in vivo</i>	
HCC-LM6	Nude mice implanted with HCC-LM6	Antisense knockdown	Suppressed migration and invasion <i>in vitro</i> , decreased lung metastases <i>in vivo</i>	Inhibiting MMP-2 and uPA expression
HCC-LM3	Nude mice implanted with Lenti OPNi-transfected HCC-LM3 cells	Stable depletion using lentiviral vectors encoding miRNA against OPN	Inhibited both <i>in vitro</i> proliferation, invasion and <i>in vivo</i> tumor growth and lung metastasis	Inhibiting MAPK and NF- κ B pathways, and MMP-2 and suppressing uPA expression
HCC-LM3 HepG2	Nude mice	shRNA gene silencing	Inhibited HCC cell growth, adhesion and invasion <i>in vitro</i> , and suppressed tumorigenicity and lung metastasis <i>in vivo</i> , enhanced sensitivity of HCC cells to chemotherapeutic drugs	Suppressing α , β 1, β 3 integrin expression, blocking NF- κ B activation, inhibiting apoptosis

HCC: Hepatocellular carcinoma; OPN: Osteopontin; siRNA: Small interfering RNA; MAPK: Mitogen-activated protein kinase; NF: Nuclear factor; MMP: Matrix metalloproteinase; VEGF: Vascular endothelial growth factor; uPA: Urokinase-type plasminogen activator; shRNA: Short hairpin RNA; Akt: Protein kinase B; miRNA: microRNA.

B virus (HBV)- or HCV-related HCC patients^[36,38,51]. In addition, OPN plasma level may be a potential diagnostic biomarker for HCC in the surveillance of patients with HBV or HCV infection. Sun *et al*^[51] have suggested that preoperative plasma level of OPN and AFP can be used as a prognostic marker for early stage HCC. A recent study conducted by Shang *et al*^[52] has also identified serum OPN as a novel marker for early HCC diagnosis because OPN was found clearly elevated 1 year before diagnosis in a pilot prospective study including 22 patients. In another two studies, a greater area under curve value of OPN than AFP was observed, suggesting superior diagnostic accuracy of OPN for HCC^[58]. HCC patients whose pretreatment OPN serum level is low and declines following transarterial chemoembolization exhibit better tumor response and longer survival^[37]. These data suggest that OPN plasma level can be used, either independently or coupled with AFP, for predicting clinical outcome in HCC patients.

OPN AS A POTENTIAL THERAPEUTIC TARGET FOR GASTRIC AND LIVER CANCERS

OPN as a therapeutic target has been explored in various tumors including cancers of breast, lung, head and neck, stomach, colon and liver. Promising results have been achieved in a series of studies^[53-57]. The strategies often

utilize OPN antibody to block its binding to receptors so as to inhibit the downstream signal transduction related to tumor growth and invasion, and deliver the small interfering RNA (siRNA) targeting OPN to tumor cells to decrease directly the expression of OPN to abrogate the effects triggered by elevated OPN.

At present, OPN-knockdown-induced tumor suppression in gastric cancer has been shown through RNA interference (RNAi)^[58-60]. *In vitro* and *in vivo* studies have demonstrated OPN-RNAi-induced inhibition of tumor growth, migration and invasion in gastric cancer^[58,59]. Moreover, Wang *et al*^[60] silenced OPN expression in gastric cancer cell line SGC7901 using lentiviral-OPN siRNA technology, and found reduced detectable tumors, fewer metastases, and longer survival time in mice implanted with OPN-SGC7901 cells. These data suggest that targeting OPN and its related signaling network is likely to provide an effective therapeutic approach for gastric cancer (Table 1).

In recent years, efforts have also been made to inhibit HCC progression and metastasis by interfering OPN^[27,31,61-63]. OPN knockdown significantly suppresses migration and invasion of HCC cells *in vitro* and decreases lung metastases *in vivo*, which is associated with decreased angiogenesis in HCC cells^[61,62]. Besides, OPN-specific antibody can effectively block HCC cell invasion *in vitro* and inhibit lung metastasis of HCC cells *in vivo*^[31]. In addition, Zhao *et al*^[63] have demonstrated that short hairpin RNA-mediated OPN depletion enhances sensitivity of HCC cells

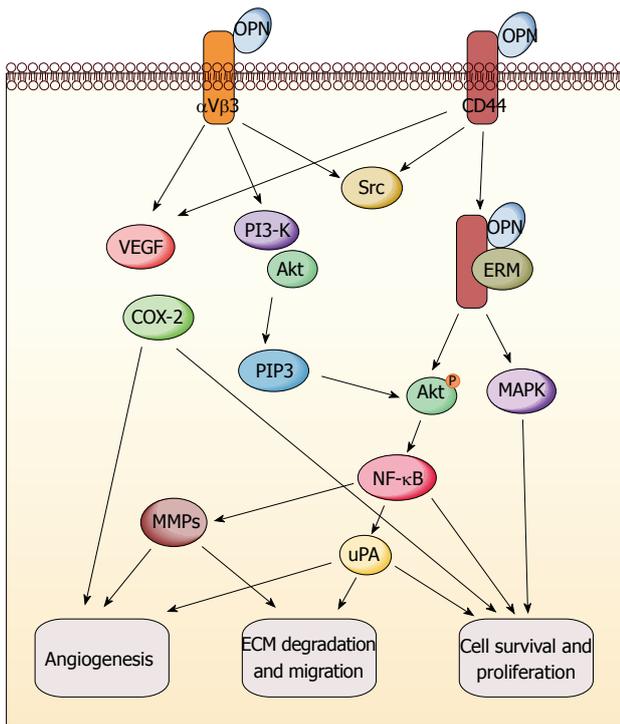


Figure 2 Molecular mechanisms of osteopontin in gastrointestinal cancers. Osteopontin (OPN) signaling leads to gastrointestinal cancer growth and metastasis through activation of various pathways, including cell survival and proliferation, angiogenesis, and extracellular matrix (ECM) degradation. VEGF: Vascular endothelial growth factor; PI3-K: Phosphoinositide 3-kinase; COX-2: Cyclooxygenase-2; MAPK: Mitogen-activated protein kinase; NF: Nuclear factor; uPA: Urokinase-type plasminogen activator; MMP: Matrix metalloproteinase; ERM: Ezrin/radixin/moesin; Akt: Protein kinase B.

to chemotherapeutic drugs through blockade of nuclear factor (NF)- κ B activation. Thus, targeting OPN and its related signaling network is likely to help develop novel therapeutic regimens for HCC (Table 1).

MOLECULAR MECHANISMS OF OPN IN GASTRIC AND LIVER CANCERS

The multifunction of OPN has been revealed in promoting tumor formation and progression (Figure 2). It exerts these functions through direct binding to integrin and/or CD44. The subsequent activation of various pathways leads to increased malignant phenotype^[64,65]. Various signaling transduction pathways triggered by OPN molecule have been reported in different cancer models such as breast cancer, melanoma, lung cancer, myeloma, prostate cancer and gastrointestinal cancers. The results indicated that OPN exerts the tumor-related functions through a complicated signaling network^[65]. Here, we only summarize the reported signaling pathways of OPN relevant to gastric and liver cancers; some of which are commonly overlapped with other cancers, but some are specific in these two types of cancers. It has been suggested that phosphoinositide 3-kinase (PI3-K)/protein kinase B (Akt) pathway and hypoxia-inducible factor-1 are involved in the tumor-promoting function of OPN,

which induces pro-survival and anti-apoptosis signaling in gastric and liver cancers after the survival pathway is activated^[63,66]. Mitogen-activated protein kinase pathway (MEK/ERK1/2) can also be triggered by OPN protein in liver cancer to promote tumor growth and metastasis, while the effect can be reversed through OPN knock-down^[62]. The NF- κ B pathway is crucial to keep cell survival through initiating the gene expression of antiapoptotic proteins, and is often induced by chemotherapeutic drugs and contributes to resistance to chemotherapy^[59,62]. Relevant tumor-promoting functions of OPN are found to be highly associated with NF- κ B pathway activation in gastric and liver cancers^[59,62,63]. The MMP family is responsible for ECM degradation and remodeling, which plays an important role in tumor invasion and metastasis. OPN-induced metastasis of gastrointestinal cancers is also involved in several MMP members such as MMP-2, MMP-9, MMP-7 and other famous invasion-related proteins such as vascular endothelial growth factor (VEGF) and urokinase-type plasminogen activator (uPA)^[54,59,62,67,68]. Recently, Lee *et al.*^[66] illustrated that OPN can enhance the survival of gastric cancer through the interaction with CD44 variant isoforms. The underlying mechanism involves Src kinase signaling upon OPN binding to CD44, followed by “inside-out” integrin activation. In addition, there may be a positive correlation between OPN and cyclooxygenase-2 (COX-2). OPN, VEGF and COX-2 could synergistically induce angiogenesis and metastasis in gastric cancer^[69]. On the other hand, the antitumor activity of COX-2 inhibitors in intestinal cancer is probably mediated through downregulation of OPN, which results from blockade of nuclear receptor subfamily 4, group A, member 2 (NR4A2) and Wnt/ β -catenin signaling, two important components of the OPN regulatory network^[70].

Several mechanisms regulating *OPN* gene expression have been revealed, but many details remain to be elucidated. *OPN* is a transcriptional target of aberrant Wnt/ β -catenin signaling^[70-72], and is also regulated by other molecules including specificity protein 1, v-ets erythroblastosis virus E26 oncogene homolog 1, runt-related transcription factor 2, v-myb myeloblastosis viral oncogene homolog, CDX2, deleted in liver cancer 1, late SV40 factor (LSF), epidermal growth factor (EGF), NR4A2 and NO^[24,73-79]. Interestingly, the activation of several downstream targets of OPN, such as Akt, LSF, NO, EGF and thrombin, can enhance OPN expression in turn, suggesting a positive feedback regulation of *OPN* gene expression^[68,73,78-82]. Moreover, the modulation of OPN mRNA stability also influences OPN expression in HCC^[83,84]. In addition, miRNA-181a decreased OPN expression in HCC cell lines, suggesting that miRNA is involved in the regulation of *OPN* gene expression^[85]. Furthermore, the expression of OPN is also affected by COX-2 and 30-kDa Tat-interacting protein^[70,86,87].

In short, OPN signaling could result in the activation of anti-apoptosis and pro-survival pathways *via* PI3-K-Akt and NF- κ B signaling molecules, angiogenesis modulation *via* VEGF induction, ECM degradation *via* MMPs

and uPA secretion, leading to tumor growth and metastasis in gastric and liver cancers.

CONCLUSION

OPN overexpression occurs frequently in patients with gastric cancer and liver cancer. Previous studies have revealed its clinicopathological correlation with tumor formation and progression in these two types of cancer, indicating its potential as an independent indicator for predicting outcome in such patients. Functional studies have shown the potential of OPN as a therapeutic target in gastric and liver cancers both *in vitro* and *in vivo*. OPN mediates multifaceted roles in the interaction between cancer cells and tumor microenvironment, in which many details need to be further explored. The various mechanisms of OPN signaling in gastric and liver cancers including evasion of apoptosis, modulation of angiogenesis, ECM degradation, activation of PI3-K-Akt and NF- κ B pathways, might induce the development and progression of gastric and liver cancers. However, no clinical trial targeting OPN is in progress for tumor treatment, although the importance of OPN has been widely investigated and demonstrated in various cancers, and many patents including antibodies or peptides against OPN have been filed to treat different tumors. OPN is an important cytokine to mediate normal physiological functions. Blocking OPN possibly results in severe adverse effects due to interference with normal OPN roles. Therefore, further understanding of the implications and roles of OPN in various tumors including gastric and liver cancers could help develop better therapeutic strategies for such patients. On the other hand, OPN as a secreted plasma protein seems to have a greater potential to be utilized as a diagnostic or prognostic marker for in relevant cancers in combination with other biomarkers or alone.

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S100A4 in esophageal cancer: Is this the one to blame?

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Abstract

Metastasis is the main reason for cancer-related death. S100A4 is one of the key molecules involved in this event. Several studies have shown that overexpression of S100A4 in non-metastatic cancer cells can make them become metastatic, and knockdown of S100A4 in metastatic cancer cells can curtail their invasive nature. A study by Chen *et al*^[2] published in the *World J Gastroenterol* 18(9): 915-922, 2012 is a typical example. This study showed *in vitro* and *in vivo* evidence that S100A4 expression level determines the invasiveness of esophageal squamous carcinoma. Considering the fact that more than half of the cancer-related deaths are caused by malignancies derived from the digestive system and esophageal cancer is the 4th top contributor to this fraction, this study warrants more attention.

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Key words: Esophageal cancer; S100A4; Metastasis

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INVITED COMMENTARY ON HOT ARTICLES

Cancer is the second leading cause of death in the world (18%), after heart disease (21%). Among about 18 million new cases of cancers diagnosed each year, about one third is skin cancer. However, 95% of skin cancer is either basal cell carcinoma or squamous cell carcinoma, which has a mortality of less than 0.5%. The majority of cancer-related deaths are actually caused by malignancies derived from the digestive system, including esophagus, stomach, small intestine, colon, rectum, anus, liver, gallbladder and pancreas^[1]. The main feature that makes these cancers deadly is metastasis, a process that cancer cells break off from their original location and invade other parts of the organ. The majority of skin cancers do not have this capacity; therefore, they can be easily treated before becoming life threatening. Esophageal cancer, on the other hand, is highly metastatic. Therefore, understanding the molecular mechanisms behind its metastasis is of great values for developing better treatment strategies. A study by Chen *et al*^[2] published in the *World J Gastroenterol* 18(9): 915-922, 2012 examined the role of S100A4, one of the well-known cancer metastatic markers, in esophageal squamous cell carcinoma (ESCC) *in vitro* and *in vivo*, in animal models as well as in clinical human specimens, and clearly demonstrated a reliance of the invasiveness of esophageal cancer on this small calcium-binding protein^[2].

A little biography of S100A4: Short but hot

S100A4 was discovered in the mid 1980s by several laboratories independently. One of these laboratories be-

longed to Daniel Nathans, MD (10/30/1928-11/16/1999) (Figure 1), the Nobel Prize winner in Physiology/Medicine 1978 for his landmark discovery of restriction enzymes. In 1983, one of his post-doctoral fellows, Daniel I Linzer, PhD, was constructing a cDNA library from serum-stimulated mouse 3T3 fibroblasts and found that a clone named 18A2 was highly up-regulated by serum exposure^[3]. There seemed to be many laboratories in the late 70s and early 80s of the 20th century which were interested in the effect of serum on gene expression. That was also how and when serum response factor (SRF) was discovered^[4,5]. In the following year, Linzer took a job at Northwest University in Illinois (now he is the Provost of this school) and continued his study on 18A2. He determined that 18A2 coded for a calcium binding protein of 101 amino acids^[6], much similar to the members of S100 family, a group of small peptides that are known to be 100% soluble in saturated ammonia sulfate. He also compared the sequence of 18A2 with 2A9, a human clone that was published a year earlier^[7], and found a 57% nucleotide and 62% amino acid homology between them. It might be due to the difference of species origin, Linzer was pretty sure that these two sequences represented different genes. Around that time and shortly thereafter, several other laboratories also published similar sequences and each of which was given a different name, including p9Ka from rat mammary cells^[8], 42A from rat neuronal cells^[9], pEL98 from mouse fibroblasts^[10], CAPL from *Aplysia* neurons^[11], mts1 from metastatic tumor cells^[12], and FSP1 from mouse fibroblasts^[13]. Despite the individuality of each of these studies, there were some common features shared among their discoveries: (1) serum inducibility; (2) around 100 amino acids; and (3) similarity to S100 calcium binding proteins. Although all of these sequences eventually turned out to be for a single molecule - S100A4, each of these studies made unique contributions to our knowledge today about S100A4. The last two studies warrant an extra attention, because one established the connection between S100A4 and cancer metastasis and the other associated it to fibroblast phenotype. Now we know that S100A4 is a prognostic marker for metastatic cancers as well as a marker for epithelial-mesenchymal transition. However, both of these studies went a little bit too far by calling this molecule metastatic-specific and fibroblast-specific, respectively. Now we know that is not entirely true, a lot of other cells (e.g., epithelial cells, endothelial cells, lymphocytes, smooth muscle cells, *etc.*) also express S100A4, just as our study reported^[14].

Functions of S100A4: Motivation to move

Up to date, S100 family includes 25 members with common characteristics such as low molecular weight, two calcium binding sites of the helix-loop-helix ("EF-hand type") conformation, and complete solubility in ammonium sulfate at pH 7. They have been implicated in regulation of protein phosphorylation, transcription factor activation, calcium homeostasis, cytoskeleton reorganization, cell migration, cell growth and death^[15].



Figure 1 Daniel Nathans, MD, Nobel Laureate (10/30/1928-11/16/1999), Department of Molecular Biology and Genetics, the Johns Hopkins University School of Medicine Baltimore, Baltimore, MD 21205, United States.

S100A4 is naturally expressed in various cell types including both cancer and normal cells, and its elevation is usually associated with cell motility. It appears that wherever cell migration is required, such as wound healing^[16], angiogenesis^[17] and cancer metastasis^[18], S100A4 is activated. Like other members of S100 family, S100A4 works like a calcium sensor. Upon calcium binding, S100A4 goes through a series of conformational changes, which allow the molecule to interact with its targets, such as nonmuscle myosin heavy chain (MHC II A) and liprin β 1, to facilitate cell migration^[19,20]. For this reason, in motile cells, S100A4 is often found in complex with these cytoskeletal components at the migrating front where a high level of calcium is accumulated. It is interesting to know that S100A4 knock-out mice do not display developmental abnormalities in the postnatal period, but 10% of them develop tumors at age of 10-14 months, possibly due to destabilization of the tumor suppressor p53^[21], as S100A4 has been shown capable to bind to the C-terminal of p53 and repress its transcriptional activity^[22,23].

Yet, the story of S100A4 is not as straightforward as it might have been anticipated. In addition to being a cytoskeletal regulator in the cytoplasm, S100A4 has also been localized to the nucleus and extracellular matrix. How it gets there and what it does in these locations remain unclear. Nevertheless, its association with transcription factors like p53 might explain some of its roles in the nucleus. It has been postulated that S100A4 binding to the tetramerization domain of p53 favors p53 oligomerization and thereby facilitates p53 nuclear translocation^[23]. On the other hand, extracellular S100A4 has been demonstrated to stimulate MMP-13 expression in chondrocytes in a receptor for advanced glycation end products (RAGE)-dependent manner^[24], while its inductivity on neuron growth was found to be RAGE irrelevant^[25]. More complicatedly, S100A4 has been found in association with cell death in a conflict way, it inhibits apoptosis in pancreatic cancer^[26] but promotes it in osteosarcoma cells^[27].

S100A4 in cancers: A facilitator, not a generator

Elevation of S100A4 has been found in almost every metastatic cancer known, including breast^[28], ovarian^[29], prostate^[30], urinary bladder^[31], lung^[32], esophageal^[33], gastric^[34], colon^[35], pancreatic^[36], liver^[37], gallbladder^[38] and

thyroid carcinomas^[39]. More direct evidence for the essential role of S100A4 in cancer metastasis perhaps comes from *in vitro* studies and animal models, which have shown that overexpression of S100A4 in non-metastatic tumor cells confers a metastatic phenotype, just as demonstrated in the study by Chen *et al*^[2] as well as several others^[40,41]; whereas, knockdown of S100A4 in metastatic tumor cells curtails their invasive capability^[2,42,43].

It should be pointed out though that S100A4 is not an oncogene product. As shown by transgenic studies^[17,44], mice carrying extra copies of *S100A4* gene develop normally as wild-type and have no increased risk of cancer. However, when these mice mated with cancer mice, their offspring showed increased number of tumors distant from their primary location^[45]. Therefore, S100A4 is not a cancer generator but a metastatic facilitator.

S100A4 has been studied extensively in other cancers, especially in breast cancer. In esophageal cancer, there are about a dozen of publications so far, mostly focusing on squamous cell carcinoma. The earliest study that can be found was done by a Japanese group^[33], showing an elevated expression of S100A4 protein in surgically resected ESCC, and a possible association with esophageal cancer progression. However, a later study reported an opposite result, showing that 11 out of 16 S100 family members examined, including S100A4, were down-regulated at transcriptional level in tumor tissues compared with adjacent normal tissues^[46]. In 2010, a Chinese research team used RNA interference technology to knock down S100A4 in metastatic esophageal tumor cells and grafted them in nude mice^[47]. They noticed that tumor growth was significantly inhibited by S100A4 deficiency, and E-cadherin expression was reciprocal to the level of S100A4. Unfortunately, the study had little impact because it was published in a local journal in Chinese. However, the idea of xenografting has recently advanced to a new cancer treatment strategy - the "avatar" mice. Principally, it is to take tumor tissue from a patient and graft it in nude mice to create a personalized colony of mice carrying exact that patient's cancer, and then test every potential treatment combinations in mice before selecting the best one to treat that patient. Manuel Hidalgo, the Director of the Spanish National Cancer Research Center in Madrid, has been practicing this approach for pancreatic cancer patients over years and showed a clear advantage in drug responses^[48,49], and now more and more researchers believe that this idea holds a great promise in cancer treatment in the future.

In the study by Chen *et al*^[2], the research team cleverly used two ESCC cell lines, EC109 (highly invasive) and TE13 (non-invasive), and successfully made these cells switch characters by down-regulation of S100A4 in EC109 and up-regulation of S100A4 in TE13. They provided *in vitro* and *in vivo* evidence that the level of S100A4 determines the metastatic status of the cancer.

There are two main subtypes of esophageal cancer: ESCC and esophageal adenocarcinoma (EAC). Although nearly 95% of esophageal cancer is ESCC, EAC has been rising by 6-fold annually in Americans and now its in-

crease rate exceeds the rate for any other type of cancers. Overexpression of S100A4 was also reported in EAC and its correlation with lymph node metastasis was found significant^[50].

Although the exact molecular mechanisms how S100A4 promotes cancer metastasis still need to be further examined, based on various studies, one possible explanation could be that S100A4 binding to liprin $\beta 1$ inhibits its phosphorylation^[19], and thereby prevents its interaction with liprin $\alpha 1$. As a result, liprin $\alpha 1$ fails to recruit leukocyte common antigen-related (LAR) protein^[51], a phosphatase, to focal adhesions. Without LAR to dephosphorylate β -catenin^[52], β -catenin becomes activated to leave E-cadherin and results in the collapse of adherens junctions, allowing cells to migrate. As found in our study, the dissociation of β -catenin from E-cadherin causes E-cadherin ubiquitination and degradation^[53], which might at least in part explains why S100A4 elevation is often found in association of E-cadherin loss, as shown in the study by Chen *et al*^[2].

S100A4 in normal situation: An innocent bystander

As discussed above, S100A4 is expressed wherever cell migration is required, regardless normal or pathological situation. However, most of S100A4 studies focus on its bad side, such as cancer metastasis and organ fibrosis. Its good side has been continually overlooked. If we go back to the story that S100A4 was discovered in an experiment of serum stimulated fibroblasts, we know that S100A4 is innocent. Cells, including fibroblasts, in our body normally do not come into a direct contact with serum unless there is an injury. Therefore, when cells are suddenly exposed to serum, as the experiment done in Nathans' lab, they naturally interpret it as a signal of a wound. Therefore, a transcriptional program for wound healing gets activated immediately to battle against injury. S100A4 is just one of the players in this battle. So is SRF, and so are many SRF-regulated genes (e.g., C-FOS, EGR-1, CCN1, CTGF, FGF10, *etc.*)^[54]. All these genes contain a common regulatory element CArG box, which SRF recognizes to bind. *S100A4* gene also contains such element in its promoter region^[55], suggesting a possible regulation by SRF. *In vivo*, S100A4 activation has been found in various wound healings, and its contributions to tissue repair and modification are indisputable^[16,56].

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NSAIDs for prevention of pancreatitis after endoscopic retrograde cholangiopancreatography: Ready for prime time?

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Abstract

Acute pancreatitis is the most common and the most fearful complication of endoscopic retrograde cholangiopancreatography (ERCP). Prevention of post-ERCP pancreatitis has therefore been of great interest to endoscopists performing ERCP procedures. So far, only pancreatic duct stenting during ERCP and rectal administration of a non-steroidal anti-inflammatory drug (NSAID) prior to or immediately after ERCP have been consistently shown to be effective for prevention of post-ERCP pancreatitis. This commentary focuses on a short discussion about the rates, mechanisms, and risk factors for post-ERCP pancreatitis, and effective means for its prevention with emphasis on the use of NSAIDs including a recent clinical trial published in *The New England Journal of Medicine* by Elmunzer *et al*^[11].

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Key words: Endoscopic retrograde cholangiopancreatography; Pancreatitis; Post-endoscopic retrograde cholangiopancreatography pancreatitis; Pancreatic stents; Non-steroidal anti-inflammatory drugs

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INVITED COMMENTARY ON HOT ARTICLES

Acute pancreatitis is the most common complication of endoscopic retrograde cholangiopancreatography (ERCP)^[1,2]. It is important to distinguish between acute pancreatitis and hyperenzymemia after ERCP. Hyperenzymemia, defined as asymptomatic elevation of serum levels of amylase and lipase, is estimated to occur in more than 75% of patients undergoing ERCP and by itself does not have any clinical consequences^[3]. Acute pancreatitis, on the other hand, is less common and can have significant clinical consequences. Although both conditions are characterized by elevation of serum levels of amylase and lipase, diagnosis of acute pancreatitis requires an additional factor, either pancreatic type pain or cross-sectional imaging confirming pancreatic inflammation^[4].

Proposed underlying mechanisms that alone or in combination can induce post-ERCP pancreatitis (PEP) are prolonged manipulation around the papillary orifice causing edema, enzymatic injury from intestinal contents or contrast, hydrostatic injury from over-injection of the pancreatic duct, and thermal injury from electrocautery. There are probably other mechanisms involved that are yet to be recognized.

In most patients, the risk of PEP is in the range of 1%-10%. In high-risk cases, the risk can be as high as 30%^[1]. Factors that convey a high risk for PEP can be

classified as patient-related, procedure-related, operator-related, and disease- or indication-related. Patient-related factors associated with a higher risk of PEP are younger age, female gender, and a normal serum bilirubin level. Procedure-related factors that have been suggested to be related to a higher risk of PEP include difficult cannulation, balloon dilatation of the biliary sphincter, and injection of contrast into the pancreatic duct particularly when acinarization occurs. Operator-related factors include lack of a good technique, lack of experience, and low case volume. The disease or indication for the ERCP is also important. For example, while the risk of PEP in patients undergoing ERCP for chronic calcific pancreatitis is very low, nearly one in 3 patients with type 3 sphincter of Oddi dysfunction undergoing ERCP will develop PEP.

Acute pancreatitis after ERCP is not a uniform disorder and varies in intensity^[1,3]. Most cases of PEP are mild and resolve with proper treatment without any permanent sequela. A minority of the cases, however, is severe. Severe PEP is a feared complication of ERCP and can result in significant morbidity and mortality. Prevention of PEP has therefore been of major interest to endoscopists and significant time and effort have been devoted to finding endoscopic or pharmacologic means of preventing PEP.

So far, only pancreatic duct stenting and use of non-steroidal anti-inflammatory drugs (NSAIDs) consistently have been shown to be effective for PEP prophylaxis.

The first randomized trials assessing pancreatic duct stenting at the time of ERCP for PEP prevention were conducted in the 1990s^[1]. Subsequent studies confirmed the effectiveness of this approach in decreasing the rate and severity of PEP, especially in high-risk patients.

Use of NSAIDs for PEP prophylaxis is relatively new. The rationale of NSAIDs administration for PEP prevention lies in their ability to inhibit substances such as prostaglandins, phospholipase A2 and neutrophil-endothelial interaction, which are believed to play an important role in severe inflammatory processes including acute pancreatitis^[6]. The first clinical trial assessing the efficacy of a rectally administered NSAID for PEP prevention was reported in 2003 by a British group^[7]. In that study, pancreatitis occurred in 6.4% of patients in the NSAID group compared to 15.5% in the placebo group. Two subsequent clinical trials by two independent Iranian research teams found that rectally administered NSAIDs were effective for PEP prevention^[8,9]. A Mexican study confirmed those results^[10].

The most recent clinical trial on use of a rectally administered NSAID for prevention of post-ERCP pancreatitis was published a few weeks ago^[11]. In this clinical trial, 602 patients at elevated risk for post-ERCP pancreatitis were randomly assigned to receive a single dose of rectal indomethacin or placebo immediately after ERCP. The incidence of post-ERCP pancreatitis was significantly reduced among those receiving rectal indomethacin (9.2%) compared to those in the placebo group (16.9%).

In conclusion, based on the current literature, two prevention modalities have proven effective for PEP prophylaxis: (1) endoscopic placement of a pancreatic duct

stent during ERCP; and (2) rectally administered NSAID immediately before or after ERCP.

Endoscopic pancreatic duct stenting for PEP prophylaxis in high-risk patients is a well-accepted strategy and is being used as a routine practice in most ERCP centers.

Although still not adopted as a routine practice, there is enough evidence to support the routine use of NSAIDs for PEP prevention at least in high-risk patients.

Although use of endoscopic and pharmacological means such as pancreatic duct stenting or rectally administered NSAIDs can decrease the rate and severity of PEP, they cannot, and should not replace the common sense. The best strategy for prevention of post-ERCP pancreatitis has been and remains avoiding unnecessary procedures. Other strategies for PEP prophylaxis include proper training of the endoscopists and assistants; adequate case volume to maintain proficiency; avoiding repeated injection to the pancreatic duct if evaluation of the pancreatic duct is not required; and referral of high-risk cases to specialized ERCP centers.

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B cell depletion in treating primary biliary cirrhosis: Pros and cons

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Abstract

Primary biliary cirrhosis (PBC) is a progressive autoimmune liver disease of unknown etiology that affects almost exclusively women. Ursodeoxycholic acid (UDCA) is currently the only approved drug by Food and Drug Administration for patients with PBC. Although the precise pathogenesis of PBC remains unclear, it has been postulated that many cell populations, including B cells, are involved in the ongoing inflammatory process, which implicates, not surprisingly, a potential therapeutic target of depleting B cell to treat this disorder. Rituximab is a chimeric anti-CD20 monoclonal antibody that has been approved for the treatment of lymphoma and some autoimmune diseases such as rheumatoid arthritis. Whether it is effective in the treatment of PBC has not been evaluated. Recently, Tsuda *et al*^[1] demonstrated that B cell depletion with rituximab significantly reduced the number of anti-mitochondrial antibodies (AMA)-producing B cells, AMA titers, the plasma levels of immunoglobulins (IgA, IgM and IgG) as well as serum alkaline phosphatase, and it was well tolerated by all the treated patients with no serious adverse events. This observation provides a novel treatment option for

the patients with PBC who have incomplete response to UDCA.

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Key words: Primary biliary cirrhosis; Rituximab; B cell depletion; Anti-mitochondrial antibodies

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INVITED COMMENTARY ON HOT ARTICLES

We read with interest the recently published paper by Tsuda *et al*^[1] describing an open-label study of rituximab treatment in six patients with primary biliary cirrhosis (PBC) who had an incomplete response to ursodeoxycholic acid (UDCA). We believe this observation provides a novel treatment option for the patients with PBC who have incomplete response to UDCA and would recommend it to the readers.

PBC is a cholestatic liver disease characterized by serological findings of anti-mitochondrial antibodies (AMA) and pathological non-suppurative destruction of biliary epithelial cells^[2,3]. PBC may lead to liver failure or

even death. However, UDCA is the only Food and Drug Administration-approved drug and its efficacy is far from satisfaction in a large proportion of patients^[4]. Recent studies have demonstrated that B cells are involved in immune mechanisms of the pathogenesis of non-suppurative cholangitis and the destruction of bile ducts in PBC^[5-7]. These findings implicate a potential treatment efficacy of B cell depletion in patients with PBC^[8-10].

Rituximab is a mouse-human chimeric anti-CD20 monoclonal antibody designed for B cell depletion in human. Its safety and efficacy as a single therapeutic agent has been demonstrated initially in the treatment of non-Hodgkin B cell lymphoma and chronic lymphocytic leukemia^[11,12]. In addition, there were also clinical trials demonstrating that rituximab significantly induced clinical remission in a number of autoimmune diseases such as granulomatosis with polyangiitis, microscopic polyangiitis, and rheumatoid arthritis (RA)^[13-15].

In the field of PBC, there were several studies in murine models investigating the treatment effect of B-cell depletion. Dhirapong *et al.*^[8] reported that B cell-depleted mice developed more aggressive PBC-like liver disease with increased infiltration of inflammatory cells around the damaged bile canaliculi in portal areas. Whereas Moritoki *et al.*^[16] showed that anti-CD20 therapy had no effect on adult dominant-negative transforming growth factor (TGF)- β R II mice (age range: 20-22 wk to 36-38 wk), and it neither alleviated liver inflammation nor exacerbated colitis. But in younger dominant-negative TGF- β R II mice aged 4-6 wk, anti-CD20 treatment significantly alleviated the liver inflammation and reduced the bile duct damage, suggesting that anti-CD20 treatment might be beneficial for patients with PBC of early disease stage.

Tsuda *et al.*^[11] used rituximab to treat six patients with PBC who had suboptimal biochemical response to UDCA. After B-cell depletion, they observed a reduction in the number of AMA-producing B cells, AMA titers, the plasma levels of immunoglobulins (IgA, IgM and IgG) as well as serum alkaline phosphatase (ALP) at week 24. As the levels of immunoglobulins, AMA titers and ALP returned to baseline levels at week 36, repeated anti-CD20 treatment was suggested to maintain the treatment effect. The necessity of repeated treatment with rituximab was also demonstrated by recent clinical trials on other autoimmune diseases such as RA and systemic lupus erythematosus, and this treatment strategy did not lead to permanent remission^[17-19]. It is noteworthy that there was also study reporting that repeated treatment with rituximab could potentially compromise host protective immune response and might cause severe infection in RA patients^[20]. In Tsuda's study on PBC patients^[11], two patients (2/6, 33.3%) experienced reactivation of varicella zoster and upper respiratory infection after the first infusion of rituximab. Though it might be arbitrary to ascribe these infections exclusively to rituximab infusion, infections remain the major concern when treating patients with anti-CD20 antibodies. In PBC and other autoimmune diseases, it remains controversial if repeated anti-CD20 treatment is beneficial in terms of safety and

efficacy, and if so, when is the optimal time for repeated therapy.

A high titer of serum AMA can be detected in 83%-95% of patients with PBC^[21]. Most studies have shown that there is no correlation between the level of serum AMA and the severity of PBC, and AMAs positivity does not predict the patient's response to treatment with UDCA^[22-25]. However, there were also some studies suggesting that AMA-positive PBC patients had more severe bile duct destruction than PBC patients with negative AMA^[26]. AMAs could induce the caspase activation of the biliary epithelial cells and subsequent cell death and bile duct damage^[27]. Tsuda *et al.*^[11] found that in the PBC patients, together with the number of peripheral B cells, the plasma levels of immunoglobulins and ALP, the level of AMA also decreased after treatment with rituximab and returned to baseline levels 36 wk after cessation of rituximab. They suggested that the depletion of the AMA-secreting plasma cells by rituximab could potentially reduce hyperactive B cell immune response and lead to the amelioration of the bile duct destruction in PBC, even though it is too early to jump to the conclusion that the level of serologic AMAs is a predicting factor for the efficacy of rituximab therapy.

Although B cell is one of the pivotal inflammatory cells in the immunopathogenesis of PBC, its precise role and the adverse events associated with B cell-depletion remain unclear^[28]. A study reported that the morbidity of severe side effects of B cell-depletion is low but not insignificant^[29]. There were also studies reporting new onset cases of inflammatory bowel disease that may be attributed to the B cell depletion in up to 40% patients with PBC^[30,31]. In dominant negative TGF- β R II mice, Moritoki *et al.*^[16] found that anti-CD20 treatment induced up-regulation of interleukin 6, which could lead to exacerbation of colitis. Paradoxically, in some studies on murine models, B cells might play a protective role in PBC and B cell depletion exacerbated the biliary pathology and caused more aggressive PBC-like liver diseases^[8,26,28]. There was also a case report showing that, after rituximab treatment, PBC developed with a high AMAs titer, intrahepatic cholestasis and steatorrhea in a RA patient^[32], though it is not exactly understood if PBC was caused by immuno-mechanism underlying RA or by rituximab itself. In Tsuda's study on PBC patients^[11], however, there was no evaluation of inflammatory bowel diseases and biliary pathology during follow-up. It should also be noted that, in their study, the number of enrolled patients and the duration of follow-up were not enough and the level of other biochemical parameters and PBC-40 scores remained unaltered. The long-term efficacy and prognosis could be the most important concern of rituximab treatment.

In conclusion, the study by Tsuda *et al.*^[11] suggests that B cell depletion with rituximab is potentially a promising treatment regimen for the PBC patients who do not have good response to UDCA. B cell depletion merits further investigation in human PBC to illuminate its safety and efficacy.

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Challenges of incorporating gene expression data to predict HCC prognosis in the age of systems biology

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Abstract

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death worldwide. The recurrence of HCC after curative treatments is currently a major hurdle. Identification of subsets of patients with distinct prognosis provides an opportunity to tailor therapeutic approaches as well as to select the patients with specific sub-phenotypes for targeted therapy. Thus, the development of gene expression profiles to improve the prediction of HCC prognosis is important for HCC management. Although several gene signatures have been evaluated for the prediction of HCC prognosis, there is no consensus on the predictive power of these signatures. Using systematic approaches to evaluate these signatures and combine them with clinicopathologic information may provide more accurate prediction of HCC prognosis. Recently, Villanueva *et al.*^[13] developed a composite prognostic model incorporating gene expression patterns in both tumor and adjacent tissues to predict HCC recurrence. In this commentary, we summarize the current progress in using gene signatures to predict HCC prognosis, and discuss the importance, existing issues and future research directions in this field.

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Key words: Gene expression signatures; Hepatocellular carcinoma; Prognosis

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INVITED COMMENTARY ON HOT ARTICLES

Hepatocellular carcinoma (HCC) is the sixth most common cancer type and the third leading cause of cancer-related death worldwide^[1]. The major risk factor of HCC is chronic infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV)^[2]. So far, curative treatments for HCC include orthotopic liver transplantation, surgical resection and percutaneous ablation. However, the recurrence rates remain high and long-term survival is poor.

There are two types of HCC recurrence: early recurrence and late recurrence with different mechanisms. Early recurrence (< 2 years after the treatment) is mostly caused by metastasis and dissemination of primary HCC; while late recurrence (\geq 2 years after the treatment) mainly

results from *de novo* tumors, as a consequence of field effect in diseased liver which is closely associated with high viral loads and hepatic inflammatory activities^[3,4]. The treatment after curative therapy varies greatly, depending on individual's profile^[5]. The traditional prognostic markers of HCC include vascular invasion (both macroscopic and microscopic) which is the most significant factor, tumor size, number of nodules, α -fetoprotein level, degree of differentiation, and satellites^[6]. Recent advancement in the field has shown that viral factors and inflammation-related conditions are apparently associated with HCC prognosis. Viral load, genotype C, viral mutations, and expression of inflammatory molecules in HBV-related HCC tissues are significantly associated with poor prognosis. Host-inflammation-related factors such as imbalance between intratumoral CD8⁺ T lymphocytes and regulatory T lymphocytes, T helper (Th)1 and Th2 cytokines in peritumoral tissues are also predictors of HBV-related HCC^[7,8]. In addition, non-coding RNA also plays a significant role in HCC progression^[9]. However, even after incorporating viral and other factors, the prediction power can not be optimized. Therefore, it is crucial to identify new prognostic markers to better approach opportunities for individualized therapeutics for HCC patients.

The application of high-throughput methods has provided new opportunities for analyzing the diversity and heterogeneity of cancers. Studies of microarray-based gene expression profiling in breast cancer have shown a great success and led to a working model for a breast cancer molecular taxonomy^[10]. Gene expression signatures succeeded in prognosis prediction and treatment responses for HCC^[11], and they are promising in developing personalized cancer medication^[12]. Gene expression profiles may add new and important prognostic information beyond those provided by the standard clinical predictors. It is important to incorporate molecular information to more accurately predict early and overall recurrence of HCC.

We read with great interest the recent article by Villanueva *et al.*^[13]. In this article, the authors developed an integrated prognostic model combining genomic and clinicopathologic data to improve outcome prediction in single-nodule early HCC patients. They analyzed the prognostic power of 22 previously reported gene signatures in a cohort of 287 early-stage HCC patients. The analysis showed that the proliferation signature was the most prevalent prediction (number of patients identified with the signature/number of total patients); and there was a substantial association among three groups of signatures: (1) signatures related to increased cell proliferation, progression in cell cycle and activation of specific pathways; (2) signatures generated in the adjacent tissues; and (3) cytokeratin-19 gene signature. They found that G3 (tumoral) signature and poor-survival (non-tumoral) signature, along with satellites were independent predictors of early tumor recurrence and overall recurrence. They also reported that genomic profiles of tumor and adjacent tissues were complementary in refining the prediction.

Advanced imaging techniques such as computed to-

mography and magnetic resonance imaging have been used to detect vascular invasion and conduct satellite evaluation before surgery, which are helpful in the pre-operative prediction of HCC prognosis. Genomic profiling using tumor and adjacent tissues obtained by fine-needle biopsy may provide complementary and/or confirmative information, thus having a great potential when combined with imaging findings in the clinical practice. Many studies have used array-based gene expression profiling obtained from tumoral or non-tumoral tissues to predict HCC prognosis. However, the number and heterogeneity of the signatures hinders their further application. The study of Villanueva *et al.*^[13] attempted to address these issues. They evaluated the prognostic predictive power of previously reported gene signatures in an independent cohort, and then developed a "composite genomic-based prognostic model". They further validated the stability of the model using samples from different sites of the same tumor nodule to test whether the genomic signature was consistent throughout different sites of a tumor^[13]. This study presents a unified approach to systematically evaluate and independently validate HCC prognostic gene signatures; and the procedure developed in this study is conducive to the future studies of other complex disease.

Cancer gene signatures may indicate specific biological traits of heterogeneous tumor sub-phenotypes that cannot be identified by traditional methods. They may be associated with tumor biology and tumor microenvironment such as chromosomal instability, wounded stroma, or invasiveness, and possibly also linked to certain signaling pathways^[14]. Gene signatures may have functional implications and may be predictive of response to specific therapeutic agents such as antiviral medications. Signatures identified in the study of Villanueva *et al.*^[13] (tumoral G3-proliferation signature and nontumoral poor-prognosis signature) reflect highly relevant biological events for outcome prediction and point out possible pathways to search for biomarkers as therapeutic targets. If used appropriately, gene signatures should be important complementary methods to current clinicopathological risk stratification systems^[15]. Integrating gene signatures in HCC prognosis prediction may potentially improve patient outcomes, obtain a better understanding of the underlying HCC biology, and identify effective therapeutic options for an individual patient.

HCC is not a single disease at the molecular level. Using gene signatures to classify HCC into molecular subtypes with similar prognostic implication can guide clinical decision-making, particularly regarding therapy. However, these signatures lack prognostic power. The assignment of a given patient to a subgroup is strongly dependent on the gene signature used and the results from studies of a specific/single gene signature cannot necessarily be generalized. Furthermore, there are few genes overlapped among gene expression signatures which reflect common cellular phenotypes and yield similar predictions. Therefore, it is not appropriate to use overlapping in gene identity to measure the reproducibility of gene-expression profiles^[16]. Thus, systematic evaluation of different gene

expression datasets and validation in independent cohorts provide basis for identifying true genomic signatures that are associated with oncogenic pathway, tumor biology and its microenvironment. Nevertheless, there are problems of using gene signatures to classify sub-phenotypes and predict HCC prognosis. In the following section, we take the paper of Villanueva *et al.*^[13] for an example to discuss several imposing issues in the field.

First, the paper does not mention whether evaluation on the quality of the different gene signatures was used. These signatures were generated from different samples with different biological background. Different studies may vary greatly in study quality, such as patient selection criteria, RNA quality, follow-up criteria, definition of prognosis, treatment after surgery, *etc.* Patient differences including different staging and underlying conditions may reflect etiological differences, thus resulting in the heterogeneity of gene signatures. Prognostic accuracy might differ in tumors with different stages. Additionally, multiple end points, such as overall recurrence, early recurrence, late recurrence, overall survival, or metastasis-free survival, used in the analyses are also the source of heterogeneity. There is also the possibility of stromal contamination, namely, gene signatures derived from analysis of tumor specimens with a high proportion of adjacent tissue contamination, and vice versa. The general reproducibility of these signatures stands out as an important issue.

Second, it is inappropriate to directly combine datasets from different platforms and different experiments because of the non-biological experimental variation or batch effects. In the study of Villanueva *et al.*^[13], gene expression data were obtained from 3 high-throughput genomic platforms, and these datasets cannot be readily put together because of their heterogeneity. Again, the authors did not mention whether any standardization procedures were applied. In addition, the method used for integration and/or standardization of different platforms is also a challenge. How to choose a robust normalization method according to the features of the dataset to reduce the batch effect is essential for further computational analyses^[17].

Third, the authors did not describe whether they applied the gene mapping procedure. Gene database updates with time, with the accumulation of information, the platform used several years ago may not be comparable to the gene database in service now. Without mapping, the genes in the 22 signatures produced at different time points may not correspond well. Accurately mapping and matching a gene across different signatures generated by different platforms at different time points is an important quality control step to enable the finding of true signatures.

Last but not least, the quality of survival analyses used to generate these signatures differs. The frequently used statistical methods, such as the significant analysis of microarray tool, the trend filter tool, and Cox's proportional Hazard model, may contribute to the great variety of gene expression signatures^[17]. Different studies also vary in terms of follow-up information collected, covariates

adjusted in multivariate analysis, and non-informative censoring. These directly affect the gene signatures generated.

For gene signatures to be used in clinical practice to accurately predict HCC prognosis, the following procedures are required. For a start, there should be a standardization of tissue composition. Without appropriate and standardized samples, the further experiments to determine a robust signature will be difficult. For example, the variable selection procedure is crucial in developing reliable and reproducible gene signature because pre-analytical variables such as stromal component and tissue processing will directly affect gene expression profiles. In addition, to enable the usage of data by different researchers and future investigators, a detailed description of data processing and analytical methods is required. A further step is to establish unified high criteria for generating gene expression signatures. Moreover, it is also important to identify gene signatures to predict early and late recurrence of HCC. HCCs are a group of diverse and heterogeneous diseases. Gene expression patterns can provide a basis to distinguish sub-phenotypes within the heterogeneity subgroups characterized by conventional clinicopathological variables, and also present important information about individualization of therapy^[18]. Viral mutations in the preS and the basal core promoter regions of HBV are significantly associated with HCC risk^[19-23]. The HBV mutations including A1762T/G1764A, preS deletion at nt.107-141, and preS2 mutations in adjacent hepatic tissues and the HCV mutation such as M91L are significantly associated with poor prognosis of HCC^[24-26]. The viral mutations should be reasonably integrated into the HCC prognosis-related gene signature.

To summarize, this paper drew our interests because gene expression signatures have shown great promise in classifying cancer subtypes and predicting prognosis. The Villanueva team has introduced an effective approach to systematically integrate different types of data for HCC prognosis prediction. With the increasing amount of data produced, there is an urgent need of standardized methods in systems biology to integrate descriptive data from cohort studies and other sources such as clinicopathological features, massive DNA and RNA parallel sequencing, and proteomics, along with functional data to guide therapeutic decisions. In addition, data on vascular features of HCC from imaging techniques may help select and validate true gene expression signatures associated with HCC prognosis. Future studies should also correlate these two non-invasive and innovative methods. It is still premature to use the current gene signatures for predicting HCC prognosis in the context of clinical practice. There is enormous work to be done for these gene signatures to be used in routine clinical practice and treatment decision making.

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Overview and developments in noninvasive diagnosis of nonalcoholic fatty liver disease

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Abstract

High prevalence of non-alcoholic fatty liver disease (NAFLD) and very diverse outcomes that are related to disease form and severity at presentation have made the search for noninvasive diagnostic tools in NAFLD one of the areas with most intense development in hepatology today. Various methods have been investigated in the recent years, including imaging methods like ultrasound and magnetic resonance imaging, different forms of liver stiffness measurement, various biomarkers of necroinflammatory processes (acute phase reactants, cytokines, markers of apoptosis), hyaluronic acid and other biomarkers of liver fibrosis. Multicomponent tests, scoring systems and diagnostic panels were also developed with the purposes of differentiating non-alcoholic steatohepatitis from simple steatosis or discriminating between various fibrosis stages. In all of the cases, performance of noninvasive methods was compared with liver biopsy, which is still considered to be a gold standard in diagnosis, but is by itself far from a perfect comparative measure. We present here

the overview of the published data on various noninvasive diagnostic tools, some of which appear to be very promising, and we address as well some of still unresolved issues in this interesting field.

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Key words: Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Liver fibrosis; Liver biopsy; Biomarkers; Transient elastography; Cytokeratin-18; Oxidative stress; Insulin resistance; Hyaluronic acid

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INTRODUCTION

Finding a means to noninvasive diagnosis of non-alcoholic fatty liver disease (NAFLD) and its entities has been the aim of many research efforts since recently, and seems to remain a very much needed goal among many clinicians and researchers in the field of hepatology. Why is it that way?

NAFLD is today considered to be the most common liver disease in adults. The prevalence of NAFLD in general population is very high, in the range of 15%-30% according to various studies, and is even increasing, due to

the rising prevalence of diabetes and obesity^[1]. Spectrum of NAFLD includes two entities with very different natural course and prognosis: simple steatosis, which mostly has a benign non-progressive course and good prognosis, and non-alcoholic steatohepatitis (NASH), which demonstrates progression of fibrosis in about 30%-40% of patients and has a proven potential to eventually lead to cirrhosis and end-stage liver disease including hepatocellular carcinoma^[2-4]. NASH seems to be present in a surprisingly high proportion of NAFLD patients, including 40% to 75% of cases with elevated aminotransferase levels, those data coming from recent studies using current histological definitions and including substantial number of patients^[2,5,6]. In studies of liver biopsy findings from apparently healthy living liver donor candidates, the proportion of NASH among patients with newly discovered NAFLD was about 30%^[7]. Even in patients with normal aminotransferase levels, proportion of NASH among NAFLD cases seems to be almost the same, and the whole spectrum of NAFLD including advanced fibrosis and cirrhosis has been observed in patients with completely normal laboratory findings^[6,8,9].

Liver biopsy is still considered the gold standard in diagnosis and the only reliable tool for distinguishing NASH from simple steatosis and for grading and staging the disease, providing important information about severity of steatosis, lobular inflammation, hepatocellular ballooning, and degree of fibrosis^[10]. Minimal histological criteria for NASH include steatosis, hepatocyte injury (in the form of ballooning or apoptosis) and lobular inflammation. Similarly to other chronic liver diseases, fibrosis is usually divided histologically in four stages: perisinusoidal fibrosis (F1), perisinusoidal and periportal fibrosis (F2), bridging fibrosis (F3) and cirrhosis (F4). Liver biopsy also has several negative aspects: it is invasive, unpleasant for patients, it usually includes hospitalization and a day or two lost at work, and the adequate interpretation of the specimen requires a pathologist with expertise in hepatopathology, which altogether makes it a costly and time-consuming procedure. Another significant drawback of liver biopsy, and in medical terms the most important one is its substantial sampling variability, which has been consistently proven for several chronic liver diseases including NAFLD. In a well-designed study by Ratziu *et al*^[11], the negative predictive value of a single biopsy for the diagnosis of NASH was calculated to be only 74%.

Considering the mentioned high prevalence of NAFLD in the general population, and the fact that every patient with NAFLD including the one with normal aminotransferases can potentially have NASH, we come to the conclusion that it would be necessary to perform liver biopsy in about one fourth of the whole Western population. This is clearly not feasible, but is it necessary? Until recently, many have advocated against the routine use of liver biopsy for patients with NAFLD because fatty liver is still considered by many to be a benign condition—although many studies have now clearly indicated a progressive course in a proportion of patients with NASH. Another reason for avoiding biopsy and definite diagno-

sis was the lack of established pharmacological treatment options which would have proven efficacy in preventing progression or leading to regression of disease.

Although there are still no generally approved treatments for NASH, several treatment options have demonstrated efficacy in various clinical trials, and for example recently published results of the randomized multicenter pioglitazone *vs* vitamin E *vs* placebo for the treatment of nondiabetic patients with nonalcoholic steatohepatitis (PIVENS) trial have provided substantial evidence for the previously suggested efficacy of vitamin E (and to a lesser extent pioglitazone) in inducing histologic improvement of NASH^[12]. Hopefully, we could soon expect to have several efficient treatment options available. All of this pretty much eliminates the validity of approach where NASH remains undiagnosed, which currently does happen in many clinical settings, e.g., in patients with accidental ultrasonographic finding of fatty liver and even patients with mildly elevated transaminase levels and known NAFLD risk factors who are very often not investigated any further.

The necessity of diagnosing NASH and the proportion of the population affected lead to a logical conclusion that a need for a reliable noninvasive tool in NAFLD diagnosis is highly urgent. Ideal noninvasive tool would be able to distinguish NASH from simple steatosis and allow for grading and staging of disease, which would largely facilitate screening of population at risk. Development of noninvasive tools would also enable monitoring of disease course and progression and evaluation of response to therapy, both in routine practice and in the setting of clinical trials, which is currently only possible with a follow-up liver biopsy. Another very important, and somewhat disregarded point, is that an efficient biomarker or set of biomarkers would accurately reflect the inflammatory and fibrotic processes on the level of the whole of liver parenchyma, thereby increasing the diagnostic accuracy and resolving the problem of sampling variability intrinsic to liver biopsy, which represents only about 1/50.000 part of the organ which is not homogeneously affected by disease features.

In the text below, we present the current level of knowledge and progress regarding the noninvasive diagnostic tools that have been studied in the context of NAFLD (Table 1).

ROUTINE LABORATORY TESTS

Patients with NAFLD are mostly asymptomatic and the disease is usually suspected based on either hyperechoic liver appearance on abdominal ultrasound or mild to moderate increases in liver enzyme levels. These are usually the only aberrations that can be encountered in this patient population (apart from signs of associated conditions like elevated glucose or lipid levels), and a large proportion of patients has completely normal laboratory findings. Hypoalbuminemia, prolonged prothrombin time, and hyperbilirubinemia are parameters of impaired liver function and occur only in patients who have already de-

Table 1 Overview of noninvasive methods in diagnosis of liver disease severity that have been evaluated in the context of non-alcoholic fatty liver disease

Routine laboratory tests
Liver enzymes
Parameters of liver dysfunction
Imaging methods
Ultrasound
Computed tomography
Magnetic resonance imaging
Magnetic resonance elastography
Liver stiffness measurement
Transient elastography (FibroScan)
Acoustic radiation force impulse shear wave imaging
Biomarkers of necroinflammation
Cytokeratin 18 fragments
High-sensitivity C-reactive protein
Interleukin-6
C-C chemokine ligand 2
Plasma pentraxin 3
Oxidative stress measurement
Tumor necrosis factor- α
Adiponectin
Insulin resistance measurement
Multicomponent tests for diagnosis of non-alcoholic steatohepatitis
Nash test
Non-alcoholic steatohepatitis clinical scoring system for morbid obesity
Model by Miele <i>et al</i> ^[61]
Biomarkers of fibrosis
Hyaluronic acid
Laminin
Type VI collagen 7S domain
Multicomponent panels for diagnosis of fibrosis
Fibrotest
Non-alcoholic fatty liver disease fibrosis score
European liver fibrosis panel/enhanced liver fibrosis panel

veloped cirrhosis. Most commonly elevated enzymes are alanine aminotransferase (ALT) and γ -glutamyltransferase (GGT), while aspartate aminotransferase (AST) elevation is less frequent and when pronounced may indicate presence of advanced fibrosis^[13]. Many studies have tried to correlate liver enzyme levels with histological severity and progression of disease, and various results have been obtained. In some cases ALT, in other AST or GGT levels demonstrated best correlation with severity of inflammation or fibrosis and their progression/regression on follow-up biopsies^[4,14-16]. Equally important, the full spectrum of NAFLD including severe inflammation and fibrosis was proven to occur with almost similar frequency in patients with completely normal liver enzymes^[8,9].

IMAGING METHODS

Imaging modalities frequently used in the diagnosis of NAFLD include ultrasound, computed tomography and magnetic resonance imaging (MRI). While they are all very sensitive (80%-100%) and specific in detection of steatosis, none of them can effectively distinguish simple steatosis from NASH or determine the degree of fibrosis^[17]. Nevertheless, MRI is more sensitive than ultrasound in detecting lesser degrees of hepatic steatosis, and new techniques in MRI are constantly being developed that

provide additional data on different tissue parameters. One of them is magnetic resonance (MR) elastography, which estimates liver tissue stiffness by imaging the propagation of induced shear waves with a modified phase-contrast MR sequence. This technique was shown to have an excellent predictive value for excluding fibrosis, while sensitivity and specificity for discriminating between mild and more severe fibrosis was around 85%^[18]. A recent study investigated the performance of MR elastography in 58 patients with NAFLD and demonstrated very high accuracy with under the receiver operated curve (AUROC) of 0.93 for discriminating patients with NASH and those with simple steatosis, with a sensitivity of 94% and a specificity 73% by using a threshold of 2.74 kPa^[19]. The future advances in MRI technology including hepatic flow parameters and diffusion-weighted MRI may hopefully provide more MR-based tools for liver fibrosis detection. Ultrasound has also demonstrated the potential for improvement in diagnosis of NAFLD and NASH. Apart from ultrasound-based elastography, use of ultrasound contrast agents has been studied in this scenario, and signal intensity after contrast administration was shown to be significantly lower in NASH when compared with simple steatosis and normal liver^[20].

LIVER STIFFNESS MEASUREMENT

Transient elastography (FibroScan[®], EchoSens, Paris, France) is a relatively novel technique which measures liver tissue elasticity by measuring the speed of propagation of probe-induced vibrations through parenchyma by ultrasound. Elasticity shows significant correlation with degree of liver fibrosis, and FibroScan is considered to produce a reliable prediction of higher degrees of liver fibrosis. The method was first assessed in population of patients with hepatitis C, and after evaluation in multiple studies it has been introduced into clinical practice. A survey performed four years ago in France showed that about a third of hepatologists was using it (mostly in evaluation of patients with hepatitis C), and the method is now gaining increasing popularity in other countries as well^[21]. More recent studies have assessed transient elastography in population of patients with NAFLD, and obtained results that are similar to those from studies in hepatitis C^[22-24]. A consistent increase in liver stiffness with increasing fibrosis stage was observed, and the largest study, performed by Wong *et al*^[22], obtained AUROC values of 0.93 for advanced fibrosis and 0.95 for cirrhosis. When the liver stiffness cut-off was set at 7.9 kPa, negative predictive value for advanced fibrosis was excellent (97%), and could be applied to 60% of the population. On the other side, positive predictive value of having advanced fibrosis or cirrhosis was at best only 72.4%, at the 9.6 kPa cut-off. Accuracy of FibroScan in detecting significant fibrosis (defined as at least perisinusoidal and portal/periportal fibrosis) was poor, as was expected from previous experience.

Several meta-analyses have assessed performance of transient elastography in fibrosis detection, consisting

mostly of studies on hepatitis C patients. They have shown generally very good diagnostic accuracy in detecting cirrhosis and somewhat lesser precision in excluding advanced fibrosis, while they demonstrated substantial heterogeneity in diagnosis of significant fibrosis ($F \geq 2$)^[25,26]. Importantly, variation in cut-off values of liver stiffness has been large and these values still require validation. In conclusion, due to the relatively low specificity, the value of transient elastography seems to remain in ruling out cirrhosis and advanced fibrosis in patients with low liver stiffness values, while patients with intermediate values would still require liver biopsy for correct classification, and the proportion of patients with high stiffness values who are misclassified is not negligible. It is also important to take into account that the population of patients that is usually encountered in clinical practice does not have advanced fibrosis or cirrhosis in large proportions, and diagnosis of lesser degrees of fibrosis is equally important in estimating the risk for liver-related morbidity and mortality. A large study that evaluated frequency and reasons of failure to obtain the elasticity measurement found that FibroScan was feasible in over 95% of the patients, and the only factor associated with failure was body mass index greater than 28^[27]. Failure occurs due to the elastic and ultrasound wave attenuation by subcutaneous fat, and while this may not be a significant issue in other chronic liver diseases, it is an important limitation in patients with NAFLD, considering the prevalence of obesity in this population. In the study by Wong *et al.*^[22], measurement could not be obtained in over 10% of cases, which significantly reduced diagnostic accuracy when the 'intention-to-diagnose' analysis was performed.

Another noninvasive method of assessing tissue stiffness, acoustic radiation force impulse (ARFI) shear wave imaging, was recently assessed in a couple of studies with NAFLD patients^[28,29]. This ultrasound-based technique estimates the tissue stiffness by measuring transient tissue deformations of several microns which are induced in the liver parenchyma by acoustic radiation force. In a study on 172 NAFLD patients by Palmeri *et al.*^[29], ARFI imaging distinguished low (Stages 0-2) from high (Stage 3-4) fibrosis stages with a sensitivity and a specificity of around 90% (AUROC of 0.90). Body mass index over 40 kg/m² was not a limiting factor for ARFI imaging, which overcomes part of the problems associated with FibroScan. When compared to FibroScan, ARFI imaging demonstrated similar diagnostic performance^[28,30].

BIOMARKERS OF NECROINFLAMMATION

Most intense research is now being focused on biomarkers, measurable serum parameters that reflect the intensity of inflammatory processes and hepatocyte necrosis, as well as the ones that reflect extracellular matrix remodeling and collagen deposition. Ideally, an excellent biomarker would be specific for liver and accurately reflect the underlying pathogenetic processes on the level of whole organ, and thus be an even more precise indicator of the

disease than liver biopsy, which is prone to sampling variability and interpretation biases as described earlier.

Cytokeratin 18 fragments

Apoptosis is an important mechanism in pathogenesis of NASH, and its initiation leads to activation of caspase family of intracellular proteases which then cleave different intracellular proteins including cytokeratin 18 (CK-18), the major intermediate filament protein in hepatocytes. By measurement of CK-18 fragments hepatocyte apoptosis can be quantified, and this method was tested as a noninvasive tool in NASH diagnosis in several studies. Initial results were very promising, as Wieckowska *et al.*^[31] demonstrated a striking increase in serum CK-18 fragment levels in patients with definitive NASH, as well as their high diagnostic accuracy for differentiating between NASH and simple steatosis or normal liver, with AUROC of 0.93 and positive and negative predictive value of 95.0% and 89.5%, respectively. However, this study included only 39 patients, and the larger validation study that was subsequently undertaken and included 139 patients obtained less favourable results: median CK-18 fragment levels in NASH cases were now only 335 U/L (compared to 765 U/L in the first study, and to about 200 U/L in non-NASH cases in both studies), and diagnostic performance was expectedly poorer (calculated AUROC was 0.83 and sensitivity for diagnosing NASH was at best 77%, with the specificity rising above 90% only at the highest tested cut-off value)^[32]. Nevertheless, CK-18 fragment levels showed very good correlation with NASH, fibrosis and NAS (NAFLD activity score), and similar results were reported from other groups as well, supporting its potential role as a noninvasive tool in NAFLD^[33-35]. Even more importantly, in the study by Diab *et al.*^[35,36] on 99 patients who underwent bariatric surgery, CK-18 fragment levels showed a significant decrease 6 mo postoperatively, and in another study changes in CK-18 fragment levels closely paralleled changes in NAS on follow-up biopsy. These findings indicate the potential use of CK-18 fragment levels in the follow-up of patients with NASH, including evaluation of response to therapy. This certainly requires further attention, as it could possibly lead us closer to the goal of eliminating the need for second liver biopsy and thus facilitating design and conductance of clinical trials, as well as enhancing patient follow-up in clinical practice.

High-sensitivity C-reactive protein

C-reactive protein (CRP) is an acute-phase reactant produced by the liver in many inflammatory conditions, and based on the hypothesis that NASH is associated with low-grade systemic inflammation, several studies have compared high-sensitivity CRP levels in patients with NAFLD. Two studies found that hs-CRP levels were significantly higher in cases with NASH compared to those with simple steatosis, and hs-CRP also correlated well with presence of advanced fibrosis^[37,38]. However, a study performed earlier concluded that measurement of hs-CRP

was not useful in predicting the histological severity of NAFLD, as there was no relationship between the levels of hs-CRP and the grades of steatosis, necroinflammation or fibrosis^[39]. Further investigation including testing of diagnostic accuracy is needed before definite conclusions can be reached about usefulness of this marker in NAFLD.

Interleukin-6 and C-C chemokine ligand 2

Interleukin-6 (IL-6) is a proinflammatory cytokine that is involved in NAFLD pathogenesis, and Wieckowska *et al*^[40] demonstrated a markedly increased IL-6 expression in liver tissue of patients with NASH as compared to simple steatosis or normal liver, with a positive correlation with severity of inflammation and fibrosis. Plasma IL-6 levels that were parallelly measured in this study correlated well with liver IL-6 expression. In another study, IL-6 was among several serum markers evaluated in 47 NAFLD patients and 30 controls, and it was significantly increased in patients with NAFLD as compared to controls, but not in NASH compared to simple steatosis^[41]. This study also evaluated serum levels of C-C chemokine ligand 2 (CCL2), a chemokine responsible for monocyte/macrophage infiltration of liver and maintaining hepatic inflammation and fibrogenesis, and found that it was significantly elevated in patients with NASH compared to simple steatosis, but diagnostic performance of CCL2 levels was not tested. In a recent study, pharmacological inhibition of CCL2 had an effect on reduction of hepatic steatosis in a murine model, and CCL2 will presumably see some further investigation in the context of NAFLD^[42].

Plasma pentraxin 3

Plasma pentraxin 3 is a novel marker of systemic inflammation from pentraxin family of acute-phase proteins that is produced by diverse cell types in response to pro-inflammatory cytokines^[43]. Yoneda *et al*^[44] have evaluated pentraxin 3 levels in 70 patients with NAFLD, and found that they were significantly higher in cases with NASH compared to non-NASH, with the AUROC value of 0.75 for NASH detection. Pentraxin 3 levels also correlated well with the stage of fibrosis. These findings should provide basis for additional evaluation of this marker in other NAFLD patient cohorts.

Oxidative stress

Oxidative stress is one of the key mechanisms in NASH pathogenesis, and several studies have measured systemic markers of oxidative stress status in NAFLD patients and compared them between cases with NASH and controls^[45-47]. Different methods for measurement of oxidative stress have been used (measurement of levels of lipid peroxidation products, levels of antioxidant defence systems like vitamin E, glutathione peroxidase and superoxide dismutase activities, antioxidant capacity of the plasma and total plasma peroxide concentrations), and studies produced disparate results. Based on the current data, there is no doubt that oxidative stress is present in

NASH, but the utility of its measurement as a noninvasive tool in NAFLD diagnosis probably does not have any clinical value.

Tumor necrosis factor- α and adiponectin

Tumor necrosis factor (TNF)- α and adiponectin are cytokines which have been proven to play important roles in NAFLD pathogenesis, and the serum levels of these cytokines were determined in patients with NAFLD and correlated to disease severity in multiple studies^[48-52]. However, diagnostic accuracy in discerning NASH from simple steatosis and the potential for noninvasive use in diagnosis were generally not evaluated, and the data on diagnostic performance of these cytokines are not available. As of the published results, most of the studies demonstrated correlation of lower adiponectin levels with presence of NAFLD compared to healthy controls, presence of NASH compared to simple steatosis, and with histological severity of the disease, while levels of TNF- α and its soluble receptor were most often not significantly different between patients with NASH and patients with simple steatosis or controls. Thus, the potential for clinical use of these cytokines as noninvasive tools for diagnosis of NASH is questionable.

Insulin resistance

Insulin resistance state leads to increased lipolysis and free fatty acid flux to the liver, and elevated plasma glucose and insulin levels promote *de novo* fatty acid synthesis and impair β -oxidation, contributing to the development of hepatic steatosis. Although it is not clear whether insulin resistance causes hepatic steatosis or the liver fat accumulation represents the primary event leading to peripheral insulin resistance, there is no doubt that it plays an important role in the pathogenesis of NAFLD. Large population studies have shown that almost all of the NAFLD patients were insulin resistant according to the homeostasis model assessment of insulin resistance (HOMA-IR)^[53]. Additionally, the potential of insulin resistance measurement as a noninvasive diagnostic tool was also evaluated. In a study by Shimada *et al*^[54], the authors tested the diagnostic performance of adiponectin, insulin resistance measured by HOMA-IR, and type IV collagen 7S in discriminating NASH from simple steatosis. While performance of each of these markers individually wasn't great, sensitivity of the combination of three markers was 94%, with a specificity of 74%.

Although insulin resistance has been usually associated with type 2 diabetes, it can also be present in type 1 diabetic patients^[55]. The euglycemic insulin clamp technique which represents the gold standard for identifying type 1 diabetic patients who are insulin resistant is impractical for routine clinical use, and insulin resistance in type 1 diabetic patients was often recognized only by higher insulin requirements. Recent introduction of a validated method for estimated glucose disposal rate (eGDR) measurement based on clinical parameters has allowed its easier assessment in a clinical setting^[56]. A recently pub-

lished study demonstrated that NAFLD markers were associated with insulin resistance measured by eGDR in type 1 diabetic patients. NAFLD associated markers (ALT, AST, alkaline phosphatase, GGT and ferritin) worsened in parallel with the decline in insulin sensitivity and after adjustment for covariates, ALT, AST and alkaline phosphatase were independent predictors of insulin resistance^[57].

Multicomponent tests

There have been several attempts at constructing a panel of clinical and laboratory parameters that would, when combined using a formula or a scoring system, result in a value that enables distinguishing between NASH and simple steatosis. The most advanced attempt was a study by the French group specialized at developing diagnostic models for various liver conditions, who constructed a complex test (NashTest) which combines 13 parameters (age, sex, height, weight, triglycerides, cholesterol, α 2-macroglobulin, apolipoprotein A1, haptoglobin, GGT, ALT, AST and bilirubin) into a patented algorithm^[58]. Their design and validation study included 257 patients and 383 controls, and the NashTest had AUROC of 0.79 [95% confidence interval (CI) 0.69-0.86], with sensitivity for NASH (using criteria by Kleiner *et al.*^[59]) of only 33% and positive predictive value of 66%. The results were somewhat better when subgroups with borderline NASH and NASH were combined, the sensitivity rising to 88% and positive predictive value to 74%. In another study, a clinical scoring system was developed based on the results of multivariate analysis in a group of morbidly obese patients that underwent intraoperative liver biopsy at bariatric surgery^[60]. The proposed NASH Clinical Scoring System for Morbid Obesity included 6 clinical variables (hypertension, diabetes, AST, ALT, sleep apnea and non-black race) and was used to stratify morbidly obese into 4 groups regarding the risk for presence of NASH (low, intermediate, high and very high). In the studied group, the proportion of patients with low-risk score who had NASH was 13%, while it was 80% in those with very high-risk score. Recently, Miele *et al.*^[61] measured several markers of liver fibrosis in a cohort of 46 patients with NAFLD, and constructed a mathematical model based on the results of multivariate analysis that included age, hyaluronic acid and tissue inhibitor of metalloproteinase 1 levels. A specific cut-off value identified patients with NASH with 86% sensitivity, and negative and positive predictive values of 96% and 60%. This model could potentially be useful in excluding patients with negative values from liver biopsy consideration if these findings are confirmed in larger independent studies.

BIOMARKERS OF FIBROSIS

As with other chronic liver diseases, the most important indicator of severity and progression of liver damage in NAFLD is the presence and degree of liver fibrosis. Estimation of fibrosis is therefore essential in the diag-

nostic workup of patients with NAFLD, and it remains one of the major reasons for performing liver biopsy in this population. After a large number of studies was undertaken in hepatitis C patients that tried either to design tests and scoring systems using combinations of readily available clinical and biochemical parameters, or to find specific biomarkers of fibrosis processes that would adequately correspond to liver biopsy findings, similar attempts were made as well in populations of patients with NAFLD. Generally, while showing good accuracy in detection of advanced fibrosis or cirrhosis, all of these tests demonstrate significantly lower sensitivities in predicting the presence of mild or moderate fibrosis. The problem lies in the fact that this is exactly the group of patients that would benefit most from therapeutic interventions, before significant fibrosis has already developed, and they therefore require early diagnosis.

Hyaluronic acid and other markers of extracellular matrix turnover

Hyaluronic acid is a component of the extracellular matrix that can be measured in serum, where it partially enters through lymphatics. Serum levels are dependent on production, which increases with increased collagen synthesis, as well as degradation, which takes course in liver sinusoidal endothelial cells after binding to specific receptors. With progression of liver fibrosis, both increased production of collagen and decreased function of sinusoidal endothelial cells lead to elevation of hyaluronic acid serum levels.

Several groups have so far evaluated the potential use of hyaluronic acid levels in diagnosis of NASH-related fibrosis. Suzuki *et al.*^[62] investigated the potential of hyaluronic acid for use in diagnosing fibrosis in a cohort of 79 patients with NAFLD and various degrees of fibrosis. The hyaluronic acid serum levels demonstrated good correlation with the degree of hepatic fibrosis, and significant difference was noted especially when comparing mild to moderate (Stages 0-2) with severe fibrosis or cirrhosis (Stages 3-4). The calculated AUROC for severe fibrosis was 0.89 (95% CI 0.81-0.97), and at the optimal cut-off value of 46.1 ng/mL sensitivity was 85% (95% CI 62%-97%) and specificity 80% (95% CI 67%-89%). When a prevalence of severe fibrosis among NAFLD patients was assumed to be 20% (approximate of usual patient population at referral centers), the corresponding positive predictive value was 51% (95% CI 39%-68%) and negative predictive value 95% (95% CI 91%-100%). Accuracy for diagnosing mild fibrosis (Stage 1) was low and the number of patients with moderate fibrosis (Stage 2) was inadequate for valid analysis. Another study evaluated hyaluronic acid and laminin levels in 50 patients with NASH, of whom 23 had some degree of fibrosis and 27 had no fibrosis on liver biopsy^[63]. Subjects with NASH and fibrosis had significantly higher hyaluronic acid and laminin levels than those without fibrosis, and AUROC and diagnostic performance of both of these markers was calculated for differentiating between presence and

absence of fibrosis, showing excellent diagnostic accuracy of hyaluronic acid at the cut-off value of 148.8 ng/mL (reported sensitivity and specificity was over 95%). In the fibrosis group, levels of hyaluronic acid significantly increased with rising fibrosis stages, however the accuracy for distinguishing different fibrosis stages was not tested due to small patient numbers. Sakugawa *et al.*^[64] investigated the levels of hyaluronic acid and type VI collagen 7S domain in a population of 112 patients with NAFLD, of whom 70 were classified as NASH. On regression analysis, both markers were independently associated with the presence of NASH or severe fibrosis, but demonstrated sensitivity and specificity for severe fibrosis in the range of 70%-80%. However, if both markers were negative in a given patient, severe fibrosis was highly unlikely to be present (negative predictive value 95.2%).

Although some of these results look very promising, studies are still lacking in power, and the proposed cut-off values and calculated diagnostic accuracies are quite heterogeneous, which may be due to other factors in addition to difference in sample size, like difference in measurement methods and studied populations. Another important aspect of clinical usefulness of hyaluronic acid and other serum markers of fibrosis that hasn't yet been investigated is the question of sensitivity to longitudinal changes in fibrosis of liver parenchyma, which could potentially enable noninvasive patient follow-up and evaluation of treatment effects.

Multicomponent panels

FibroTest is a copyrighted panel developed by the French group who originally conceived it for diagnosis of liver fibrosis in hepatitis C, where it has subsequently been extensively studied. It includes 5 biochemical parameters (α 2-macroglobulin, apolipoprotein A1, haptoglobin, total bilirubin and GGT) that are incorporated into a patented formula. More recently, the authors of the panel conducted a study that thoroughly evaluated diagnostic performance of FibroTest in the setting of NAFLD by including 267 patients and a large number of healthy controls^[65]. Fibrosis stage was determined according to Kleiner *et al.*^[59], and advanced fibrosis included stages F2-F4 (perisinusoidal and portal/periportal fibrosis, bridging fibrosis and cirrhosis). Mean FibroTest value steadily increased with increasing fibrosis stage, and calculated AUROC for advanced fibrosis was 0.86 (95% CI 0.77-0.91), while it was 0.92 (95% CI 0.83-0.96) for bridging fibrosis or cirrhosis (F3-F4). A FibroTest cut-off score of 0.30 had 77% sensitivity and 90% negative predictive value, and score of 0.70 had 98% specificity and 76% positive predictive value for advanced fibrosis. As expected, performance was even better in detection of F3-F4, with 92% sensitivity and 98% negative predictive value. In addition to one third of patients having the value that fell between these two cut-offs and thus a nondiagnostic test result, other causes of FibroTest failure were analyzed and included Gilbert's syndrome, acute inflammation, and abnormal apolipoprotein A1 that was related to dyslipidaemia.

Another large multicenter study by a different group was undertaken and included a total of 733 patients divided in 2 groups in an attempt to develop and validate a noninvasive scoring system that would separate NAFLD patients with and without advanced liver fibrosis^[66]. The score was named NAFLD fibrosis score and included a formula with 6 variables (age, hyperglycemia, body mass index, platelet count, albumin and AST/ALT ratio), selected based on results of multivariate analysis. Biopsy was also scored according to Kleiner *et al.*^[59], but the diagnostic goal of advanced fibrosis included only Stages F3 and F4. AUROC values were 0.88 in estimation and 0.82 in validation set, and two cut-off points were determined similarly to the previously mentioned study. Using the low cut-off point, negative predictive value of the score was 93% in estimation group and 88% in validation group, while with the high cut-off point positive predictive value was 90% in estimation group and 82% in validation group. Only 25% of patients had score values between the cut-offs and would thus be considered as "indeterminate" and still require liver biopsy after this noninvasive test was performed.

After the European Liver Fibrosis Group developed an algorithm that included age, tissue inhibitor of matrix metalloproteinase 1, hyaluronic acid and aminoterminal peptide of pro-collagen III and tested its performance in diagnosing significant fibrosis in a large cohort of patients with various chronic liver diseases, another study was undertaken more recently that investigated performance of this panel specifically in NAFLD patients^[67,68]. The original panel was modified by excluding age and naming it enhanced liver fibrosis panel (ELF), and its diagnostic performance was tested in a cohort of 192 patients. The ELF panel had very good performance in distinguishing severe fibrosis (Stage F3-F4) with an AUROC of 0.90 (95% CI 0.84-0.96), while AUROC for detecting moderate and severe fibrosis together (F2-F4) was 0.82 (95% CI 0.75-0.88). Diagnostic accuracy varied with various cut-off points tested, and if cut-offs with 90% sensitivity and specificity for severe fibrosis detection were selected, 86% of study patients would have avoided a liver biopsy, with 76% correctly classified. The study also suggested that the addition of simple parameters, the ones included in previously mentioned NAFLD fibrosis score, could augment the diagnostic performance of the ELF panel, although additional studies with larger sample size would be required to confirm this.

CONCLUSION

Due to the very high prevalence of the disease and numerous difficulties related to establishing the diagnosis, NAFLD remains undiagnosed or incompletely defined in a large number of cases. Therefore, the search for the means to noninvasive diagnosis of different forms of NAFLD is a matter of uttermost importance. It is gaining even greater significance in the light of recent advances in the treatment of NASH, as the research efforts are finally starting to provide us with definite treatment

options. Recently published study with vitamin E and pioglitazone, as well as other current treatment trials place the necessity of establishing a correct diagnosis and not missing NASH in a whole different perspective^[12]. Furthermore, given the proportion of population with fatty liver and the fact that the presence of NASH in a given patient is often not linked with elevation in liver enzymes, the number of patients in need of a screening becomes daunting. After the insight in all of the aforementioned studies, we can see that some have indeed come very close and demonstrated very good diagnostic performance of certain noninvasive tools. However, the gold standard used in almost all of the studies is liver biopsy, and the question that remains is whether we are actually able to accurately assess the performance of noninvasive methods when the gold standard by itself has significant flaws. These flaws were very clearly demonstrated in a study of sampling variability in NAFLD by Ratziu *et al*^[11]. One can also pose the question: have we maybe found an excellent noninvasive tool already, but are ignorant of the fact due to our incapability to actually see “the absolute truth”? This question has been addressed in a study by Mehta *et al*^[69], who calculated the AUROC for a hypothetical liver histology surrogate marker against the biopsy for a range of possible performances of both tests. The authors found that an ideal marker (99% accuracy) could in the best possible setting (sensitivity and specificity of biopsy 90%, prevalence of significant disease 40%) have an AUROC of not more than 0.90. This may mean that, unless an alternative gold standard is found, we might as well be in pursuit of something that isn't there.

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Orotate phosphoribosyl transferase mRNA expression and the response of cholangiocarcinoma to 5-fluorouracil

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Abstract

AIM: To determine whether expression of certain enzymes related to 5-fluorouracil (5-FU) metabolism pre-

dicts 5-FU chemosensitivity in cholangiocarcinoma (CCA).

METHODS: The histoculture drug response assay (HDRA) was performed using surgically resected CCA tissues. Tumor cell viability was determined morphologically with hematoxylin and eosin- and terminal deoxynucleotide transferase-mediated dUTP nick-end labeling-stained tissues. The mRNA expression of thymidine phosphorylase (TP), orotate phosphoribosyl transferase (OPRT), thymidylate synthase (TS), and dihydropyrimidine dehydrogenase (DPD) was determined with real-time reverse transcriptase-polymerase chain reaction. The levels of gene expression and the sensitivity to 5-FU were evaluated.

RESULTS: Twenty-three CCA tissues were obtained from patients who had been diagnosed with intrahepatic CCA and who underwent surgical resection at Srinagarind Hospital, Khon Kaen University from 2007 to 2009. HDRA was used to determine the response of these CCA tissues to 5-FU. Based on the dose-response curve, 200 µg/mL 5-FU was selected as the test concentration. The percentage of inhibition index at the median point was selected as the cut-off point to differentiate the responding and non-responding tumors to 5-FU. When the relationship between TP, OPRT, TS and DPD mRNA expression levels and the sensitivity of CCA tissues to 5-FU was examined, only OPRT mRNA expression was significantly correlated with the response to 5-FU. The mean expression level of OPRT was significantly higher in the responder group compared to the non-responder group (0.41 ± 0.25 vs 0.22 ± 0.12 , $P < 0.05$).

CONCLUSION: OPRT mRNA expression may be a useful predictor of 5-FU chemosensitivity of CCA. Whether OPRT mRNA could be used to predict the success of 5-FU chemotherapy in CCA patients requires confirmation in patients.

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Key words: Histoculture drug response assay; 5-fluorouracil; Cholangiocarcinoma; Orotate phosphoribosyl transferase; Chemosensitivity

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INTRODUCTION

Cholangiocarcinoma (CCA), a bile duct epithelial tumor, has poor prognosis owing to the absence of an early diagnostic method and effective treatments. The only curative therapy is surgical resection, but most CCA patients are diagnosed at an unresectable stage. Therefore, chemotherapy is the only practical treatment^[1].

5-fluorouracil (5-FU) is one of the most common anticancer agents and is used to treat a variety of solid tumors. 5-FU alone or 5-FU-based regimens are widely used to treat CCA patients. As reviewed by Thongprasert^[2], the overall response rate and median survival time following treatment with 5-FU for CCA are 10% and 6.5 mo, respectively. In addition, a combination of 5-FU and leucovorin yields a response rate of 32% and a median survival time of 6 mo^[2]. Combination therapy of 5-FU with cisplatin consistently yields response rates of 10%-40%, and median survival times are better than those observed with 5-FU alone^[2]. Combinations of 5-FU with taxanes or etoposide, however, have not shown convincing superiority over 5-FU alone for CCA treatment^[3,4]. Thus, data obtained from these clinical studies revealed relatively poor response rates of CCA to 5-FU-based regimens.

After entering cells, 5-FU is converted to 5-fluorodeoxyuridine monophosphate (FdUMP) through intermediary molecules by thymidine phosphorylase (TP) and orotate phosphoribosyl transferase (OPRT)^[5-7]. FdUMP then forms a complex with thymidylate synthase (TS), leading to inhibition of DNA synthesis^[5]. In addition, 5-FU can be phosphorylated by OPRT to form 5-fluorouridine monophosphate, and then to 5-fluorouridine triphosphate, which is subsequently incorporated into RNA, resulting in RNA dysfunction^[8]. In the degradation pathway, 5-FU is metabolized by dihydropyrimidine dehydrogenase (DPD) to an inactive metabolite, 5-fluorodihydrouracil, and subsequently excreted in the urine^[9]. The resistance of several cancer types to 5-FU may be due to alterations in the expression of several genes that are involved in the metabolism and action of this drug^[10]. Moreover, the expression and activities of these enzymes

have been proposed as markers to predict the response to 5-FU of several cancers such as gastric cancers and metastatic colorectal cancer^[11-14].

Because 5-FU is the main chemotherapeutic agent for treatment of CCA, elucidating the molecular mechanism involved in the response to 5-FU may be useful for the treatment of CCA. In the present study, we examined the correlation between mRNA expression of target genes involved in 5-FU metabolism and the chemosensitivity of cancer tissues to 5-FU in 23 CCA patients.

MATERIALS AND METHODS

Chemicals

Hank's balanced salt solution (HBSS), RPMI 1640 medium, fetal bovine serum, penicillin, and streptomycin were purchased from GIBCO BRL (Grand Island, NY, United States). Collagen gel sponges were purchased from Pharmacia and Upjohn (Kalamazoo, MI, United States). 5-FU was provided by Fresenius Kabi Oncology Ltd. (Hayarna, India). The DeadEnd Colorimetric Terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) system was purchased from Promega (Madison, WI, United States). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, United States). The TaqMan[®] gene expression assay kit and TaqMan Universal polymerase chain reaction (PCR) master mix with AmpErase UNG were purchased from Applied Biosystems (Foster City, CA, United States).

CCA tissues

Twenty-three samples of CCA tissues were obtained from patients who had been diagnosed with intrahepatic CCA and who underwent surgical resection at the Department of Surgery, Srinagarind Hospital, Khon Kaen University between 2007 and 2009. The histological types of the CCA tissues were classified according to the World Health Organization classification. Of these patients, 69.6% were men. The median age of the CCA patients was 61 years (range: 42-70 years). Written informed consent was obtained from all patients before the collection of tumor tissues. The study protocol was approved by the Khon Kaen Ethics Committee for Human Research, Khon Kaen University, Thailand (HE500501).

Histoculture drug response assay

After surgery, the tumor tissues were immediately transferred to the laboratory in HBSS containing 100 IU/mL penicillin and 100 µg/mL streptomycin. Histoculture drug response assay (HDRA) was performed as described^[13] with some modifications. Cubes of collagen gel sponge (1 cm³) were immersed in 1 mL RPMI 1640 containing 20% fetal bovine serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 5-FU at final concentrations of 100, 200, and 400 µg/mL in a 24-well plate. After washing six times in HBSS, the tumor tissues were aseptically cut into small pieces using biopsy punches (3 mm diameter), placed on the collagen gel sponges, and cultured at 37 °C for 4 d

in a 5% CO₂ atmosphere. Duplicate tissue cultures were performed for each drug concentration. Wells containing culture medium without 5-FU were used as controls.

After 4 d of culture, the viability of tumor cells in the cultured tissues was examined with histology. Hematoxylin and eosin (HE) and TUNEL staining were used to assess cell viability^[15]. In brief, tissues were fixed in 4% formaldehyde and embedded in paraffin, and 4- μ m tissue sections were cut. Deparaffinized sections were rehydrated, stained with HE and TUNEL, and examined under a microscope. *In situ* TUNEL was carried out according to the manufacturer's instructions. TUNEL-positive cells were quantified in at least four high-power fields ($\times 40$) of randomly selected tissue sections. The total live tumor cells showing anaplastic characteristics with hyperchromatic nuclei/cytoplasm were counted and scored as the percent of the total tumor cells. TUNEL-stained tumor cells were identified as dead cells. The efficacy of 5-FU was calculated and expressed as the inhibition index (% I.I.) using the following formula: % I.I. = $(1 - \% \text{ living tumor cells in 5-FU-treated tumor tissue} / \% \text{ living tumor cells in control tissue}) \times 100$. The % I.I.s at various concentrations of 5-FU ranging from doses of 100-400 μ g/mL were determined.

Determination of mRNA expression

The mRNA expression of target genes including those encoding TS, DPD, TP, OPRT and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in CCA tissues was determined by reverse transcription and quantitative real-time PCR. In brief, total RNA of each CCA tissue was isolated using TRIzol reagent according to the manufacturer's protocol. Reverse transcription was performed as described^[16].

The mRNA expression of the target gene was determined using the TaqMan[®] gene expression assay kit according to the manufacturer's instructions. Real-time PCR was performed in 20- μ L PCR reactions containing TaqMan Universal PCR master mix, target-specific primers, one TaqMan[®] MGB FAM[™] dye-labeled probe, and 50 ng cDNA. Each PCR was carried out in duplicate. The PCR conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All data were analyzed using the ABI PRISM 7500 Real-time PCR system sequence detection software v.1.4 (Applied Biosystems). The quantity of target cDNA or GAPDH PCR product was calculated using the corresponding standard curve, and the amount of target cDNA in a given sample was normalized to that of GAPDH cDNA.

Statistical analysis

The Student's *t*-test was used to compare the % I.I. values between the responder and non-responder groups.

RESULTS

Microscopic examination of CCA tissues after HDRA

HE- and TUNEL-stained sections of control and 5-FU treated CCA tissues are shown in Figure 1. After 4 d of culture on the collagen gel sponge, the CCA tissue archi-

tecture including cell-to-cell contact was well maintained. Most of the tumor cells were alive and showed anaplastic characteristics with hyperchromatic nuclei/cytoplasm (short arrows). On the other hand, cells with eosinophilic cytoplasm, shrunken (condensed) nuclei (pyknotic nuclei), and fragmented nuclei (karyorrhexis, indicated by long arrows) were found in 5-FU-treated cells. TUNEL-stained tumor cells were identified as dead cells. The mean percentage of living tumor cells in control tissues at day 4 was $83.3\% \pm 14.2\%$ of those observed at day 0. In addition, the proportion of viable tumor cells in control condition gradually decreased on days 5, 6 and 7 of culture; therefore HDRA of CCA tissues was performed for 4 d.

Response of CCA tissues to 5-FU

The responses of CCA tissues to various concentrations of 5-FU ranging from 100-400 μ g/mL were determined using CCA tissues obtained from five patients. Dose-dependent responses of CCA tissues to 5-FU were observed (Figure 2). From these results, a 5-FU concentration of 200 μ g/mL was selected as the test concentration. The % I.I. values for 5-FU (200 μ g/mL) treatment of 23 CCA tissues are shown in Figure 3. The median % I.I. value was selected as the cut-off to classify CCA tissues as responders or non-responders (Figure 3).

Relationship between the expression of target genes and the sensitivity to 5-FU

For each CCA tissue, mRNA expression was quantified with real-time PCR using specific TaqMan probes for genes encoding enzymes involved in the 5-FU metabolic pathway, including TS, DPD, TP and OPRT. Moderate variability in the expression levels of TS, TP and OPRT mRNA normalized to GAPDH expression among individual samples was observed (24-fold, range: 0.05-1.22; 33-fold, range: 0.13-4.27, and 17-fold, range: 0.06-1.02, respectively), whereas high variability was observed for DPD expression (135-fold, range: 0.05-6.76).

Scattered, overlapping expression levels of these genes were observed in the responder and non-responder groups. However, the mean expression level of OPRT was significantly higher in the responder group compared to the non-responder group (0.41 ± 0.25 vs 0.22 ± 0.12 , $P < 0.05$, Figure 4). The mean expression levels of TS, DPD, and TP appeared higher in the responder group compared to the non-responder group, but the differences were not statistically significant (0.26 ± 0.32 vs 0.18 ± 0.12 , $P = 0.43$; 1.73 ± 1.96 vs 0.74 ± 0.50 , $P = 0.12$; and 1.60 ± 1.16 vs 1.02 ± 0.72 , $P = 0.16$, respectively; Figure 4).

DISCUSSION

5-FU is phosphorylated in cells to become an active metabolite that inhibits DNA synthesis and induces RNA dysfunction^[5,8]. Intra-tumoral gene expression and activities of several enzymes related to 5-FU metabolism correlate with sensitivity to this drug for the treatment of several cancers^[11-14]. Of the genes we studied, OPRT seems to have predictive power and may be a promising

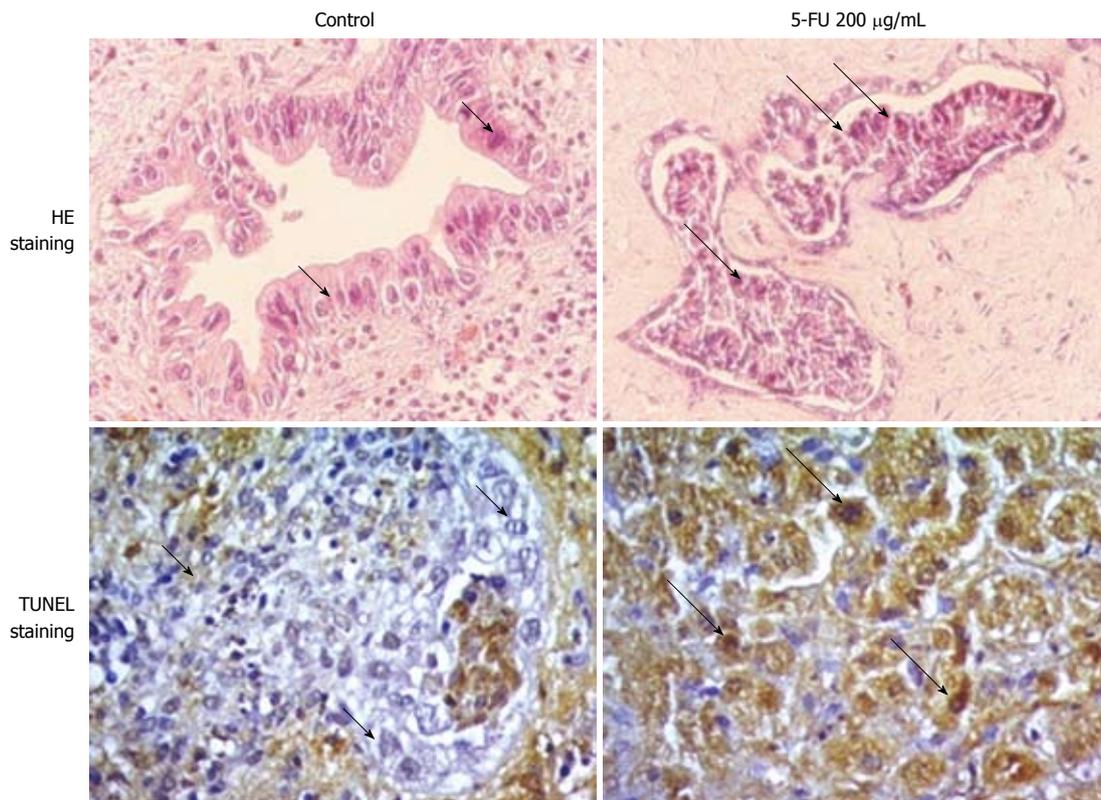


Figure 1 Photomicrographs (40 ×) of hematoxylin and eosin-stained and terminal deoxynucleotide transferase-mediated dUTP nick-end label-stained sections of cholangiocarcinoma tissues cultured on collagen gel sponges for 4 d in the absence or presence of 5-fluorouracil. Tumor cells with anaplastic characteristics showing hyperchromatic nuclei/cytoplasm are indicated with short arrows. Cells with eosinophilic cytoplasm with shrunken (condensed) nuclei (pyknotic nuclei) and fragmented nuclei (karyorrhexis) are indicated with long arrows. 5-FU: 5-fluorouracil; HE: Hematoxylin and eosin; TUNEL: Terminal deoxynucleotide transferase-mediated dUTP nick-end label

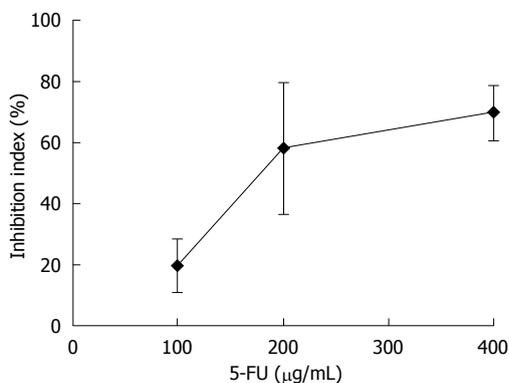


Figure 2 Dose-response-curve of cholangiocarcinoma tissues to 5-fluorouracil. Sensitivities of five cholangiocarcinoma tissues to 5-fluorouracil (5-FU) are shown as the percent of inhibition index (% I.I.), which was evaluated at doses of 100 µg/mL, 200 µg/mL, and 400 µg/mL using histoculture drug response assay. Data are the mean ± SD of two independent experiments.

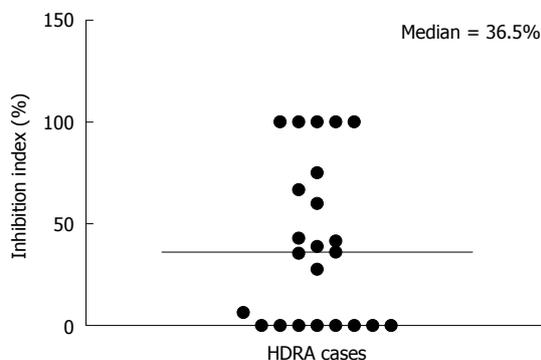


Figure 3 Distribution of percent of inhibition index of 23 cholangiocarcinoma tissues to 200 µg/mL 5-fluorouracil. Sensitivities of 23 cholangiocarcinoma (CCA) tissues to 5-fluorouracil (5-FU) were evaluated at 200 µg/mL 5-FU using histoculture drug response assay (HDRA). The median value of % I.I. (36.5%) was selected as the cut-off for classifying these tissues as responders and non-responders. Each circle represents one CCA sample. The median is shown by the long, thin horizontal line.

marker. High enzymatic activity of OPRT in tumor tissues is associated with high sensitivity of urinary bladder cancer^[17] and colorectal cancer^[18] to 5-FU. In addition, OPRT mRNA or the ratio of OPRT/DPD mRNA is associated with prolonged survival of metastatic colon cancer patients receiving oral tegafur-uracil and leucovorin^[11] and colorectal liver metastasis patients receiving intra-arterial chemotherapy with 5-FU^[19]. In addition, combined

expression of OPRT and TS in pre-chemotherapeutic fresh-frozen samples obtained from primary tumors may predict the response to S-1, an oral DPD-inhibiting fluoropyrimidine, in metastatic gastric cancer patients^[20]. Consistent with the previous studies, we observed here that intratumoral expression of OPRT mRNA in the responder group was significantly higher than expression

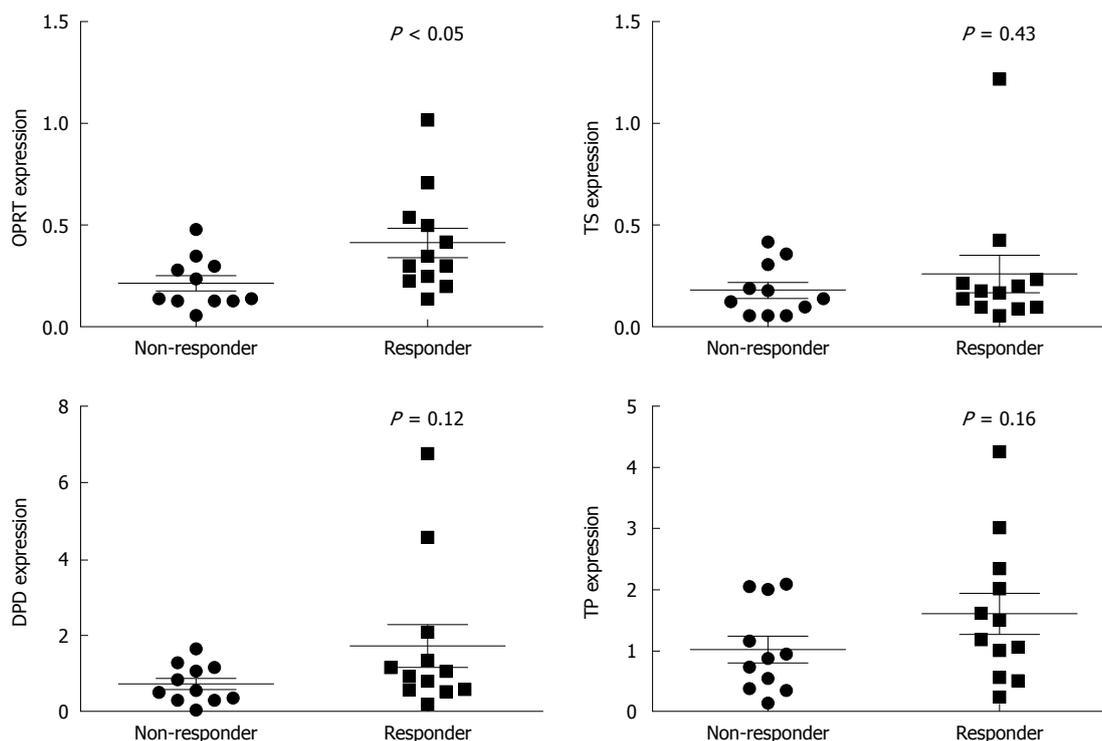


Figure 4 Relationship between chemosensitivity to 5-fluorouracil and mRNA expression of thymidine phosphorylase, orotate phosphoribosyl transferase, thymidylate synthase, and dihydropyrimidine dehydrogenase in 23 cholangiocarcinoma tissues. Chemosensitivity to 5-fluorouracil (5-FU) and mRNA expression were analyzed for 23 cholangiocarcinoma (CCA) tissues using histoculture drug response assay and quantitative real-time reverse transcription-polymerase chain reaction. The responders and non-responders were classified using the median value of the % I.I. as the cut-off point. The mRNA expression of orotate phosphoribosyl transferase (OPRT) was significantly higher in the responder ($P < 0.05$) group. The mRNA levels of thymidylate synthase (TS), and dihydropyrimidine dehydrogenase (DPD) and thymidine phosphorylase (TP) in the non-responder and responder groups, however, were not significantly different. The long, thin lines represent the mean, and the error bars represent the SD values. Each symbol (circles and squares) represents one CCA tissue sample.

in the non-responder group. These results suggest that the OPRT mRNA level in CCA tissues may be a promising predictor of an *in vitro* sensitivity to 5-FU using the HDRA technique. It should be noted that we observed overlapping expression levels of each gene among responder and non-responder tumors.

We observed moderate variability in the expression levels of TS, TP, and OPRT mRNA among individual samples (24-fold, 33-fold and 17-fold, respectively). The moderate differences in OPRT and TS expression levels observed in the present study were similar to those previously observed in gastric carcinoma and colorectal tissues^[21,22]. Similar to the variability reported in colorectal^[22] and esophageal carcinoma^[23], we observed high variability in the expression of DPD among CCA tissues. Genetic polymorphisms of genes encoding TP, DPD, TS and OPRT are well known^[24-27]. Variable expression of DPD, TP, TS and OPRT may explained by genetic polymorphisms in these genes.

TP is the first enzyme involved in the metabolic activation pathway of 5-FU to 5-fluorodeoxyuridine (FdUR), which can be phosphorylated by thymidine kinase to form FdUMP^[28]. In this study, no relationship was found between the intratumoral expression of TP mRNA and the sensitivity to 5-FU. In addition to TP, that the rate of conversion of 5-FU to FdUR could be influenced by the availability of the TP cofactor, deoxyribose-1-phosphate

(dRib-1-P) in cells^[29]. It has been previously demonstrated that the addition of dRib-1-P greatly increases the incorporation of thymidine into DNA and increases the potency of the growth-inhibitory actions of 5-FU^[30]. Thus, it may be possible that the amount of dRib-1-P in CCA tissues may be limited. Similar observations have been reported in colorectal tumor that there was no relationship between TP expression and 5-FU sensitivity^[31,32].

TS is a key enzyme that catalyzes the methylation of deoxyuridine monophosphate to deoxythymidine monophosphate, an important step in DNA synthesis^[33]. Colon cancer patients with high TS expression are reportedly un-responsive to 5-FU and show poor prognosis^[34]. In contrast, tumoral expression of TS mRNA is associated with response to protracted infusions of 5-FU-based chemotherapy and survival in patients with disseminated colorectal cancer^[35]. Consistent with a report on colorectal tumors^[36], no relationship between TS expression and sensitivity to 5-FU in CCA *in vitro* was observed in our present study.

DPD is a major enzyme in the metabolism of 5-FU to an inactive metabolite. Some reports have suggested a negative correlation between 5-FU sensitivity and DPD activity in human stomach cancer cells^[37]. In patients with advanced colorectal cancer treated with 5-FU/leucovorin, patients who responded to the treatment exhibited low levels and a narrow distribution range of DPD mRNA

expression compared to the non-responder group^[32]. In the present study, DPD mRNA expression was apparently higher in the responders than in the non-responders. However, no clear relationship was found between DPD mRNA expression and 5-FU sensitivity.

In conclusion, we developed HDRA as an *in vitro* screening of the response of CCA tissues to 5-FU. We found that the level of OPRT mRNA may be a promising predictor of CCA sensitivity to 5-FU. Whether the OPRT mRNA level could be used as a predictor of the success of 5-FU chemotherapy in CCA patients needs to be confirmed further in patients.

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COMMENTS

Background

Cholangiocarcinoma (CCA) has a poor prognosis owing to the absence of effective treatments. Data from clinical studies have revealed that the response rate of CCA to 5-fluorouracil (5-FU) or 5-FU-based regimens is relatively poor. Therefore, a method to identify patients who may benefit from 5-FU is required.

Research frontiers

The histoculture drug response assay (HDRA) was used to determine the response of 23 CCA tissues to 5-FU. By determining the relative expression levels of several genes involved in the action and metabolism of 5-FU, they found that the orotate phosphoribosyl transferase (OPRT) mRNA level was significantly correlated with the response of CCA to 5-FU.

Innovations and breakthroughs

This is the first report to show a relationship between OPRT mRNA expression and the sensitivity of CCA tissues to 5-FU.

Applications

The HDRA may be useful as an *in vitro* test for determining the sensitivity of CCA tissues to anticancer agents. Based on the results from this study, OPRT mRNA expression may be a useful predictor of the chemosensitivity of CCA to 5-FU.

Peer review

This is a good descriptive study in which authors analyze whether expression of certain enzymes related to 5-FU metabolism predicts 5-FU chemosensitivity in CCA. This is an interesting paper of the basic research data of CCA using HDRA.

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Increased expression of chondroitin sulphate proteoglycans in rat hepatocellular carcinoma tissues

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Abstract

AIM: To investigate the expression of chondroitin sulphate proteoglycans (CSPGs) in rat liver tissues of hepatocellular carcinoma (HCC).

METHODS: Thirty male Sprague Dawley rats were randomly divided into two groups: control group ($n = 10$) and HCC model group ($n = 20$). Rats in the HCC model groups were intragastrically administered with 0.2% (w/v) N-diethylnitrosamine (DEN) every 5 d for 16 wk, whereas 0.9% (w/v) normal saline was administered

to rats in the control group. After 16 wk from the initiation of experiment, all rats were killed and livers were collected and fixed in 4% (w/v) paraformaldehyde. All tissues were embedded in paraffin and sectioned. Histological staining (hematoxylin and eosin and Toluidine blue) was performed to demonstrate the onset of HCC and the content of sulphated glycosaminoglycan (sGAG). Immunohistochemical staining was performed to investigate the expression of chondroitin sulphate (CS)/dermatan sulphate (DS)-GAG, heparan sulphate (HS)-GAG, keratan sulphate (KS)-GAG in liver tissues. Furthermore, expression and distribution of CSPG family members, including aggrecan, versican, biglycan and decorin in liver tissues, were also immunohistochemically determined.

RESULTS: After 16 wk administration of DEN, malignant nodules were observed on the surface of livers from the HCC model group, and their hepatic lobule structures appeared largely disrupted under microscope. Toluidine blue staining demonstrated that there was an significant increase in sGAG content in HCC tissues when compared with that in the normal liver tissues from the control group [0.37 ± 0.05 integrated optical density per stained area (IOD/area) and 0.21 ± 0.01 IOD/area, $P < 0.05$]. Immunohistochemical studies demonstrated that this increased sGAG in HCC tissues was induced by an elevated expression of CS/DS (0.28 ± 0.02 IOD/area and 0.18 ± 0.02 IOD/area, $P < 0.05$) and HS (0.30 ± 0.03 IOD/area and 0.17 ± 0.02 IOD/area, $P < 0.01$) but not KS GAGs in HCC tissues. Further studies thereby were performed to investigate the expression and distribution of several CSPG components in HCC tissues, including aggrecan, versican, biglycan and decorin. Interestingly, there was a distinct distribution pattern for these CSPG components between HCC tissues and the normal tissues. Positive staining of aggrecan, biglycan and decorin was localized in hepatic membrane and/or pericellular matrix in normal liver tissues; however, their expression was

mainly observed in the cytoplasm, cell membranes in hepatoma cells and/or pericellular matrix within HCC tissues. Semi-quantitative analysis indicated that there was a higher level of expression of aggrecan (0.43 ± 0.01 and 0.35 ± 0.03 , $P < 0.05$), biglycan (0.32 ± 0.01 and 0.25 ± 0.01 , $P < 0.001$) and decorin (0.29 ± 0.01 and 0.26 ± 0.01 , $P < 0.05$) in HCC tissues compared with that in the normal liver tissues. Very weak versican positive staining was observed in hepatocytes near central vein in normal liver tissues; however there was an intensive versican distribution in fibrosis septa between the hepatoma nodules. Semi-quantitative analysis indicated that the positive rate of versican in hepatoma tissues from the HCC model group was much higher than that in the control group (33.61% and 21.28%, $P < 0.05$). There was no positive staining in lumican and keratan, two major KSPGs, in either normal or HCC liver tissues.

CONCLUSION: CSPGs play important roles in the onset and progression of HCC, and may provide potential therapeutic targets and clinical biomarkers for this prevalent tumor in humans.

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Key words: Hepatocellular carcinoma; Proteoglycan; Chondroitin sulphate; Heparan sulphate; Keratan sulphate

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INTRODUCTION

Proteoglycans (PGs) are remarkably complex macromolecules consisting of one or more glycosaminoglycan (GAG) chains, which are covalently attached to different core proteins. Depending upon the structures of their GAG side-chains, PGs can be categorized as different groups such as heparan sulphate PGs (HSPGs), chondroitin/dermatan sulphate PGs (CS/DS PGs) and keratan sulphate PGs (KSPGs)^[1]. According to their different structures of the core proteins, CS/DS PGs can be further categorized as large aggregating PGs (aggrecan, versican), and small leucine-rich PGs (biglycan and decorin), which have been found to be widely expressed in many tissues including liver.

CS/DS PGs are major components of the cell surface and extracellular matrix (ECM)^[1,2]. They perform

a myriad of functions ranging from structural roles in the ECM to control of growth factor gradients and the regulation of certain cell processes such as cell adhesion, growth, receptor binding, migration, and interactions with other ECM constituents^[3-5]. These, especially the latter two functions, are largely mediated through specific interactions between their charged GAG chains and proteins such as growth factors, cytokines, chemokines, proteinases and their inhibitors^[6,7]. In addition, emerging data have revealed that the core proteins of PGs can also form complexes with other proteins, such as integrins and regulate their signaling^[7]. Because CS/DS PGs are at the crossroads of many signaling events and the abilities to regulate cell behaviors, they are being extensively investigated for their potential as therapeutic targets for cancers. What has become clear to date is that the functional effects of CS/DS PGs on cancers can range from stimulatory to inhibitory influences^[5], depending on the core protein and GAG structures^[1,8], the types and stages of cancers and the localizations of the tumors^[9].

PGs have been found widespread and abundant in liver tissues^[10]. Interestingly, in rats, fetal and early neonatal liver exhibits a completely different PG expression pattern when compared with adult liver tissues, where the synthesis of heparan sulphate (HS) comprises more than 80% and CS less than 5% of total GAGs^[11]. In contrast, CS is the major type of GAG synthesized in fetal liver, representing above 50% of total sulfated GAG (sGAG). Moreover, the overall PG production in fetal liver is enhanced two-fold when compared with that in the adult liver tissues. Thus, the synthesis of CS is elevated nearly 30-fold in fetal liver as compared with the adult counterpart^[12]. Immediately after birth CS formation decreases rapidly to the adult levels between the 10th and 15th day of postnatal life^[13], whereas the production of HS is almost unchanged during perinatal liver development due to a relatively low fractional synthesis of HS GAG in fetal liver^[12]. This phenomenon illustrates that CSPG synthesis and expression in liver tissues are cell-type dependent, and the undifferentiated liver cells trend to produce more CSPGs compared with the differentiated hepatocytes in the adult liver tissues.

Interestingly, the expression patterns of PGs including HSPGs and CSPGs are markedly changed under pathological conditions^[14]. For example, PGs are abnormally expressed in a wide variety of malignant tumors^[9]. In liver, hepatocellular carcinoma (HCC) and hepatic parenchyma adjacent to tumor contain abnormally higher concentrations of CS GAGs than the corresponding healthy tissues, but only with mild alteration in HS expression, indicating that the increase in CS GAGs is a characteristic abnormality in HCC tissues^[15-17]. This indicates that the expression of CSPGs plays a pivotal role in the occurrence, progression and metastasis of HCC, and therefore CSPG expression may be a potential marker and treatment target for HCC.

In this study, the expression patterns of different CSPGs including aggrecan, versican, decorin, biglycan in

the liver tissue from a rat HCC model established using N-diethylnitrosamine (DEN) were investigated using histological and immunochemical staining analyses.

MATERIALS AND METHODS

All chemicals were obtained from Xi'an Chemical Reagent Factory (Xi'an, China) unless otherwise stated and were of analytical grade or better.

Animal model preparation

The rat HCC model experimentation was approved by the Animal Ethics Committee, Medical School of Xi'an Jiaotong University. Use of animals in this study was in accordance with the China National Institute of Health publication 85-23 "Guide for Care and Use of Laboratory Animals" (National Research Council, 1996). Thirty male Sprague Dawley rats weighing 248.18 ± 12.32 g (3-4 mo old) were purchased from the Laboratory Animal Center of Medical School, Xi'an Jiaotong University. Rats were acclimated for 7 d before experimentation. Rats were randomly divided into two groups: control group ($n = 10$) and HCC model group ($n = 20$). Rats in the HCC model group were intragastrically administered with 0.2% (w/v) DEN (Sigma, United State) in saline (10 ng DEN per gram body weight) every 5 d for 16 wk, whereas 0.9% (w/v) normal saline was administered to the rats in the control group. All the rats had free access to distilled water. Electrolyte balance between the two groups was maintained through their common dietary food intake.

Sample collection

The weights of the rats were measured every week. After 16 wk from the initiation of the experiment, all the rats were killed under general anesthesia. Hepatic tissues were collected and fixed in 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS, 0.16 mol/L NaCl, 0.003 mol/L KCl, 0.008 mol/L Na_2HPO_4 , 0.001 mol/L KH_2PO_4 , pH 7.3) immediately. The tissues were embedded in paraffin and sectioned at 8 μm thickness.

Histological staining

Sections were deparaffinized and hydrated and either stained with hematoxylin and eosin or Toluidine blue as previously described^[18]. After dehydration, sections were mounted using DPX mounting medium (Thermo Fisher Scientific, Loughborough, United Kingdom). Representative regions were photographed under bright field optics using a Leica DMRB light microscope (Leica, Wetzlar, Germany) equipped with digital image acquisition.

Immunohistochemical staining

Immunohistochemical staining was performed using Mouse on Mouse™ Vectastain® Elite® ABC Kits (Vector labs, Peterborough, United Kingdom) according to the manufacturer's protocols. Briefly, sections were incubated with 0.3% (v/v) hydrogen peroxide for 30 min at room temperature to quench endogenous peroxidase activity. After blocking

with mouse immunoglobulin (Ig) blocking reagent for 1 h at room temperature, sections were incubated with rat anti-Versican (Abcam, Cambridge, United Kingdom), mouse anti-Aggrecan, mouse anti-Decorin, mouse anti-Keratocan, mouse anti-Lumican, mouse anti-Biglycan (in house) primary antibodies^[19] for 60 min, respectively. For the negative control, the primary antibody was replaced by PBS or 2 $\mu\text{g}/\text{mL}$ mouse or rat IgG (DAKO, Ely, United Kingdom). Sections were then incubated with biotinylated goat anti-mouse or rat IgG for 30 min at room temperature. After washing, sections were incubated with Mouse on Mouse™ ABC reagent for 5 min. Sections were then visualized using Vector® NovaRED™ kit (Vector labs, Peterborough, United Kingdom) according to the manufacturer's protocols. Cell nucleuses were counterstained with hemotoxylin. After dehydration, sections were mounted using DPX mounting medium. Representative regions were then photographed under bright field optics using a Leica DMRB bright field microscope (Leica, Wetzlar, Germany) equipped with digital image acquisition.

Semi-quantitative analysis for versican positive rate in liver tissues

Positive staining rate for versican in liver tissue sections was quantitatively analyzed. Sections of 4 liver tissues from 4 individual rats in each experimental group were taken for analysis. For each liver tissue specimen, three sections were randomly selected, and the positive and negative stained cells in these sections were counted using Image J software (NIH, United States). The percentage of positive cells was then calculated using the equation below: the percentage of positive cells = (positive stained cells)/(positive stained cells + negative stained cells) \times 100%.

Semi-quantitative analysis for the intensity of positive staining in tissues

The intensity of positive staining in tissue sections was analyzed by integrated optical density (IOD) using the Image-Pro Plus 5.1 software (Media Cybernetics, United States) as described previously^[20] with minor modification. Briefly, four 20 \times TIFF-format images from four individual rats in each group were analyzed in a blinded manner. All of the images were taken using the same microscope and camera sets. Image-pro Plus software was used to calculate the average IOD per stained area (μm^2) (IOD/area) for positive staining.

Statistical analysis

Data were presented as mean \pm SE, with samples derived from 4 animals in each group. D'Agostino and Pear omnibus normality test was used for normality and equal variances test. Student *t* test plus Bonferroni's post-test was carried out using GraphPad Prism 4.0 software (GraphPad Software Inc., California, United States). The comparisons of the staining results were performed only between rats from the HCC model and the control groups, but not between the tumor nodules and its adjacent normal liver

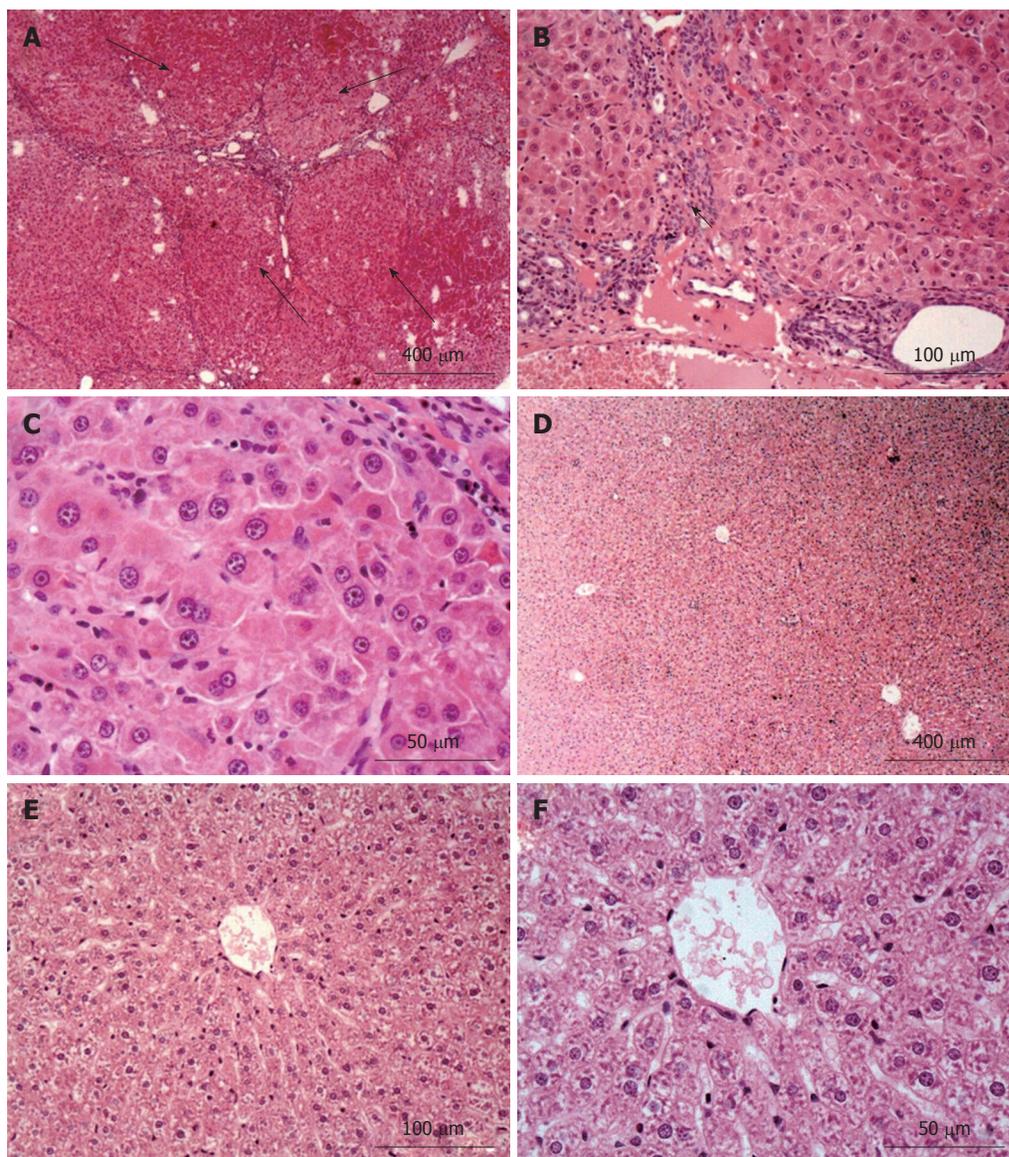


Figure 1 Hematoxylin and eosin staining results for liver tissues from the hepatocellular carcinoma model (A-C) and the control group (D-F). Normal liver structure and cell morphology were observed in the control group. However, apparent hepatoma nodules (long black arrows, A) and fibrosis (short black arrow, B) in the hepatocellular carcinoma model group were observed when compared with that in the control group.

tissues of rats from the same group. Differences were considered significant at $P < 0.05$.

RESULTS

Rat HCC model establishment

After 16 wk administration of DEN, malignant nodules were observed on the surface of the livers in the HCC model group but not in the control group. The average number of macroscopic nodules bigger than 3 mm and 5 mm on the surface of a single liver was 33.4 and 4.9, respectively, with the biggest nodule being approximately 1.5 cm × 1.0 cm × 0.8 cm (Table 1). H and E staining was used to identify and classify the cancerous nodules pathologically according to Edmondson *et al*^[21]. As expected, the normal hepatic lobule structure was disrupted and hepatoma nodules (long black arrows) were evident in the tis-

Table 1 Number and size of malignant nodules in rat livers

Group	<i>n</i>	Nodules ≥ 3 mm	Nodules ≥ 5 mm	The biggest nodule (mm ³)
Control group	10	0	0	0
Model group	14	33.4 ± 7.9	4.9 ± 1.9	122.8

sues from the HCC model group (Figure 1A), which were separated by fibrosis septa (short black arrows, Figure 1B), suggesting fibrosis formation around the tumors. The differentiation of HCC cells was also investigated according to method described by Edmondson. All of the cancer cells in the HCC model group were classified as grade III, and there was no hepatic plate-like structure present in the tumor tissues (Figure 1C). In contrast, there was no evidence of macroscopic tumor nodules in the livers from

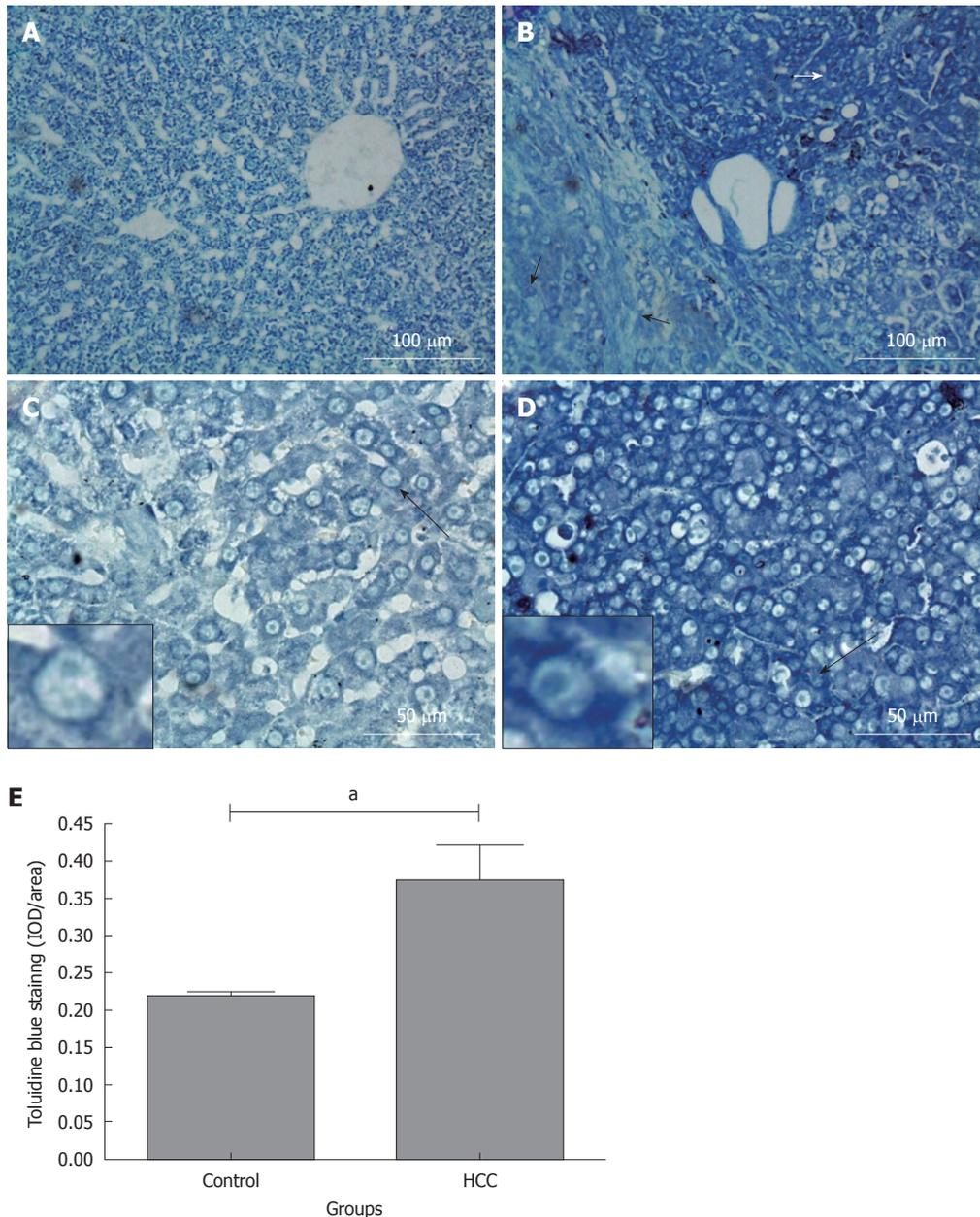


Figure 2 Toluidine blue staining in rat liver tissue sections. Rats were treated with N-diethylnitrosamine for 16 wk to establish a hepatocellular carcinoma (HCC) animal model. Sulphated glycosaminoglycan content in tissues were stained with Toluidine blue. A and C: Control group; B and D: HCC model group. Long black arrows: The cells are magnified in the small boxes. Short white arrow: Hepatoma tissues with intensive Toluidine blue staining; short black arrow: Weaker Toluidine blue staining fibrosis and “relative normal” liver tissues adjacent to the hepatoma nodules; E: Comparison of average integrated optical density (IOD) of toluidine blue staining in liver tissue between control and HCC model group ($^*P < 0.05$). IOD/area: Integrated optical density per stained area.

rats in the control group (Figure 1D), where a normal morphology was observed for hepatic cells, composing a normal liver tissue structure (Figure 1E and F). These results indicated the successful establishment of HCC in rats treated with DEN.

Increased sGAG content in HCC tissues

The contents of sGAG were investigated using Toluidine blue staining, which was evident in liver tissues from both control (Figure 2A and C) and HCC model groups (Figure 2B and D). Positive staining was found in the cytoplasm, cell membrane and/or pericellular matrix

(Figure 2C and D; the cells identified by long black arrows are magnified in the small boxes). Noticeably, Toluidine blue staining in hepatoma tissues (white short arrow, Figure 2B) was stronger than that in the fibrosis and “relative normal liver tissue” (black short arrows, Figure 2B) adjacent to the tumor nodules. Semi-quantitative IOD analysis indicated that there was more Toluidine blue positive staining in the tissues from the HCC model group when compared with the tissues from the control group (0.37 ± 0.05 IOD/area and 0.21 ± 0.01 IOD/area, $P < 0.05$, Figure 2E). This finding demonstrates elevated sGAG content in HCC tissues compared to that in the normal liver tissues. To

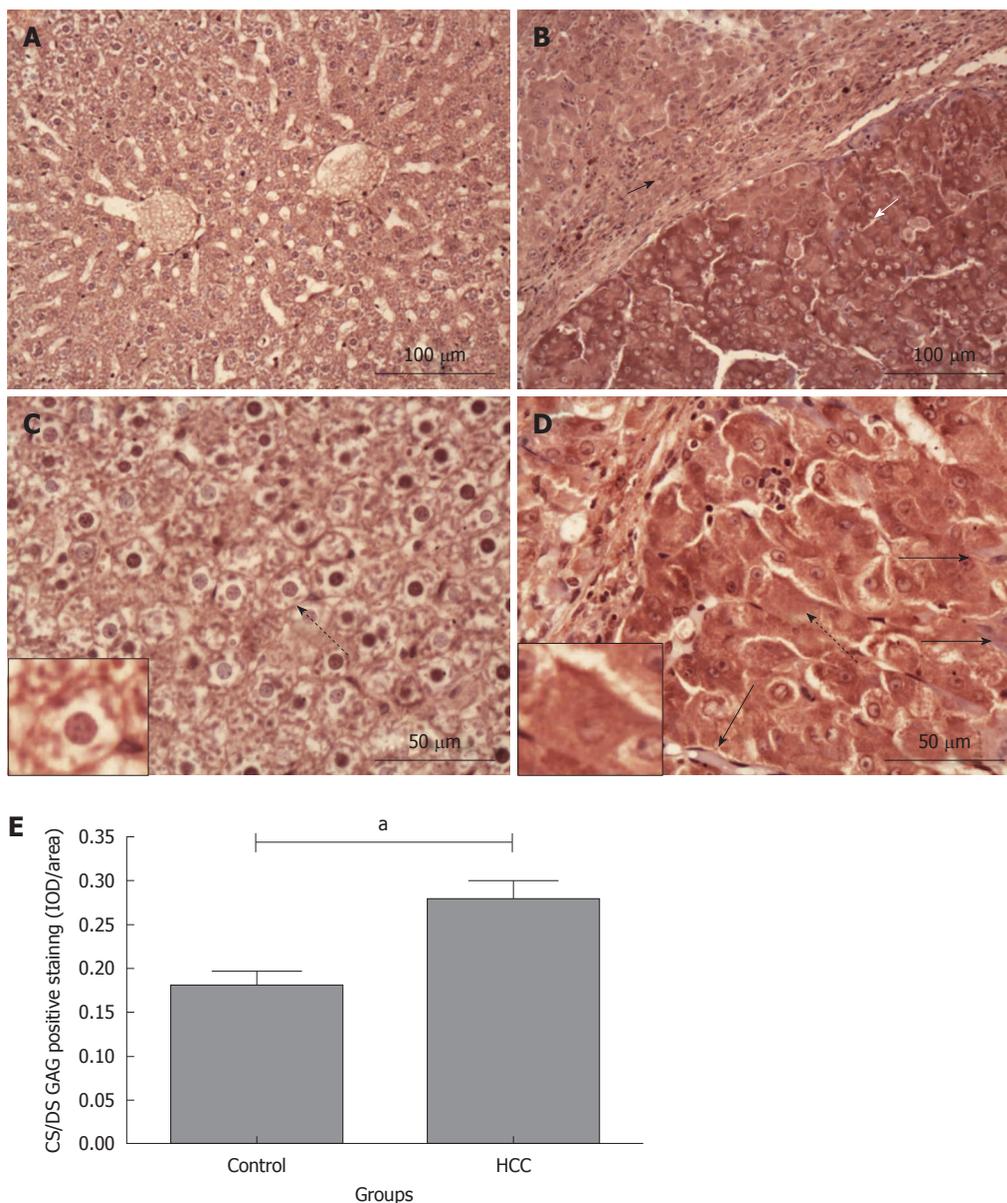


Figure 3 Chondroitin sulphate/dermatan sulphate glycosaminoglycan immunohistochemical staining in rat liver tissues. Chondroitin sulphate (CS)/dermatan sulphate (DS) sulphated glycosaminoglycan (sGAG) content in liver tissues was stained using 2B6 (+) antibody (dark red). A and C: Control group; B and D: Hepatocellular carcinoma (HCC) model group. Long black arrows: Perisinusoidal cells negatively stained by 2B6 antibody; dotted arrows: The cells are magnified in the small boxes; short white arrow: Hepatoma tissues with intensive CS/DS GAG staining; short black arrow: Weaker CS/DS GAG staining in fibrosis and "relative normal" liver tissues adjacent to the hepatoma nodules; E: Comparison of the average integrated optical density (IOD) in CS/DS GAG positive staining in liver tissues between the control and HCC model groups ($P < 0.05$). IOD/area: Integrated optical density per stained area.

further identify the specific expression of sGAG in HCC model tissue, immunohistochemical staining for CS/DS, HS and keratan sulphate (KS) GAG were performed.

Increased CS/DS GAG expression in HCC tissues

The expression of CS/DS GAG chains in normal and HCC tissues was investigated using 2B6 (+) monoclonal antibody^[11,19]. As shown in Figure 3, positive staining for 4-sulphated CS/DS GAGs was observed on cell membranes and/or pericellular matrix in the normal liver tissues from the control group (Figure 3A and C; the cell identified with dotted arrow is magnified in the small box). In contrast, 2B6 (+) positive staining was observed in the cytoplasm, cell membrane and/or pericellular ma-

trix (Figure 3B and D; the cell pointed with dotted arrow is magnified in the small box). The expression of 4-sulphated CS/DS GAGs was variable across the HCC tissues, with a stronger staining in the hepatoma nodules (white short arrow, Figure 3B) but a relative weaker staining in its adjacent tissues such as fibrous septa and relatively normal hepatocytes (black short arrow, Figure 3B). Interestingly, these variable distribution patterns in 4-sulphated CS/DS were also observed inside the hepatoma nodules, i.e., several perisinusoidal cells were negatively stained in CS/DS expression (Figure 3D, black long arrows), although most of the hepatoma cells were positively stained. Semi-quantitative IOD analysis for the intensity of positive staining indicated that there was more 4-sulphated CS/DS expres-

sions in the hepatoma nodules when compared with that in the normal tissues from the control group (0.28 ± 0.02 IOD/area and 0.18 ± 0.02 IOD/area, $P < 0.05$, Figure 3E).

Increased HS GAG staining in HCC tissues

We also investigated the expression of HS GAG chains in the tissues from rats in the control and HCC model groups. Similar to the CS/DS GAG staining, HS positive staining was evenly distributed across the normal liver tissue sections from the control group (Figure 4A and D) and mainly localized on the cell membrane and/or pericellular matrix (Figure 4D; the cell identified with a black short arrow is shown at a higher magnification in the small box). However, this “normal tissue” HS distribution pattern was altered and became uneven in the tissues obtained from rats in the HCC model group. In some hepatoma nodules, intensive HS positive staining was observed in the hepatoma cytoplasm, cell membrane, pericellular matrix and even in cell nuclei (Figure 4E; the cell identified with a black short arrow is shown at a higher magnification in the small box). There was no HS positive staining in the fibrous tissue septa (black long arrows; Figure 4B and E). Semi-quantitative IOD analysis indicated that there was a stronger HS staining in these hepatoma nodules than that in the normal liver tissues from the control group (0.30 ± 0.03 and 0.17 ± 0.02 , $P < 0.01$, Figure 4G). However in some hepatoma nodules, a relative weaker HS positive staining was observed on the hepatoma cell membrane and/or pericellular matrix (Figure 4C and F; the cell identified with a black short arrow is shown at a higher magnification in the small box), similar to that observed in the normal liver tissues from the control group. In this case, there was no significant difference in the average density of HS positive staining between the HCC model and the control groups ($P = 0.1169$).

KS GAG expression was not altered in HCC tissues

KS is another important sGAG side chains attached to the core proteins of several matrix PGs. In contrast to the CS and HS GAG staining described above, the positive staining of KS GAG chains was weak and there was no difference between control and HCC model groups (data not shown).

Collectively, the results described above demonstrate that there is a significant elevation in the expression of CS/DS and HS but not KS GAG chains in the HCC model tissues when compared with the normal liver tissues. Therefore, we further investigated the expression patterns of different PG core proteins with CS GAGs, including aggrecan, versican, biglycan and decorin.

Increased aggrecan expression in HCC tissues

Aggrecan is a common CSPG found in many musculoskeletal tissues especially in hyaline articular cartilage. Interestingly, aggrecan expression in the liver at the gene level has been reported previously^[14,22]. In this study, aggrecan expression in liver tissues was immunohistochemically investigated using a monoclonal antibody [anti-IGD (6B4)]

recognizing the interglobular domain of aggrecan core protein^[19,23]. Positive staining for aggrecan was observed in both control and HCC model groups (Figure 5A and B). However, their distribution patterns were different. In the control group where aggrecan positive staining was mainly localized on cell membrane and/or pericellular matrix (Figure 5C; the cell identified with a black short arrow is magnified in the small box). In contrast, there was more intensive aggrecan positive staining in hepatoma cytoplasm, cell membrane and/or pericellular matrix in the tissues from the HCC model group (Figure 5D; the cell identified with a black short arrow is magnified in the small box). Noticeably, there was no or very weak aggrecan positive staining in the fibrous tissue septa between hepatoma nodules (black long arrows, Figure 5B and D). Interestingly, the differences in staining intensity and patterns for aggrecan described above were also observed between the hepatoma tissues and its adjacent “relative normal liver tissues” (Figure 5E; the areas inside the black or red boxes are magnified on the left column). Semi-quantitative IOD analysis indicated that there was more aggrecan positive staining in the tissues from the HCC model group when compared with that in the control group (0.43 ± 0.01 IOD/area and 0.35 ± 0.03 IOD/area, $P < 0.05$, Figure 5F). These results demonstrated that DEN-induced HCC in rat liver increases the aggrecan expression in cells at the protein level, suggesting that there may be a correlation between HCC and aggrecan expression.

Increased versican expression in HCC tissues

Versican is another member of the large aggregating CSPGs family and its expression in rat liver tissues was also investigated by immunohistochemical staining. In contrast to the aggrecan staining, most of the hepatocytes were negatively stained for versican in the liver tissues from the control group (Figure 6A and D). However, a weak versican positive staining was observed on the cell membrane and/or pericellular matrix of some hepatocytes around the central vein (Figure 6D; the cell identified with a black short arrow is magnified in the small box). In the liver tissues from rats in the HCC model group, versican positive staining was observed in some hepatoma cells (Figure 6B and E), and mainly localized in the cytoplasm, cell membrane and/or pericellular matrix (Figure 6E; the cell identified with a black short arrow is magnified in the small box). Statistical analysis indicated that versican positive staining rate in the HCC model group was much higher than that in the control group ($33.61\% \pm 4.90\%$ and $21.28\% \pm 1.79\%$, $P < 0.05$, Figure 6G), although a large number of cells were still negatively stained. Interestingly, the strongest versican positive staining was observed in the pericellular matrix of fibrous tissue septa and portal areas (Figure 6C and F), indicating a different versican distribution pattern between the control and HCC model groups.

Increased biglycan expression in HCC tissues

In normal liver tissues, a moderate positive staining of biglycan was evenly distributed on hepatic membrane

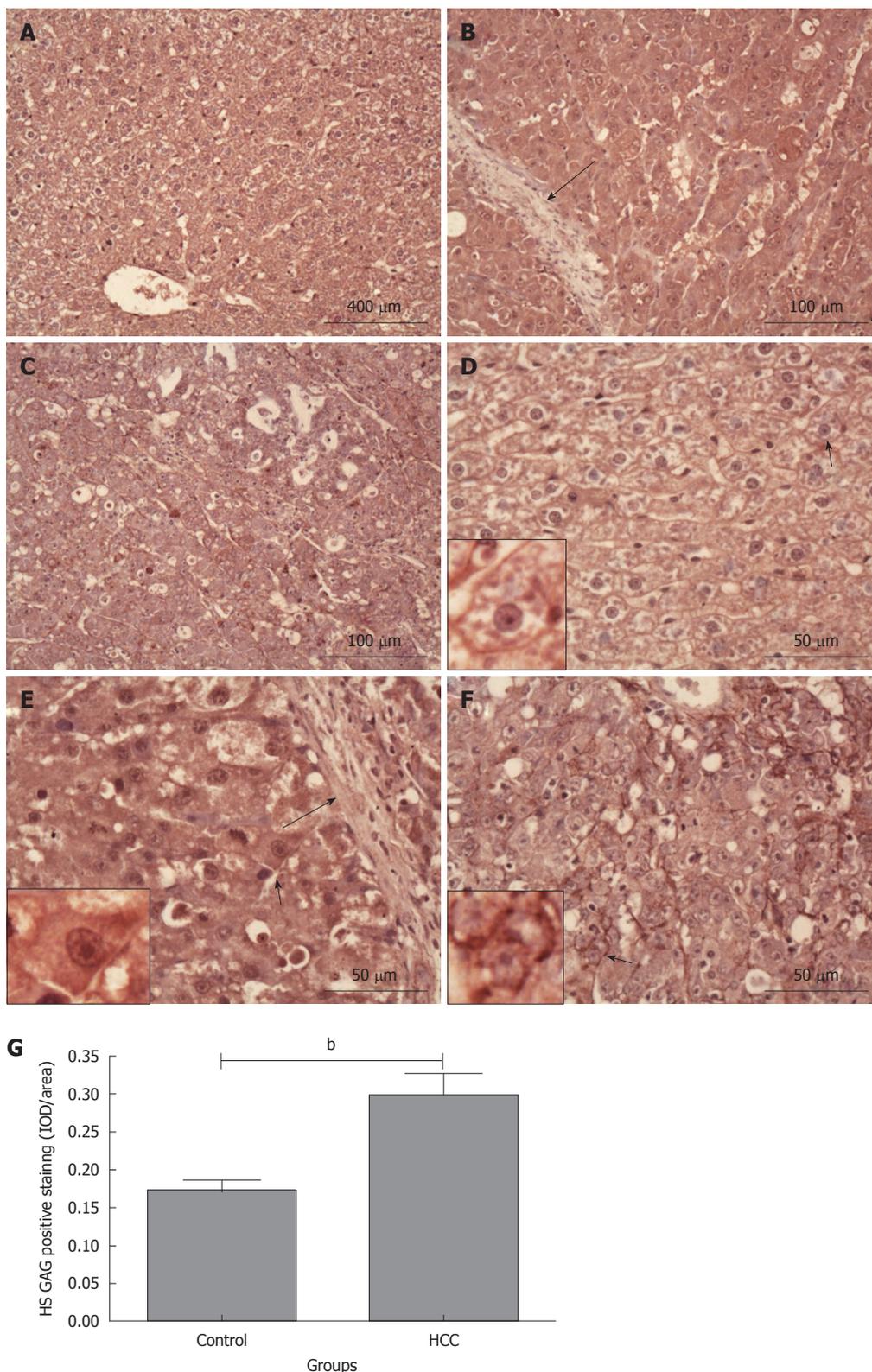


Figure 4 Heparan sulphate glycosaminoglycan staining in rat liver tissues. Heparan sulphate (HS) glycosaminoglycan (GAG) content in tissue was stained using 10E4 antibody (dark red). A and D: Control group; B and E: Hepatocellular carcinoma (HCC) tissues with intensive HS GAG staining; C and F: HCC tissues with relative weaker HS GAG staining. Long black arrows: Fibrous tissue septa; short black arrows: The cells are magnified in the small boxes; G: Comparison of the average integrated optical density (IOD) in HS GAG positive staining in liver tissues between the control and HCC model groups ($P < 0.01$). IOD/area: Integrated optical density per stained area.

and/or pericellular matrix in the liver tissues of rats from the control group (Figure 7A). There was limited positive

staining in hepatocyte cytoplasm and nuclei (Figure 7C; the cell identified with a black short arrow is magnified in

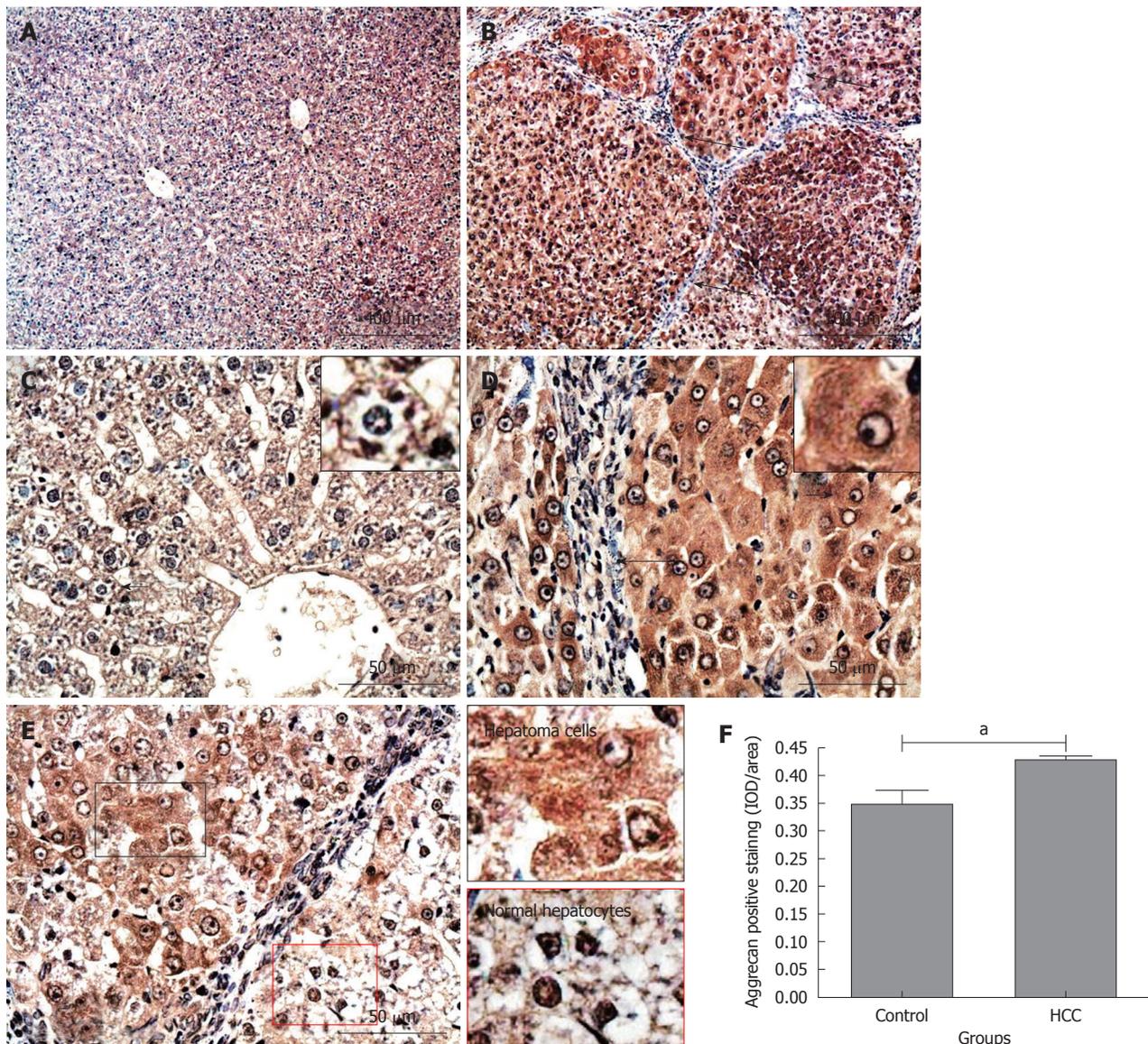


Figure 5 Immunohistochemical staining for aggrecan in rat liver tissues. Aggrecan positive staining was dark red. A and C: Control group; B, D and E: Hepatocellular carcinoma (HCC) model group. Long black arrows: Fibrous tissue septa; short black arrows: The cells are magnified in the small boxes; E: Areas in black and red boxes are magnified in left column; F: Comparison of the average integrated optical density (IOD) in aggrecan positive staining in liver tissues between the control and HCC model groups ($^a P < 0.05$). IOD/area: Integrated optical density per stained area.

the small box). In contrast, strong biglycan staining was observed in almost all hepatoma cells in tumor nodules from the HCC model group (Figure 7B). The staining was not only on the cell membrane and/or pericellular matrix but also in the cytoplasm (Figure 7D; the cell identified with a white short arrow is magnified in the small box). Interestingly, the differences in staining intensity and patterns for biglycan were also observed between hepatoma cells and its adjacent “relatively normal hepatocytes” (Figure 7E; the areas inside the black or red boxes are magnified on the left column). Semi-quantitative IOD analysis showed that there was significantly more biglycan expression in HCC tissues when compared with that in the normal liver tissues (0.32 ± 0.01 and 0.25 ± 0.01 , $P < 0.001$, Figure 7F). There was no intensive biglycan staining in the portal areas and fibrous tissue septa between hepatoma nodules (white long arrow, Figure 7D).

Increased decorin expression in HCC tissues

Similarly to biglycan, decorin positive staining was evenly distributed on hepatic cell membrane and/or pericellular matrix across the whole liver tissue sections from the control group (Figure 8A and C; the cell identified with a black short arrow is magnified in the small box). In the HCC model tissues, intensive decorin positive staining was observed in almost all hepatoma cells (Figure 8B), mainly localized in the cytoplasm, cell membrane and/or pericellular matrix (Figure 8D; the cell identified with a black short arrow is magnified in the small box). This difference described above was also observed between hepatoma cells and its adjacent “relatively normal hepatocytes” (Figure 8E; the areas inside the black or red boxes are magnified on the left column). Semi-quantitative IOD analysis indicated that there was significantly more decorin expression in hepatoma nodules when compared

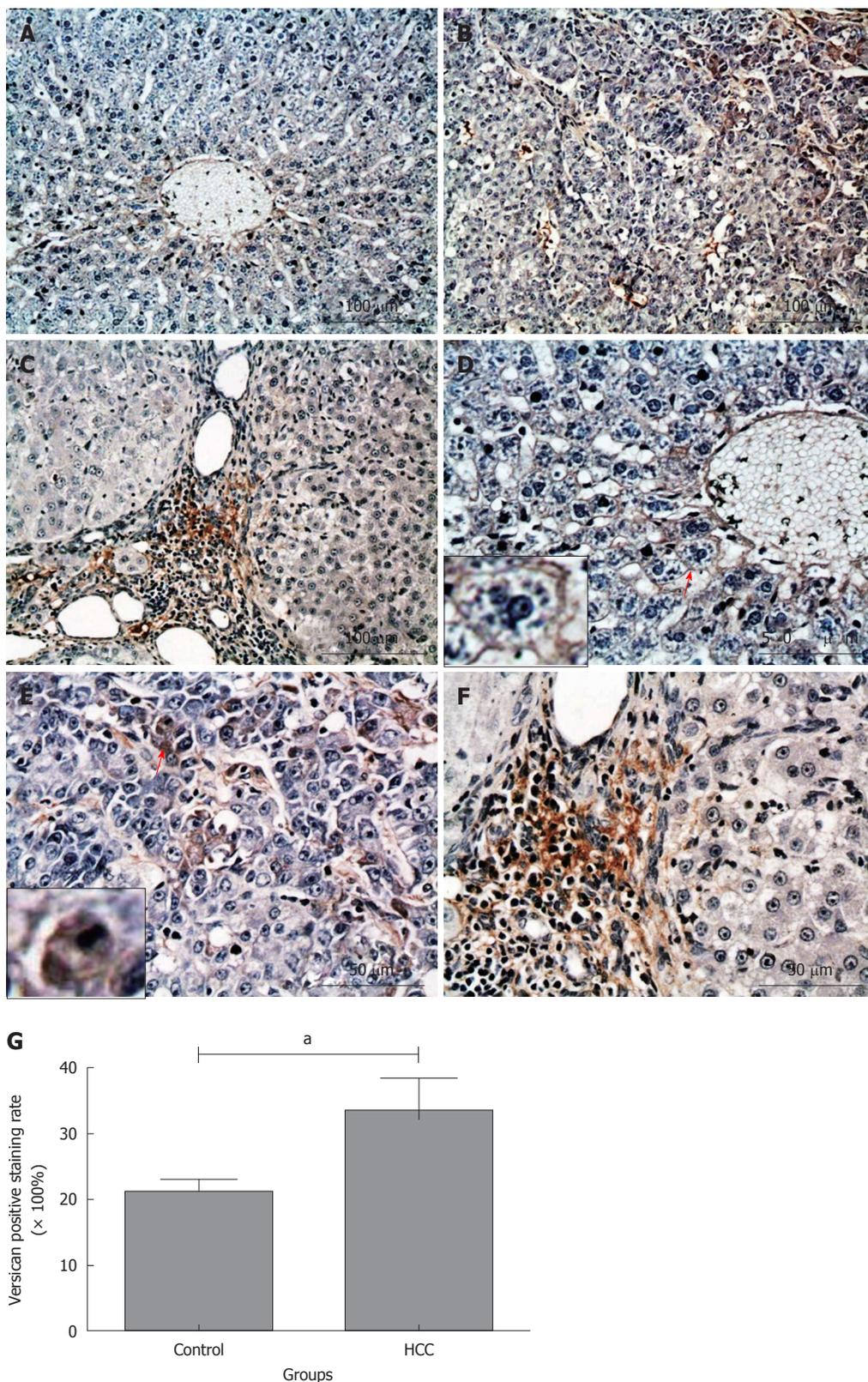


Figure 6 Immunohistochemical staining for versican in rat liver tissues. Versican positive staining was dark red. A-F: Most of hepatocytes were negatively stained in control group (A and D), whereas more hepatoma cells in hepatocellular carcinoma (HCC) nodules were positively stained (B and E); however, the strongest versican positive staining was observed in the fibrosis septa between hepatoma nodules (C and F). Short red arrows: The cells are magnified in the small boxes; G: Comparison of the positive rate for versican staining in liver tissues between the control and HCC model groups. ^aP < 0.05.

with that in the normal liver tissues (0.29 ± 0.01 and 0.26 ± 0.01 , $P < 0.05$, Figure 8F). Interestingly, there was no

decorin positive staining in the portal areas and fibrous tissue septa between the tumor nodules (Figure 8B and D).

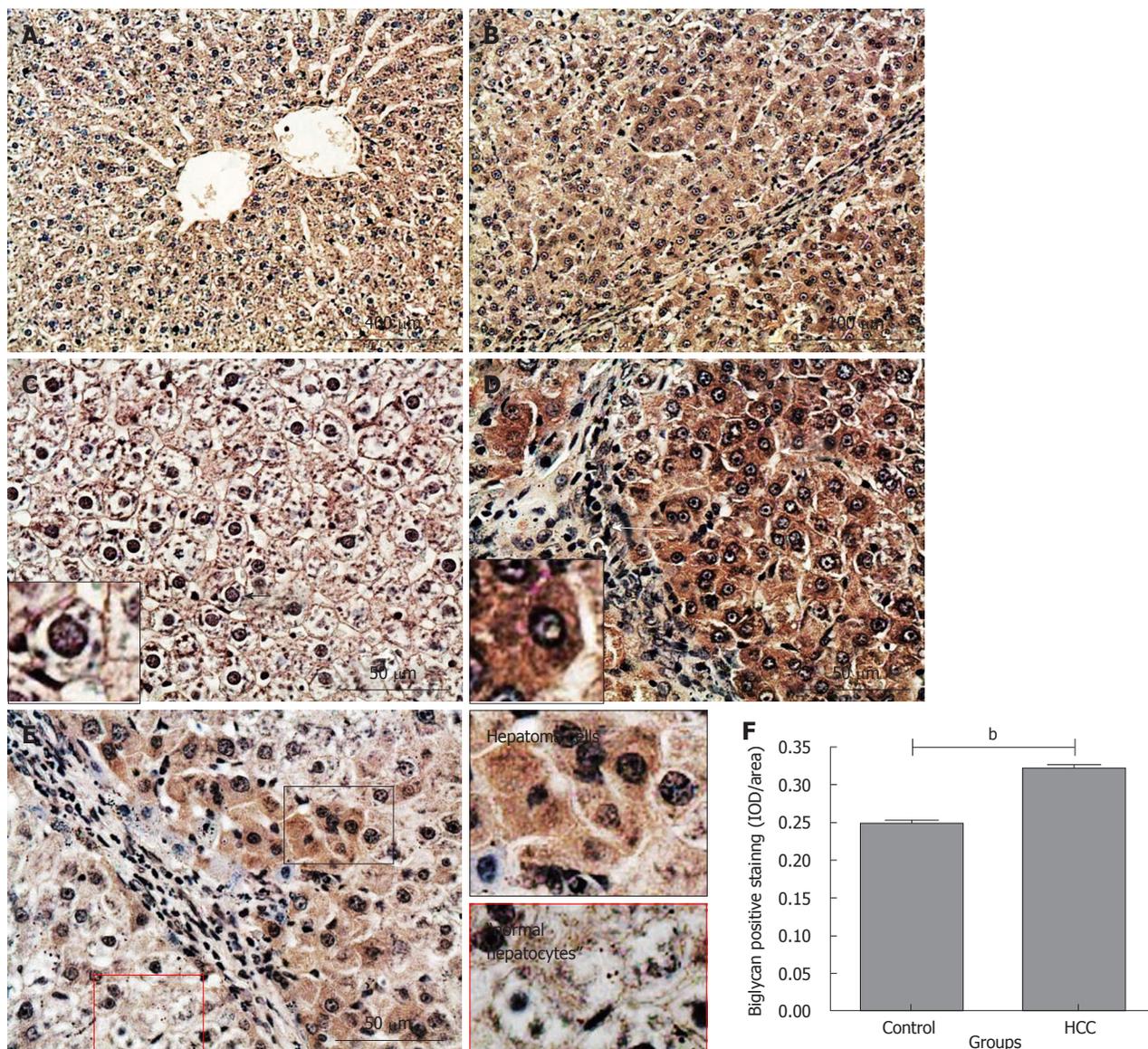


Figure 7 Immunohistochemical staining for biglycan in rat liver tissues. Biglycan positive staining was dark red. A and C: Control group; B, D and E: Hepatocellular carcinoma (HCC) model group. Long white arrow: Fibrous tissue septa; short black and white arrows: The cells are magnified in the small boxes; E: Areas in black and red boxes are magnified in left column; F: Comparison of the average integrated optical density (IOD) in biglycan positive staining in liver tissues between the control and HCC model groups ($^bP < 0.001$). IOD/area: Integrated optical density per stained area.

There was no positive staining for keratocan or lumican in rat liver tissues

The expression of keratocan and lumican was also investigated. Consistent with the KS negative staining results, there was no or very weak keratocan or lumican staining in these liver tissues, either from the control or HCC model group (data not shown).

DISCUSSION

The abnormally high expression of CS GAGs in HCC tissues has been known for a long time^[16,17] although little is known about the biological mechanisms underlying their increased presence. Interestingly, an accumulation of CS GAG expression has also been observed in other physiological and pathological processes involved in liver development and metabolism. For example, in neonatal

liver where premature hepatocytes (hepatic stem cells) still remain as an undifferentiated phenotype, much higher CS GAGs were observed when compared with that in the postnatal liver tissues^[13]. Similarly, there is a transient accumulation of CS GAGs during liver regeneration after partial hepatectomy^[13] and active fibrosis^[24]. All of these examples demonstrate that CSPGs are involved in embryogenesis, regeneration and carcinogenesis of liver. One of the crucial events occurring within these biological processes is the epithelial mesenchymal transition (EMT), a complex molecular and cellular transformation of cell phenotype from differentiated characteristics (mature epithelial cells) to undifferentiated mesenchymal (stem/progenitor cells) features. During this process, cells acquire motility, enhanced migratory capacity/invasiveness^[25], and become more stem cell-like^[26]. Interestingly, increased production of ECM components such as CS

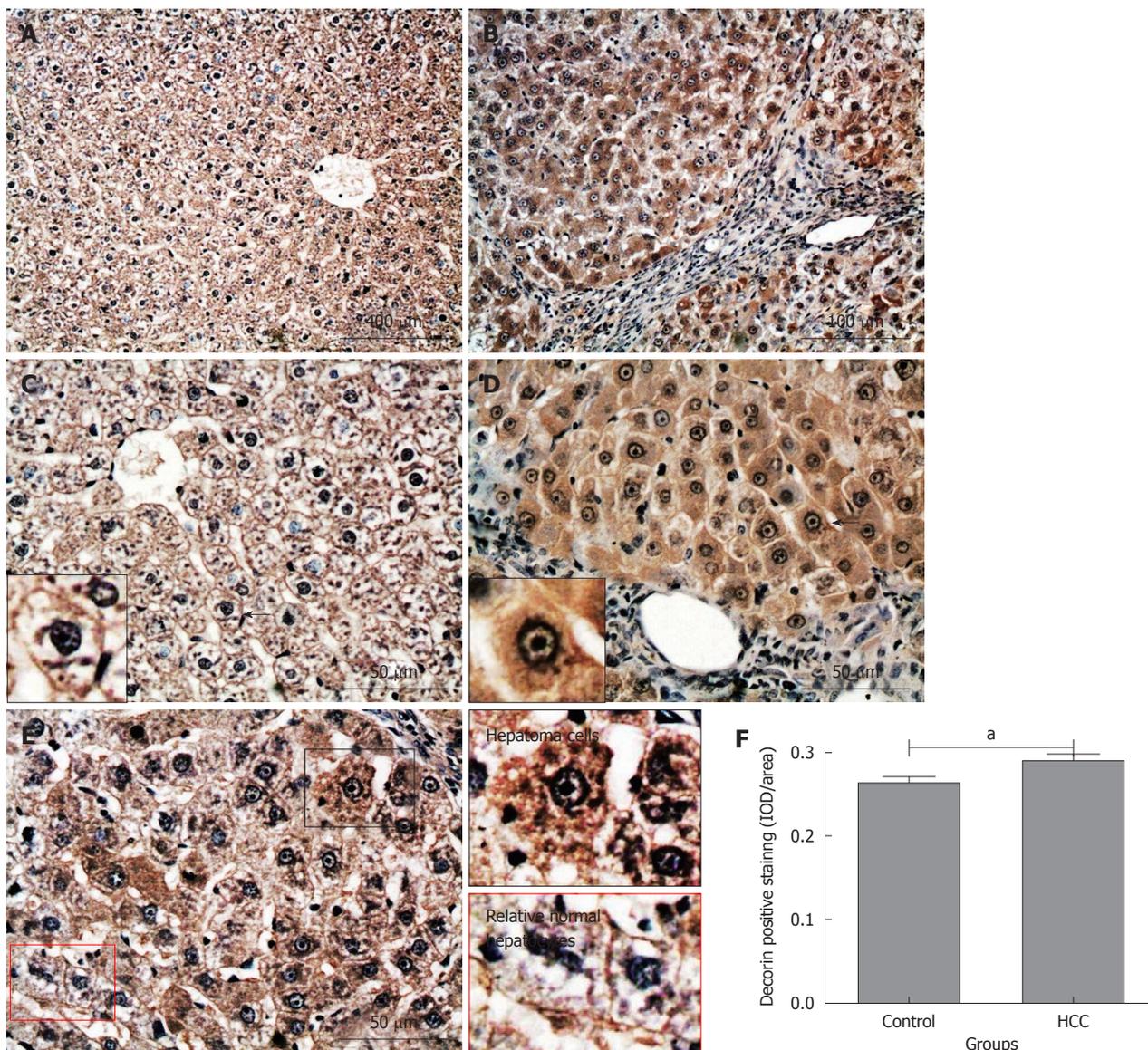


Figure 8 Immunohistochemical staining for decorin in rat liver tissues. Decorin positive staining was dark red. A and C: Control group; B, D and E: Hepatocellular carcinoma (HCC) model group. Short black arrows: The cells are magnified in the small boxes; E: Areas in black and red boxes are magnified in left column; F: Comparison of the average integrated optical density (IOD) in decorin positive staining in liver tissues between the control and HCC model groups ($^*P < 0.05$). IOD/area: Integrated optical density per stained area.

GAGs has been observed during this process^[8,27], demonstrating that *de novo* CSPG expression may play a pivotal role during the cell phenotype transformation, notably initiating the development of HCC. However, it is still unclear what the precise CSPG expression patterns are in the hepatoma cells and their relationship with HCC development.

In this study, the Toluidine blue staining results indicated that there was more sGAG content in HCC tissues than that in the normal liver tissues from the control group, which is consistent with previous studies^[16,17]. Our further immunostaining with CS/DS, HS and KS antibodies demonstrated that there was a significant increase in CS/DS and HS but not KS GAG expression in HCC tissues. The weak expression of KS GAG in both normal liver and HCC tissues is consistent with the very low staining patterns for keratan and lumican, two major

KSPGs expressed in the other tissues. A recent study performed on human HCC tissues^[28], indicated that KSPGs were not involved in HCC development. Therefore, the increased sGAG content in HCC tissues must be induced by the enhanced production and accumulation of CS/DS and/or HSPGs, which is confirmed by our CS/DS and HS GAG staining. Previous studies have reported the expression of several HSPGs including glypican-3^[29], syndecan-4^[30] and perlecan^[31] are increased in HCC tissues, which coincides with our HS staining results. However, little is known for the expression patterns of specific CSPGs in HCC, therefore our following investigation was mainly focused on CSPG expression.

Aggrecan gene expression has been previously reported in liver tissues^[14,22]. Our results demonstrate, for the first time at a protein level, the positive expression of aggrecan in liver tissues, which was mainly localized on

the cell membrane and/or pericellular matrix in normal hepatocytes. However, in the HCC model group there was much stronger aggrecan staining in the cytoplasm, the cell membranes and/or pericellular matrix in HCC hepatoma cells, indicating an elevated aggrecan production and accumulation in HCC tissues. The function of this increased aggrecan presence is not clear but a previous study has suggested that the expression of aggrecan in tumors may be a result of EMT^[27]. Moreover, aggrecan production is mediated by different growth factors such as transforming growth factor β in hepatocytes^[22,32], which has been identified as a promoter for both HCC-related fibrosis and angiogenesis^[32,33]. Interestingly, there was very low aggrecan expression in the fibrous tissue septa between hepatoma nodules, consistent with previous studies showing that the formation of fibrous septa arises from myofibroblasts^[34], which have a low expression of CS/DS PGs^[35].

In contrast to the increased aggrecan expression, versican content in hepatoma cells was variable with the most intensive staining mainly localized in the ECM of fibrous septa between hepatoma nodules. This finding is novel as little is known about versican expression in HCC tissues. Interestingly, versican expression was observed around the central veins and portal areas, illustrating there may be a close relationship between versican and HCC metastases. This is consistent with previous studies, where an elevated expression of versican was observed in the ECM of other tumor tissues including breast^[36] and prostate^[37], and correlated with metastases^[38]. The mechanism as to how versican promotes metastases is not clear. However, the deposition of versican in the tumor stroma, particularly in the hyaluronic acid rich region, will lead to the structural aggregation of tumor matrix and modulation of cellular attachment and motility, therefore supporting cancer cell growth, proliferation, migration and differentiation, all processes vital for tumor development and metastases^[5,36].

Both biglycan and decorin are the members of the small leucine-rich proteoglycan family. They are usually associated with growth factor binding^[4] and collagen fibrillogenesis^[39,40]. Therefore, it is not surprising that an elevated biglycan and decorin expression was observed during liver fibrosis^[10,41,42]. However, our results showed that the major positive staining of decorin and biglycan was localized in the hepatoma cells instead of fibrosis septa between hepatoma nodules, suggesting that the expression of biglycan and decorin may play different roles in HCC occurrence and liver fibrosis. The association between biglycan and HCC has not been previously reported; however, elevated expression of biglycan may correlate with the aggressiveness and poor prognosis of the other cancers^[43]. Varied evidence for the changes in decorin expression in HCC tissues has been previously reported. Kovalszky *et al.*^[17] and Lai *et al.*^[44] have found that decorin expression was elevated in HCC tissues. In contrast, Miyasaka *et al.*^[45] showed that there was a decline in decorin gene expression in HCC. The difference may arise from the different stages of HCC, as previ-

ous studies have showed that decorin can be either pro-angiogenic or anti-angiogenic in tumors^[46]. The precise contributions of biglycan and decorin metabolism during HCC occurrence and metastases have not yet been elucidated. However, the ability of these proteins to interact with the other matrix components and induce ECM remodeling^[47] as well as increasing cell proliferation and migration^[48] highlights them as an important PG subsets involved in tumor formation and metastases. Alternatively, the increased biglycan and decorin expression may also be a consequence of EMT of hepatoma cells, because a recent study reported higher biglycan and decorin expression levels during a Ras-induced EMT in MDCK cells^[49]. Clearly, further studies for the roles of biglycan and decorin in hepatocarcinogenesis are warranted.

Much less is known about the precise role that CSPGs play in the HCC induction and metastases. CSPGs are ubiquitous components of ECM and cell surface, therefore can predominantly interact with a wide variety of key molecules, such as growth factors, cytokines, chemokines, adhesion molecules, and lipoproteins. These interactions regulate biological processes including signaling, cell differentiation, cell-cell or cell-matrix interactions and morphogenesis^[50]. In this study, using histological staining, we found a significant increase in the sGAG content in DEN-induced HCC tissues when compared with the normal rat liver tissues from the control group and this increased sGAG content in tumor tissue was mainly induced by elevated expression in CS/DS and HS but not KS GAGs. We further demonstrated that the expression of several CSPGs including aggrecan, versican, biglycan and decorin was elevated in HCC tissues. To our knowledge, this is the first systematical study demonstrating the elevated CSPGs expression in HCC tissues. The experimental data shown here expands our knowledge of the relationships between CS/DS PGs and HCC, and other liver diseases.

COMMENTS

Background

Proteoglycans (PGs) are macromolecules consisting of one or several polysaccharide chains of the glycosaminoglycan (GAG), which covalently attached to a variety of core proteins. They are widely expressed in cells and extracellular matrix in various tissues including liver. According to the difference in GAG side chains, PGs can be categorized as chondroitin sulphate PG (CSPG) and heparan sulphate PG, etc. PGs have been found to play a critical role in different malignant tumor progression. However, the effect of PGs on cancer is variable, which can range from stimulatory to inhibitory, depending on their core proteins and GAG types, the sources and stages of cancers and the tumor localizations.

Research frontiers

Previous studies have shown that the expression of CS GAG was increased in hepatocellular carcinoma (HCC) tissues, and inhibition of CS GAG expression in HCC cell line partially abrogates cell ability of migration *in vitro*. This illustrated that CSPGs may play a pivotal role in the occurrence, progression and metastasis of HCC and thereby they may be used as potential markers and treatment target for HCC. The hotspot in this area is their temporal and spatial expression and the mechanism how they are involved in the onset, development and metastasis of HCC.

Innovations and breakthroughs

The authors investigated the expression pattern of different CSPGs including aggrecan, versican, decorin, biglycan in the liver tissues from a rat HCC model

established using N-diethylnitrosamine (DEN). This is the first systematical study demonstrating the elevated CSPGs expression in HCC tissues.

Applications

The study results suggest that the CSPGs could be potential therapeutic targets and clinical biomarkers for HCC in humans in the future.

Terminology

PG is a kind of macromolecule units consists of a "core protein" with one or more covalently attached GAG chain(s); GAGs are long unbranched polysaccharides consisting of a repeating disaccharide unit; Chondroitin sulfate is a sulfated GAG composed of a chain of alternating sugars (N-acetylgalactosamine and glucuronic acid).

Peer review

This is a good descriptive study in which authors investigate the expression of PGs in rats with DEN-induced HCC. The results are interesting and suggest that CSPGs are potential therapeutic targets and clinical biomarkers for HCC.

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Effects of *Lactobacillus plantarum* on gut barrier function in experimental obstructive jaundice

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Abstract

AIM: To investigate the mechanisms of *Lactobacillus plantarum* (*L. plantarum*) action on gut barrier in pre-operative and postoperative experimental obstructive jaundice in rats.

METHODS: Forty rats were randomly divided into groups of sham-operation, bile duct ligation (BDL), BDL + *L. plantarum*, BDL + internal biliary drainage (IBD), and BDL + IBD + *L. plantarum*. Ten days after *L. plantarum* administration, blood and ileal samples were collected from the rats for morphological examination, and intestinal barrier function, liver function, intestinal oxidative stress and protein kinase C (PKC) activity

measurement. The distribution and expression of the PKC and tight junction (TJ) proteins, such as occludin, zonula occludens-1, claudin-1, claudin-4, junction adhesion molecule-A and F-actin, were examined by confocal laser scanning microscopy, immunohistochemistry, Western blotting, real-time fluorescent quantitative polymerase chain reaction assay.

RESULTS: *L. plantarum* administration substantially restored gut barrier, decreased enterocyte apoptosis, improved intestinal oxidative stress, promoted the activity and expression of protein kinase (BDL vs BDL + *L. plantarum*, 0.295 ± 0.007 vs 0.349 ± 0.003 , $P < 0.05$; BDL + IBD vs BDL + IBD + *L. plantarum*, 0.407 ± 0.046 vs 0.465 ± 0.135 , $P < 0.05$), and particularly enhanced the expression and phosphorylation of TJ proteins in the experimental obstructive jaundice (BDL vs BDL + *L. plantarum*, 0.266 ± 0.118 vs 0.326 ± 0.009 , $P < 0.05$). The protective effect of *L. plantarum* was more prominent after internal biliary drainage (BDL + IBD vs BDL + IBD + *L. plantarum*, 0.415 ± 0.105 vs 0.494 ± 0.145 , $P < 0.05$).

CONCLUSION: *L. plantarum* can decrease intestinal epithelial cell apoptosis, reduce oxidative stress, and prevent TJ disruption in biliary obstruction by activating the PKC pathway.

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Key words: *Lactobacillus plantarum*; Protein kinase C; Intestinal mucosal barrier; Phosphorylation; Obstructive jaundice

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INTRODUCTION

Biliary tract surgery in patients with obstructive jaundice is associated with high a morbidity and mortality rate^[1]. Evidence accumulated over the past several years indicates that the absence of bile in the gastrointestinal tract promotes bacterial overgrowth and increases intestinal permeability, leading to significant translocation of bacteria and endotoxin following bile duct obstruction^[2,3]. The mechanism underlying the increased intestinal permeability in obstructive jaundice has been obscure. However, recent experimental studies have shown that the regional decrease in tight junction (TJ)-associated protein levels in the intestinal epithelium^[4], increased apoptosis of enterocytes in intestinal crypts^[5], and intestinal oxidative stress^[6] are the key factors in the pathogenesis of hepatic and intestinal injury in obstructive jaundice^[7].

Probiotic bacteria have been shown to have beneficial effects in the intestinal barrier function. For example, live *Bifidobacterium lactis* has been shown to inhibit toxic effects in epithelial cell culture^[8]. *Lactobacillus plantarum* (*L. plantarum*) has been found to inhibit epithelial barrier dysfunction and interleukin-8 secretion induced by tumor necrosis factor- α ^[9] and prevent cytokine-induced apoptosis in intestinal epithelial cells^[10]. *L. plantarum* stabilizes the cellular TJ, thereby preventing enteropathogenic *Escherichia coli*-induced redistribution of integral TJ proteins^[11]. Based on the excretion of orally administered ¹⁴C, White *et al.*^[12] demonstrated that enteral administration of the probiotic bacterium *L. plantarum* 299 reduced intestinal hyperpermeability associated with experimental biliary obstruction. However, these authors did not clarify the mechanism for the protective effect of the probiotics on the intestinal barrier in obstructive jaundice. A recent clinical study reported that preoperative oral administration of synbiotics could enhance immune responses, attenuate systemic post-operative inflammatory responses, and improve the intestinal microbial environment after hepatobiliary surgery for obstructive jaundice^[13].

TJs, which represent the uppermost basolateral connection between neighboring enterocytes, are important components of the epithelial barrier^[14]. TJ assembly and paracellular permeability are regulated by a network of signaling pathways that involves different protein kinase C (PKC) isoforms^[15]. A substantial body of experimental

data indicates that PKC regulates paracellular permeability of the epithelial barrier^[16,17]. PKC regulates the assembly of TJ proteins through phosphorylation of zonula occludens-1 (ZO-1)^[15]. Seth *et al.*^[18] suggested that PKC β I activation may be one of the initial events in the probiotic-mediated protection of TJs. PKC ϵ may play a role in the downstream events of the signaling pathway involved in this process. These data suggest that PKC plays a crucial role in the mediation of intestinal epithelial TJ proteins.

This study aims to investigate the effects of *L. plantarum* on the intestinal mucosal barrier, oxidative stress, epithelial TJ-protein structure and phosphorylation, especially its impact on the expression and activity of PKC.

MATERIALS AND METHODS

Reagents

Rabbit polyclonal anti-occludin, rabbit polyclonal anti-junction adhesion molecule (JAM)-A, rabbit polyclonal anti-claudin-1, mouse monoclonal anti-claudin-4, and rabbit polyclonal anti-phosphoserine antibodies were supplied by Zymed (Invitrogen, Carlsbad, CA, United States). Rabbit polyclonal anti-ZO-1 and rabbit polyclonal anti-PKC were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, United States). Fluorescein isothiocyanate (FITC)-phalloidin was obtained from Sigma (St. Louis, United States). FITC-conjugated secondary antibodies were supplied by Zymed (Invitrogen). Biotin-labeled goat anti-rabbit immunoglobulin G (IgG) and horseradish peroxidase (HRP)-labeled streptavidin were supplied by DAKO (Glostrup, Denmark). All other reagents of analytical grade were purchased from Sigma (St. Louis, United States).

L. plantarum

The *L. plantarum* (strain CGMCC No. 1258) used in this study was a gift from Dr. Xiao-Ming Hang (Onlly Institute of Biomedicine, Shanghai Jiao Tong University, Shanghai, China). *L. plantarum* cultures were prepared exactly as described previously^[11].

Animals

Forty male albino Wistar rats weighing 250-320 g were purchased from Fudan University Medical Animal Center (Shanghai, China). They were housed in stainless-steel cages, three rats per cage, at controlled temperature (23 °C) and humidity and with a 12 h/12 h dark/light cycle. They were maintained on a standard laboratory diet with tap water ad libitum, except for an overnight fast before surgery. The study was conducted according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, and it was approved by the Ethics and Research Committee of Shanghai Sixth People's Hospital, Shanghai, China.

Experimental design

Animals were randomly divided into five groups of eight rats each as described below.

Group I, sham-operation: A 2.0-cm upper midline abdominal incision was made, and the common bile duct (CBD) was freed from the surrounding tissues without ligation or transection.

Group II, bile duct ligation: The CBD was double ligated in its middle third with a 4-0 silk suture and transected between the two ligatures.

Group III, bile duct ligation + *L. plantarum*: After bile duct ligation (BDL), a volume of 10 mL live *L. plantarum* (activity, 2×10^8 CFU/mL) divided into two equal doses was administered daily to the rats by gavage for 10 d. After 10 d, the animals were sacrificed under ketamine anesthesia.

Group IV, BDL + IBD: The CBDs of animals were ligated and isolated. A polyethylene tube PE-10 (American Health and Medical Supply International Corp. Co., Ltd., Scarsdale, New York, United States) was inserted into the proximal CBD in a cephalad direction and fixed. The drainage end was tied and positioned in the right hepatorenal recess. After 5 d of obstructive jaundice, the abdomen was reopened through the previous incision. After releasing the biliary obstruction by transecting the tube, a distal 3-cm segment of the catheter was inserted into the duodenum for internal biliary drainage. The animals were sacrificed after another 5 d.

Group V, BDL + IBD + *L. plantarum*: Ten days after BDL, live *L. plantarum* was infused as described for Group III.

All non-*L. plantarum* control groups, including sham-operation (SHAM), were gavaged with the same volume of the same vehicle (Dulbecco's phosphate buffered saline) used for the *L. plantarum* groups. The animals were sacrificed after 10 d.

All surgical procedures were performed under strict sterile conditions and ketamine anesthesia. At the end of the experiment on day 10, 4-5 mL blood sample was collected from each animal by puncturing the portal vein.

Serum total bilirubin and alanine aminotransferase measurement

The serum total bilirubin and alanine aminotransferase (ALT) levels were determined using a kit (Jiancheng Biological Co., Ltd., Nanjing, China) and a Hitachi Model 7600 series automatic analyzer (Hitachi Co., Tokyo, Japan).

Plasma endotoxin measurement

Endotoxin concentrations were determined using a quantitative chromogenic Limulus Amebocyte Lysate test kit (Shanghai Med. and Chem. Institute, Shanghai, China). Samples were processed according to the manufacturer's instructions^[19].

Plasma D-lactate and plasma diamine oxidase measurement

Plasma D-lactate levels were measured by an enzymatic spectrophotometric assay^[20] using a serum D-lactate quan-

titative colorimetric detection kit according to the manufacturer's instructions (GMS19038.6, Genmed, Boston, MA, United States). Results were expressed as mol/mL. Plasma diamine oxidase (DAO) activities were determined with an enzymatic spectrophotometric assay^[21] using a Serum DAO detection kit according to the manufacturer's instructions (Jiancheng Biological Co., Ltd., Nanjing, China). Results were expressed as U/L.

Plasma reduced glutathione/oxidized glutathione measurement

Plasma glutathione (GSH) and glutathione (GSSG) were determined by an enzymatic spectrophotometric assay^[22] using the GSH and GSSG detection kits according to the manufacturer's instructions (Jiancheng Biological Co., Ltd.). Results were expressed as mol/mL.

Detection of superoxide dismutase and malondialdehyde in the ileum

Superoxide dismutase (SOD) activity was detected using Sun *et al*'s^[23] nitroblue tetrazolium method. Malondialdehyde (MDA) levels were measured using the thiobarbituric acid test according to Ohkawa *et al*'s^[24]. Intestinal tissue samples were thawed, weighed, and homogenized 1:9 (w/v) in 0.9% saline. The homogenates were centrifuged at $3000 \times g$ for 10 min at 4 °C, and the supernatant was removed for the measurement of MDA content, SOD activity, and total protein. Total intestinal protein concentration was determined by a Coomassie blue method, with bovine serum albumin (BSA) as a standard. SOD activity and MDA levels were detected with kits according to the manufacturer's instructions (Jiancheng Bioengineering Ltd., Nanjing, China). Results were expressed as U/mg protein and nmol/mg protein.

Light microscopy

Samples 1 cm in length were collected from the terminal ileum. To avoid mucosal damage, the intestinal lumen was carefully cannulated and gently washed with normal saline before sampling. Specimens were fixed by immersion in 10% buffered formaldehyde solution and embedded in paraffin. Sections (5 µm thick) were cut and stained for routine light microscopy using HE.

Transmission electron microscopy

Samples 3-4 mm in length were collected from the terminal ileum. These samples were longitudinally cut and immersed in 2.5% phosphate-buffered glutaraldehyde solution for 24 h at 4 °C. Specimens were then washed with phosphate-buffered solution (PBS), fixed in 1% osmium tetroxide for 2 h at 4 °C, dehydrated in ethanol and propylene oxide, and embedded in Epon 812 for 48 h. Sections (1 µm thick) were cut and stained with methylene blue. Ultrathin sections were then cut with a diamond knife, stained with uranyl acetate and lead citrate, and observed under transmission electron microscopy (TEM).

Terminal deoxyuridine nick-end labeling assay

Four-µm thick sections were collected on poly-L-lysine-

coated glass slides. The nuclear DNA fragmentation of apoptotic cells was labeled *in situ* by the terminal deoxynucleotidyl transferase nick-end labeling immunohistochemical method^[25] using an ApopTag[®] Plus Peroxidase In Situ Apoptosis Detection Kit (CHEMICON, Billerica, MA, United States) according to the manufacturer's instructions.

Immunofluorescence microscopy

Terminal ileum tissues were fixed in 3% paraformaldehyde for 3 h, washed with PBS, and embedded in paraffin. Sections (5 μ m thick) were cut and attached to glass slides. After deparaffinization and rehydration, sections were permeabilized with 0.2% Triton X-100 in PBS for 20 min. Slides were washed with PBS extensively and blocked with 5% normal goat serum PBS containing 0.05% Tween-20 and 0.1% BSA for 20 min at room temperature. Primary antibodies were added to the slides and incubated overnight at 4 °C in a humidity chamber. After washing, sections were incubated with FITC-conjugated specific secondary antibody (Sigma) at room temperature for 2 h in the dark. The slides were again washed extensively and then mounted with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, United States). Sections were observed under a confocal laser microscope (LSM 510, Zeiss, Jena, Germany).

Expression of PKC by immunocytochemical staining using labelled streptavidin biotin method

After the rats were sacrificed, terminal ileum tissues were excised and fixed in Bouin's solution and embedded in paraffin. Immunohistochemistry was performed on 5- μ m thick paraffin sections. After deparaffinization and dehydration, endogenous peroxidase was blocked with 30 mL/L H₂O₂ for 15 min. After blocking of nonspecific binding sites with 5% normal goat serum, slides were incubated with specific primary antibody overnight at 4 °C. Primary antibodies were diluted 1:50 (rabbit polyclonal anti-human PKC, Santa Cruz Biotechnology, Inc., United States) in PBS. Next, the slides were washed three times for 5 min each with PBS and incubated with biotinylated goat anti-rabbit IgG at 37 °C for 30 min, washed as before, and developed with HRP-labeled streptavidin. The incubation and the subsequent washing were exactly the same as done before. Finally, diaminobenzidine chromogen, a peroxidase substrate, was added for color development. The reaction was stopped with a tap water rinse. The sections were counterstained with hematoxylin and mounted for examination.

Western blotting analysis

Terminal ileum samples were homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer [150 mmol NaCl, 50 mmol Tris·HCl (pH 7.4), 0.5 mmol phenylmethylsulfonyl fluoride, 2.4 mmol EDTA, and 1 mmol sodium orthovanadate with 1% nonidet-40 (NP-40) and Sigma protease inhibitor cocktail (1:100)] for 30 min at 4 °C. After centrifugation at 10 000 \times g for 10 min at 4 °C, the protein concentration of each sample was quantified by the Bradford method. An equal amount of

total protein was separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and then transferred to a nitrocellulose membrane. After blocked overnight in tris-buffered saline (TBS) containing 0.05% tween (TBS-T) and 5% dry powdered milk, membranes were washed three times for 5 min each with TBS-T and incubated for 2 h at room temperature in primary antibody (rabbit anti-claudin-1, rabbit anti-occludin, rabbit anti-JAM-A, or rabbit anti-ZO-1). After three washes with TBS-T, the membranes were incubated for 1 h with HRP-conjugated secondary antibody. Following two washes with TBS-T and one wash with TBS, the membranes were prepared for visualization of protein by the addition of enhanced chemiluminescence (ECL) reagent (Amersham, Princeton, NJ, United States). Densitometric analysis was performed using an Alpha Imager 1220 system (AlphaImotech Co., San Leandro, CA, United States).

Real-time reverse transcription-PCR

The levels of occludin, ZO-1, claudin-1, claudin-4, JAM-A and UGT1A mRNA were measured by real-time reverse transcription-PCR (RT-PCR) using SYBR1 green^[26]. Total RNA was isolated from terminal ileum samples with the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Real-time RT-PCR was performed with an ABI prism 7000 Real-Time PCR System (Applied Biosystems, Foster City, CA, United States). The primers were designed using the Primer Express[®] Program (Applied Biosystems). Their sequences are shown in Table 1. The following procedure used 2 μ g of RNA. In a sterile RNase-free microcentrifuge tube, 1 μ L of 20 μ M oligo (dT) 15 primer was added to a total volume of 15 μ L water. The tubes were heated to 70 °C for 5 min, cooled immediately on ice, and spun briefly. The following reagents were added to the annealed primer/template: 5 μ L of 5 \times M-MLV reaction buffer, 1.25 μ L of 10 mmol dNTPs and 25 units of RNasin RNase inhibitor, and 200 units of M-MLVRT RNase H were added to the reagent to yield a 25 μ L total reaction volume. All were mixed gently and then incubated for 60 min at 42 °C before the reaction was terminated at -20 °C.

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene expression was used as a house-keeping gene control. Separate PCR reactions (25 μ L) were conducted for each transcript and contained 2.0 μ L cDNA, 12.5 μ L of 2 \times SYBR Premix Ex Taq[™] (TaKaRa, Ltd., Shiga, Japan), and 0.5 μ L each of 10 μ mol/L gene-specific forward and reverse primers. PCR conditions were optimized to 95 °C (30 s), followed by 40 cycles (45 s each) at 95 °C, 60 °C (5 s), and 72 °C (30 s), and the reaction was completed at 37 °C for 30 s. Five serial dilutions of cDNA were analyzed for each target gene and used to construct linear standard curves. To compensate for variations in the RNA input and in the efficiency of the real-time RT-PCR, we used a normalization strategy based on the house-keeping gene *GAPDH*.

Immunoprecipitation and immunoblotting assays

The terminal ileum tissues were homogenized and ex-

Table 1 Sequences of oligonucleotide primers and conditions for real-time polymerase chain reaction

Gene target	Genbank number (mRNA)	Oligonucleotide ¹ (5'- to 3'-)	Annealing temperature (°C)	Product size (bp)
<i>Occludin</i>	NM-031329	F: GCTCAGGAATATCCACCTATC R: TTCTCCAGCAACCAGCATC	60	344
<i>ZO-1</i>	NM-001106266	F: CCACAGACATCCAACCAGC R: AGCCCAAAGAACAGAAGACC	60	247
<i>Claudin-1</i>	NM-031699	F: GCCTCCAATGCCGTCT R: TGCCTGCGTCCCTCTTG	60	317
<i>Claudin-4</i>	NM-001012022	F: GTTCCCGCCAGCAACTATG R: CCTTCAGCCCCGTATCCA	60	282
<i>JAM-A</i>	NM-053796	F: CCTCCATCCAAGCCGACA R: AAAAGACCAAATCCCCTGAC	60	211
<i>prkC</i>	NM-001105713	F: GATGGACGGGTCACGA R: CGCTTGGCAGGGTGTIT	60	165
<i>β-actin</i>	NM-031144	F: CAGGTCATCACTATCGGCAAT R: GAGGTCCTTACGGATGTCAAC	60	144

¹Primers were designed based on sequences of rat-corresponding genes from the GenBank database. *JAM-A*: Junction adhesion molecule-A; *ZO-1*: Zonula occludens-1.

tracted with the buffer used for Western blotting assays for 30 min at 4 °C. After centrifugation at 10 000 × *g* for 10 min at 4 °C, the protein concentration of each sample was quantified by the Bradford method. The supernatant was treated with protein G plus protein A agarose beads (Sigma) and incubated overnight at 4 °C with rabbit anti-occludin antibody (Zymed) and protein G + protein A agarose beads. The beads were washed with PBS and ice-cold RIPA buffer, and immunoprecipitated proteins were separated on 10% SDS-polyacrylamide gel electrophoresis gels and transferred onto nitrocellulose membranes (Invitrogen). The membranes were blocked with 1% BSA in PBS overnight at 4 °C and then incubated with rabbit anti-phosphoserine antibodies (Zymed) for 2 h at room temperature, followed by HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., United States). The reaction was visualized by an enzyme chemiluminescence kit from Pierce (Rockford, IL, United States). Western blotting was performed with an anti-occludin rabbit polyclonal antibody (Zymed) followed by an anti-rabbit secondary antibody coupled with peroxidase (Santa Cruz Biotechnology, Inc.) and ECL. For Western blotting of *ZO-1*, the same protocol was used with the rabbit polyclonal anti-*ZO-1* antibody and a rabbit anti- β -actin antibody (both from Santa Cruz Biotechnology, Inc.).

PKC activity assay

The PKC activity assay was conducted according to the instructions of the PepTag non-radioactive PKC assay kit (Promega, Madison, WI, United States). Briefly, terminal ileum tissues were homogenized and lysed in cold lysis buffer, containing 20 mmol/L tris-HCl, 0.5 mmol/L ethylene glycol tetraacetic acid, 2 mmol/L ethylenediamine-tetraacetic acid, 2 mmol/L phenylmethanesulfonyl fluoride, and 10 mg/L leupeptin (pH 7.5). Assays were then performed at 30 °C in a total volume of 25 μ L containing 5 μ L PKC reaction 5 × buffer, 5 μ L PLSRTPLSVAAK peptide, 5 μ L PKC activator, 1 μ L peptide protection solution, and 9 μ L sample. Reactions were initiated by the addition of the 9 μ L sample and terminated after 30 min

by incubation of the reaction mixture at 95 °C for 10 min. After added with 1 μ L of 80% glycerol, each sample was separated by 0.8% agarose gel electrophoresis at 100 V for 15 min. The intensity of fluorescence of phosphorylated peptides reflected the activity of PKC. All experiments were carried out in triplicate, with each data point representing the results from a separate culture.

Image analysis

Quantification of the immunohistochemical and immunofluorescence staining was performed on stored images of completely scanned tissue sections. Images were acquired with an AxioCam MRc (Carl Zeiss, Jena, Germany) connected to an Axioplan 2 fluorescence microscope (Carl Zeiss), at × 40 magnification. Each microscopic field was individually autofocused before acquisition. Five fields were selected from each slide and a total of five slides per group were examined. All image acquisition and processing were done using custom-written macros in KS400 image analysis software (version 3.0, Carl Zeiss).

Statistical analysis

Results were presented as mean ± SD of three experiments. The data were analyzed using GraphPad PRISM (GraphPad Software Inc., San Diego, CA, United States) and SPSS 11.0 (SPSS Inc., Chicago, IL, United States). All data were analyzed using one-way analysis of variance with Bonferroni/Dunnett T3 *post-hoc* test for multiple comparisons to determine differences between two experimental groups. *P* values < 0.05 were considered to be significant.

RESULTS

General observations

All animals survived throughout the experiment. Bile duct ligated rats were clinically jaundiced within 3 d. At reoperation on day 3, the ligation and division of the CBD were successful in all cases and resulted in significant dilatation of the CBD remnant proximal to the liga-

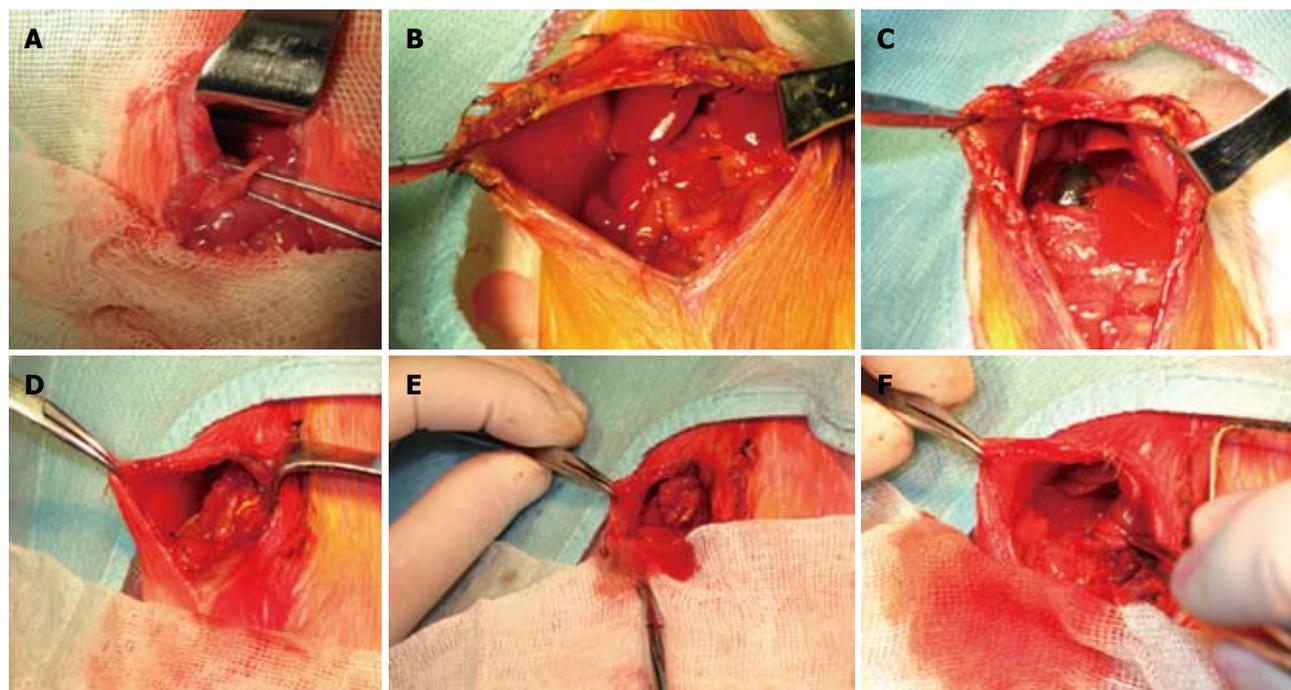


Figure 1 Images of experimental obstructive jaundice and internal biliary drainage. A: Dissection revealing the common bile duct; B: Reoperation after 3 d of common bile duct ligation. Light yellow abdominal ascites were present in the right side of the abdominal cavity; C: The proximal bile duct showed a remarkable expansion (dark blue color) after 3 d of common bile duct ligation; D: The PE-10 polyethylene tube was positioned with the end tied in the right hepatorenal recess; E: Brown bile flowed out while the catheter end was open; F: The distal 3 cm segment of the catheter was inserted into the duodenum for internal biliary drainage.

	Group I	Group II	Group III	Group IV	Group V
Endotoxin (ng/mL)	0.58 ± 0.09	17.12 ± 1.09 ^a	14.25 ± 0.68	3.05 ± 0.78 ^c	1.91 ± 0.54
TBIL (μmol/L)	3.0 ± 1.63	153.83 ± 25.73 ^a	132.0 ± 23.09	23.75 ± 5.42 ^c	9.0 ± 1.87
ALT (U/L)	60.8 ± 5.69	543.83 ± 184.09 ^a	218.38 ± 91.09	118.63 ± 19.72 ^c	97.0 ± 10.37
AST (U/L)	130.9 ± 27.42	980.5 ± 663.25 ^a	512.75 ± 156.76	437.88 ± 42.41 ^c	271.0 ± 28.93

^a*P* < 0.05 vs Group III; ^c*P* < 0.05 vs Group V. Group I : Sham-operation; Group II : Bile duct ligation (BDL); Group III : BDL + *L. plantarum*; Group IV : BDL + internal biliary drainage (IBD); Group V : BDL + IBD + *L. plantarum*. TBIL: Total bilirubin; ALT: Alanine transaminase; AST: Aspartate transaminase.

ture with obvious cholestatic appearance of the liver. At reoperation on day 10, the CBD diameter had returned to a normal size in the BDL + IBD group, and the cholestatic livers also appeared improved visually (Figure 1).

Serum total bilirubin and ALT levels

Obstructive jaundice led to significantly elevated serum levels of total bilirubin [153.73 mmol/L vs 3.0 ± 1.63 mmol/L] and ALT [543.83 U/L vs 60.8 ± 5.69 U/L, *P* < 0.05]. Administration of *L. plantarum* significantly reduced levels of total bilirubin (132 ± 23.9 mmol/L vs 9.0 ± 1.87 mmol/L) and ALT (218.38 ± 91.09 U/L vs 97 ± 10.37 U/L) in the portal serum (Table 2).

Portal endotoxin concentrations

Group II (BDL + *L. plantarum*) animals presented with significantly elevated endotoxin concentrations in portal blood compared with those in Group I (SHAM) (*P* < 0.05). Treatment with *L. plantarum* in the BDL + *L. plantarum* and BDL + IBD + *L. plantarum* groups significantly

reduced endotoxin levels in the portal serum (Table 2).

Plasma D-lactate and plasma DAO measurement

Plasma D-lactate levels increased significantly in the BDL group compared with the SHAM group. Administration of *L. plantarum* significantly decreased the plasma D-lactate levels in the BDL + *L. plantarum* and IBD + *L. plantarum* groups (Table 3).

DAO activity in the BDL group was significantly higher than that in the SHAM group. Plasma DAO activity became significantly lower after the use of probiotics in the BDL + *L. plantarum* and IBD + *L. plantarum* groups (Table 3).

Glutathione redox state

Plasma GSH was significantly reduced in Group II (BDL) animals compared with those in Group I (*P* < 0.05). Administration of *L. plantarum* significantly increased the levels of GSH in the Group IV (BDL + IBD) animals, whereas GSSG was found to be significantly increased in BDL animals. Administration of *L. plantarum* significantly

Table 3 Levels of *D*-lactate, diamine oxidase, superoxide dismutase, malondialdehyde, glutathione and glutathione

	Group I	Group II	Group III	Group IV	Group V
D-lactate (mmol/L)	1.723 ± 0.106 ^a	4.236 ± 0.050 ^f	3.599 ± 0.181	3.152 ± 0.123 ^e	2.800 ± 0.129
DAO (U/L)	2.829 ± 0.438 ^a	18.925 ± 1.485 ^f	12.928 ± 1.544	10.198 ± 0.584 ^e	7.109 ± 0.590
SOD (U/mg protein)	65.002 ± 4.397 ^a	26.782 ± 1.979 ^f	35.396 ± 1.328	43.916 ± 1.720 ^e	53.066 ± 3.203
MDA (nmol/mg protein)	0.408 ± 0.054 ^a	1.253 ± 0.154 ^f	0.914 ± 0.108	0.672 ± 0.054 ^e	0.540 ± 0.029
GSH (μmol/L)	21.091 ± 0.452 ^a	7.235 ± 0.479 ^f	8.431 ± 0.537	10.504 ± 0.481 ^e	19.082 ± 0.455
GSSG (μmol/L)	2.974 ± 0.260 ^a	23.753 ± 2.895 ^f	12.795 ± 1.360	4.944 ± 0.207 ^e	3.537 ± 0.343

^a*P* < 0.05 vs Group II; ^f*P* < 0.05 vs Group III; ^e*P* < 0.05 vs Group V. Group I: Sham-operation; Group II: Bile duct ligation (BDL); Group III: BDL + *L. plantarum*; Group IV: BDL + internal biliary drainage (IBD); Group V: BDL + IBD + *L. plantarum*. DAO: Diamine oxidase; SOD: Superoxide dismutase; MDA: Malondialdehyde; GSH: Glutathione; GSSG: Glutathione.

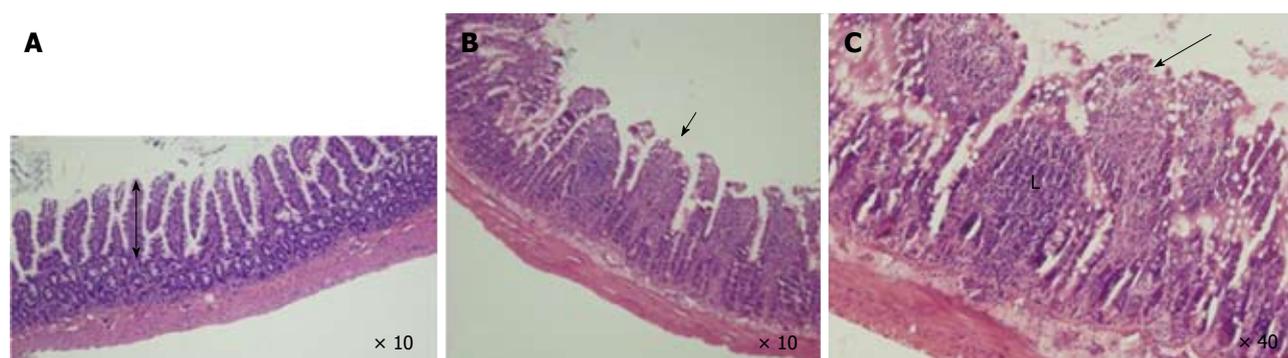


Figure 2 Light microscopic micrographs of samples stained with haematoxylin and eosin. A: Normal structure of villi (two-way arrow); B: Blunting of villi (short arrow); C: Existing subepithelial edema (long arrow) and the lymphocyte and plasma cell infiltration (L). Images shown represent at least three regions observed on the same slide.

reduced the levels of GSSG in the BDL + IBD group (Table 3).

Intestinal mucosal SOD and MDA

Ileum mucosal MDA was significantly increased in BDL group compared with SHAM. Administration of *L. plantarum* significantly decreased the levels of MDA in the BDL + IBD group. The trend in ileum mucosal SOD levels among the groups was opposite to the results of MDA (Table 3).

Morphological studies

Specimens collected from the terminal ileum in the BDL group showed subepithelial edema and blunting of the villi, mostly located at the tip of the villi, with a large number of lymphocytes and plasma cells infiltrated in the intestinal mucosa (Figure 2). Under TEM, cell ultrastructure was disordered, with loss or disruption of microvilli and large dense secondary lysosomes that resembled partially degraded bacteria within the enterocyte cytoplasm. Inflated vacuolization of the cells, swollen mitochondria with partial or complete absence of interior cristae, disruption of desmosomes and formation of oedematous spaces, and expansion of endoplasmic reticulum were observed. Cells often showed serious damage to the plasma membrane and complete loss of junctional specialization between adjacent cells. Additionally, spherical and rod-shaped bacteria in the ileum were seen near enterocytes. These features are typical of pre-necrotic and necrotic injury of the intestinal mucosa. However, in the BDL +

L. plantarum group, cells were aligned regularly, with less swelling of mitochondria, no expansion of the endoplasmic reticulum, while the morphology was nearly normal in the IBD + *L. plantarum* group (Figure 3).

Apoptosis in the intestinal mucosal epithelium

Apoptotic nuclei were significantly more abundant in the markedly atrophic villi in the BDL group than in the SHAM group. The apoptotic nuclei were mostly distributed at the top of villi (Figure 4A). Administration of *L. plantarum* significantly decreased the number of apoptotic nuclei in the BDL + IBD + *L. plantarum* group (Figure 4B).

Expression of PKC illustrated by immunocytochemistry

PKC appeared as brown spots in the perinuclear structure. Its expression was decreased in the BDL group compared with the SHAM group. Administration of *L. plantarum* significantly enhanced the expression of PKC in the BDL + *L. plantarum* and IBD + *L. plantarum* groups (Figure 5).

Effects of *L. plantarum* on TJ protein localization (fluorescence microscopy)

Confocal imaging was performed to assess the distribution of the TJs in each group. TJ-associated proteins were continuously distributed in bright green or red color along the membrane of the cells. The F-actin staining showed a continuous line at the cell borders and along the cytoskeleton. Their borders were very clear in the SHAM group, where TJ-associated proteins were present at the apical

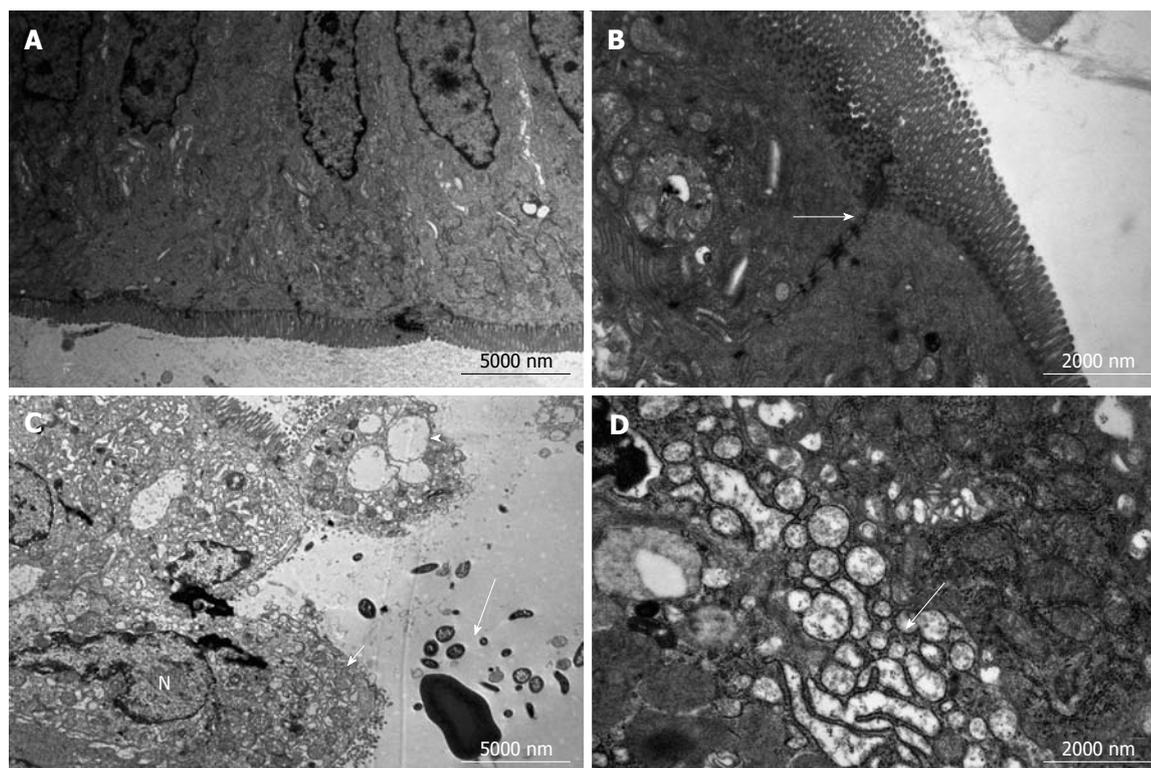


Figure 3 Ultrastructural assessment of enterocytes in the terminal ileum. A: Normal cell ultrastructure; B: The positions of tight junctions (arrow) and desmosomes; C: Enterocytes showed vacuolar degeneration (short arrow) and mitochondrial swelling, disruption of their microvilli (arrowhead), large dense secondary lysosomes, complete loss of the junctional specialization between adjacent cells (long arrow), and karyopyknosis (N); D: Expansion of the endoplasmic reticulum (arrow). Images shown represent at least three regions observed on the same slide. Group I : Sham-operation; Group II : Bile duct ligation (BDL); Group III : BDL + *Lactobacillus plantarum* (*L. plantarum*); Group IV : BDL + internal biliary drainage (IBD); Group V : BDL + IBD + *L. plantarum*.

intercellular borders in a belt-like manner, encircling the cells and delineating the cellular borders. In the BDL group, the fluorescence was dispersed and even became punctate, with loss of membrane fluorescence as against the uniform membrane staining in controls. Administration of *L. plantarum* enhanced the expression of TJ-associated proteins in the BDL + *L. plantarum* and IBD + *L. plantarum* groups (Figure 6).

Effects of *L. plantarum* on TJ and PKC protein levels (Western blotting)

Western blotting analyses were performed to determine the relative protein levels of the target proteins occludin, claudin-1, claudin-4, JAM-A, ZO-1 and PKC in the terminal ileum. The intensity of the whole-cell proteins was determined from ratios of the integrated intensity of the target protein bands to the integrated intensities of the β -actin bands in the same sample. Compared with samples obtained from rats in the SHAM group, levels of target proteins were decreased in protein extracts from ileal mucosal scrapings of rats subjected to BDL. Administration of *L. plantarum* significantly enhanced the expression of TJ-associated proteins in the BDL + *L. plantarum* and IBD + *L. plantarum* groups (Figure 7).

Levels of mRNA in TJ and PKC determined by real-time PCR assays

Intragastric administration of *L. plantarum* resulted in

changes in the levels of occludin, ZO-1, claudin-1, claudin-4, JAM-A and PKC. This result raised the question whether these altered protein levels were a consequence of changes in mRNA levels. We, therefore, used real-time RT-PCR to determine the levels of mRNA in the terminal ileum in each group. The levels of mRNA of occludin, ZO-1, claudin-1, claudin-4, JAM-A, PKC and UGT1A1 were found significantly lower in the BDL group than in the SHAM group. Administration of *L. plantarum* significantly increased the mRNA levels of target proteins in both the BDL + *L. plantarum* and IBD + *L. plantarum* groups (Table 4).

Phosphorylation of occludin and ZO-1

We examined the phosphorylation status of occludin and ZO-1 using immunoprecipitation and immunoblotting assays. Occludin and ZO-1 were phosphorylated at serine residues. BDL lowered p-occludin and p-ZO-1 proteins levels compared with the SHAM group. Administration of *L. plantarum* improved the expression of the p-occludin and p-ZO-1 proteins from the terminal ileum in the BDL + *L. plantarum* and BDL + IBD + *L. plantarum* groups (Figure 8).

Effects of *L. plantarum* on PKC activity

As shown in Figure 9, PKC activity was significantly decreased in the BDL group compared with SHAM group. Intragastric administration of *L. plantarum* partly restored

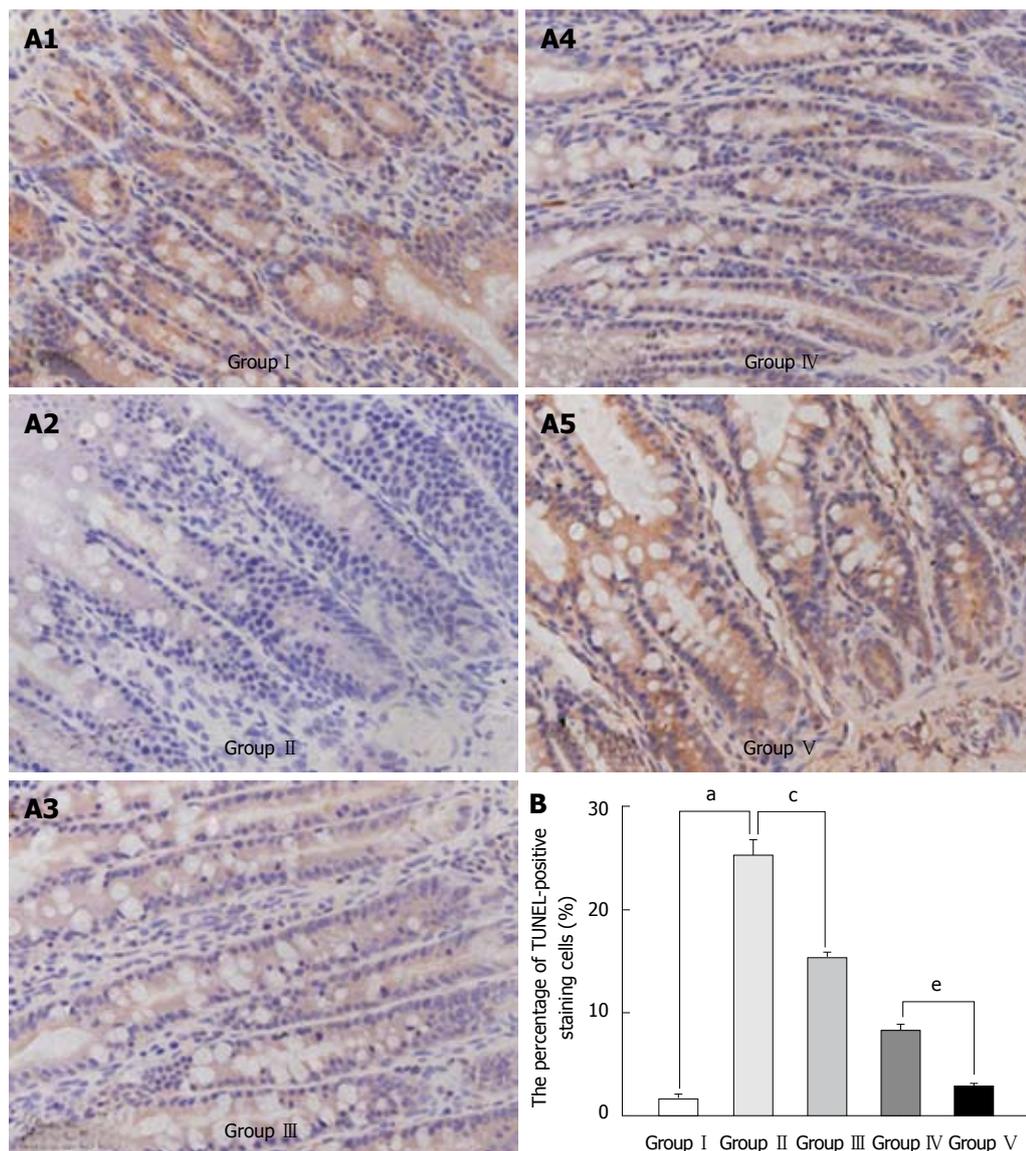


Figure 4 Effects of *Lactobacillus plantarum* on the apoptosis in the intestinal mucosal epithelium. A: Ileum sections from each group stained using the terminal deoxyuridine nick-end labeling (TUNEL) method. TUNEL-positive cells were stained dark brown. A significantly higher number of TUNEL-positive cells was detected in tissues from group II animals compared with group III; and the number of TUNEL-positive cells in group IV was higher than in group V. Images shown represent at least three regions observed on the same slide; B: Statistical evaluation of effects of *Lactobacillus plantarum* (*L. plantarum*) on the apoptosis in the intestinal mucosal epithelium. Data in the bar graph represent mean \pm SD of a minimum of three slides per group. ^a*P* < 0.05 Group I vs Group II; ^c*P* < 0.05 Group II vs Group III; ^e*P* < 0.05 Group IV vs Group V. Group I: Sham-operation; Group II: Bile duct ligation (BDL); Group III: BDL + *L. plantarum*; Group IV: BDL + internal biliary drainage (IBD); Group V: BDL + IBD + *L. plantarum*.

Table 4 Expression (mRNA) ratio (studied genes/ β -actin) for tight junction and protein kinase C in terminal ileum tissues of each experimental group (mean \pm SD)

Genes	Group I	Group II	Group III	Group IV	Group V
<i>Occludin</i>	2.5458 \pm 0.2260	0.4881 \pm 0.0426 ^a	0.9792 \pm 0.2066	1.4902 \pm 0.0720 ^c	1.8976 \pm 0.1049
<i>ZO-1</i>	7.2420 \pm 0.4025	0.9541 \pm 0.1629 ^a	1.4064 \pm 0.1632	2.8843 \pm 0.1641 ^c	4.0727 \pm 0.2059
<i>Claudin-1</i>	1.9751 \pm 0.0615	0.0546 \pm 0.0336 ^a	0.4741 \pm 0.0897	0.9092 \pm 0.1295 ^c	1.4793 \pm 0.2119
<i>Claudin-4</i>	42.8680 \pm 7.5291	0.3546 \pm 0.0916 ^a	5.3245 \pm 1.1801	8.7719 \pm 1.4659 ^c	15.9592 \pm 2.8815
<i>JAM-A</i>	3.3259 \pm 0.3704	0.4712 \pm 0.1107 ^a	0.9456 \pm 0.1101	1.6270 \pm 0.2153 ^c	2.1006 \pm 0.1534
<i>PKC</i>	6.6958 \pm 0.9349	1.7959 \pm 0.2992 ^a	2.8281 \pm 0.3287	3.7178 \pm 0.5110 ^c	4.7235 \pm 0.4958

^a*P* < 0.05 vs Group III; ^c*P* < 0.05 vs Group V. Group I: Sham-operation; Group II: Bile duct ligation (BDL); Group III: BDL + *L. plantarum*; Group IV: BDL + internal biliary drainage (IBD); Group V: BDL + IBD + *L. plantarum*. *JAM-A*: Junction adhesion molecule-A; *PKC*: Protein kinase C.

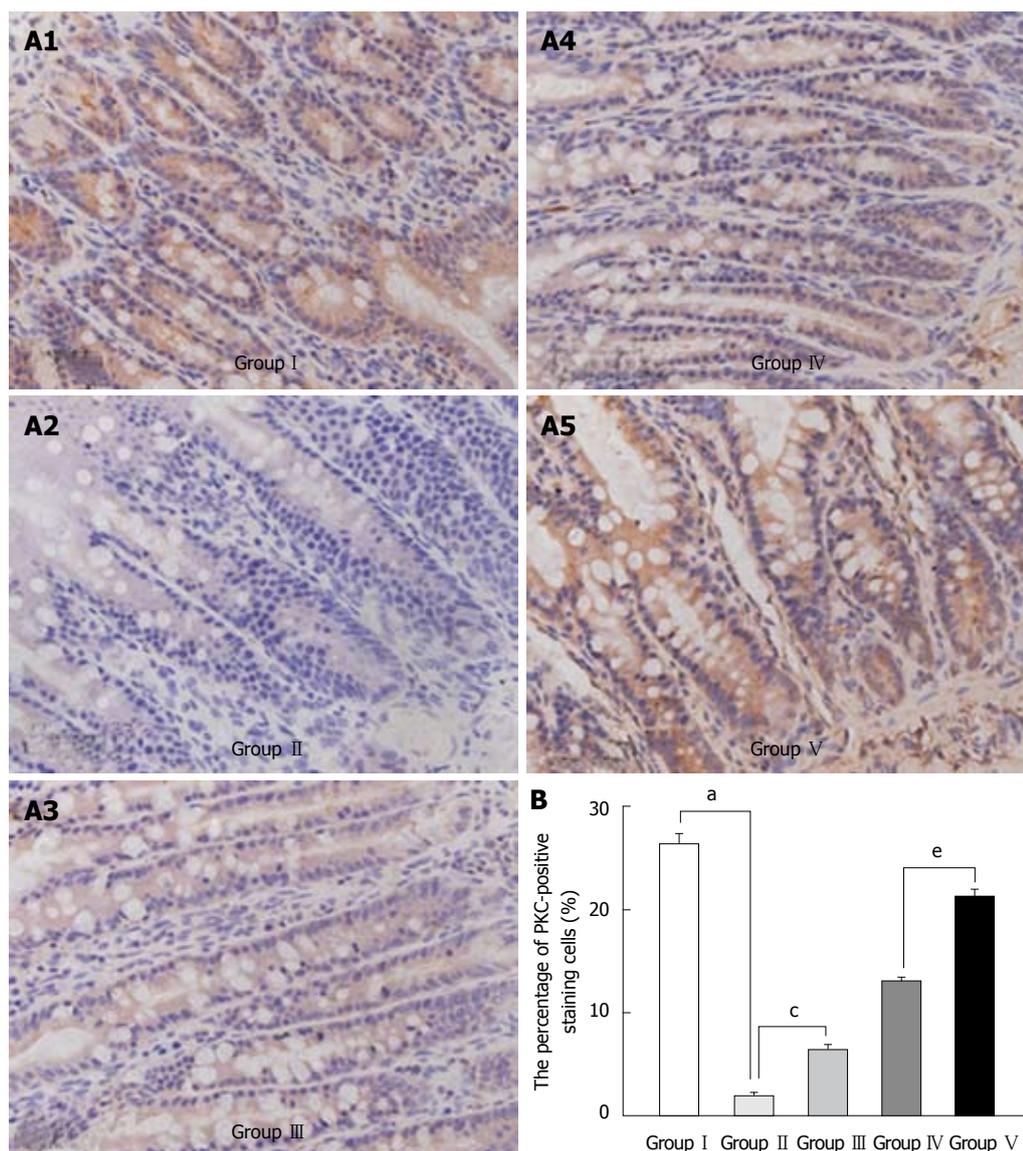


Figure 5 Effects of probiotics on the expression of protein kinase C in the mucosa of the terminal ileum. A: Probiotics effects on biliary obstruction-induced expression of protein kinase C (PKC) as determined by immunohistochemistry. Images shown are representative of at least three regions observed on the same slide; B: Statistical evaluation of effects of addition of probiotics (*Lactobacillus plantarum*) on the expression of PKC in the intestinal mucosal epithelium. Data in the bar graph represent mean \pm SD of the three separate experiments. ^a $P < 0.05$ Group I vs Group II; ^c $P < 0.05$ Group II vs Group III; ^e $P < 0.05$ Group IV vs Group V. Group I : Sham-operation; Group II : Bile duct ligation (BDL); Group III : BDL + *Lactobacillus plantarum* (*L. plantarum*); Group IV : BDL + internal biliary drainage (IBD); Group V = BDL + IBD + *L. plantarum*.

PKC activity in both the BDL + *L. plantarum* and BDL + IBD + *L. plantarum* groups.

DISCUSSION

The present study demonstrated that oral administration of *L. plantarum* significantly reduced bilirubin, ALT and endotoxin levels in the systemic circulation in an experimental obstructive jaundice animal model with internal biliary drainage for 10 d. Moreover, oral *L. plantarum* administration to the rats in the BDL + *L. plantarum* group and the BDL + IBD + *L. plantarum* group not only reduced the serum endotoxin levels, but also substantially improved liver function. This result is consistent with the conclusions reported in previous literature^[12].

In our study, oral *L. plantarum* administration also

significantly decreased the serum DAO activity and D-lactate level in both the BDL + *L. plantarum* group and the BDL + IBD + *L. plantarum* group. These findings indicate that *L. plantarum* plays an important role in intestinal integrity. Previous *in vitro* studies have confirmed that probiotics exert direct protective effects in intestinal epithelial cell TJJs *via* a PKC-kinase-dependent mechanism and inhibiting epithelial cell apoptosis in cell culture experiments^[11,18,10]. The current experiments focused on *L. plantarum* effects, while several previous studies have reported the protective effects of other lactobacilli and probiotics. For example, Moorthy *et al*^[27] reported that pretreatment with a combination of *Lactobacillus rhamnosus* (*L. rhamnosus*) and *Lactobacillus acidophilus* had a significant protective effect on TJ proteins (claudin-1 and occludin) in a *Shigella dysenteriae* 1 infection rat model. Khailova

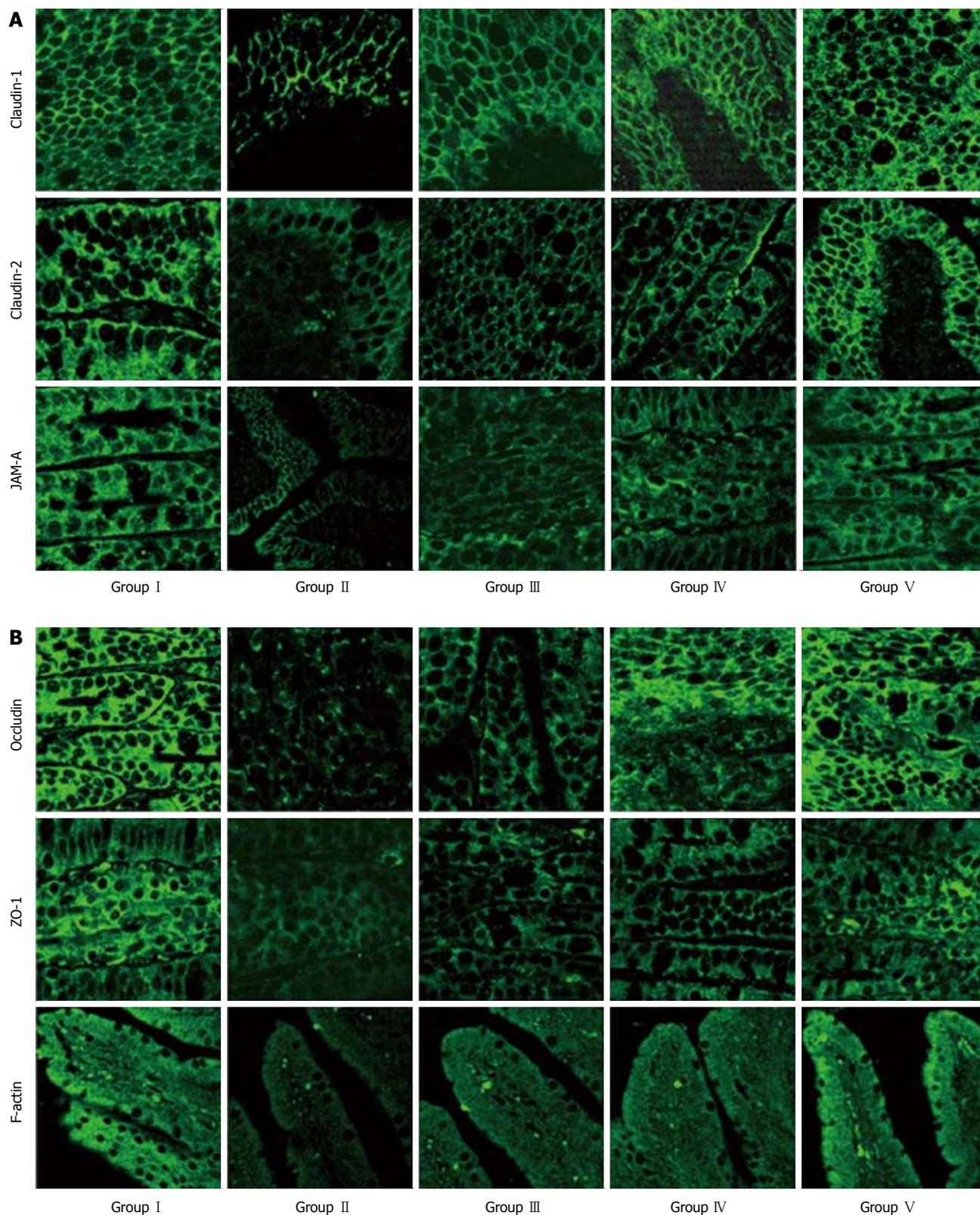


Figure 6 Immunofluorescence expression of claudin-1, claudin-4 and junction adhesion molecule-A (A) and occludin, ZO-1 and F-actin (B) in the mucosa of the terminal ileum. Images shown are representative of at least three regions observed on the same slide. Group I : Sham-operation; Group II : Bile duct ligation (BDL); Group III: BDL + *Lactobacillus plantarum* (*L. plantarum*); Group IV: BDL + internal biliary drainage (IBD); Group V = BDL + IBD + *L. plantarum*. JAM-A: Junction adhesion molecule-A.

et al^[28] reported that *Bifidobacterium bifidum* improved intestinal integrity [composition of TJ and adherens junction (AJ) proteins] in a rat model of necrotizing enterocolitis.

Mennigen *et al*^[29] found that the probiotic mixture VSL#3 protected the epithelial barrier by maintaining TJ protein expression and preventing apoptosis in a murine model of

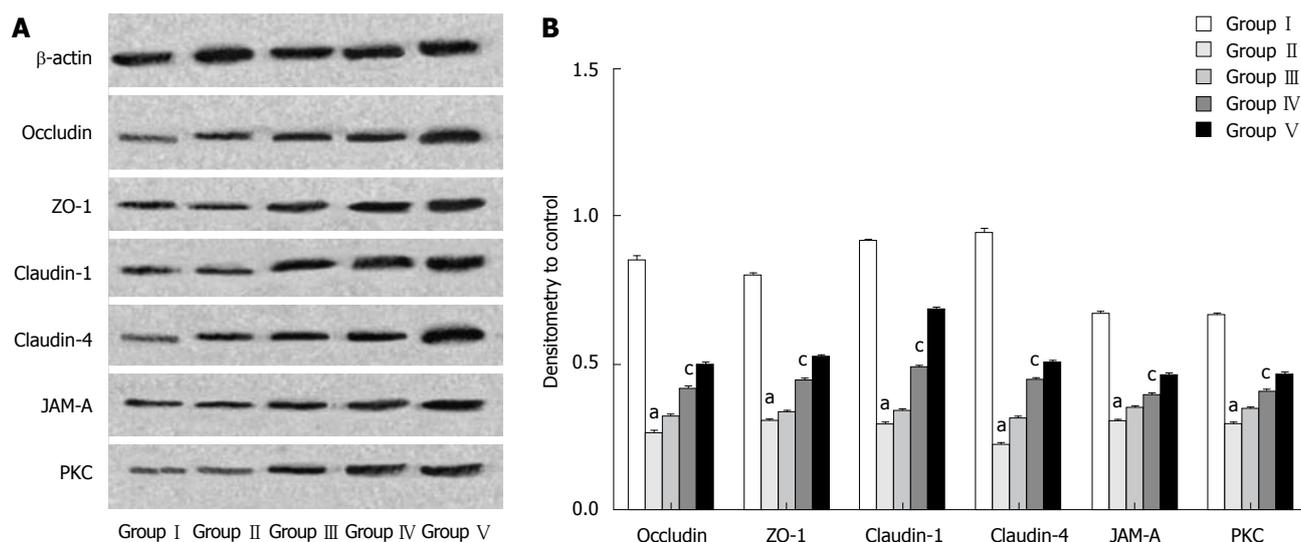


Figure 7 Effects of probiotics on the levels of tight junction proteins and protein kinase C proteins in the mucosa of the terminal ileum. A: Western blotting analysis of occludin, ZO-1, claudin-1, claudin-4, junction adhesion molecule (JAM)-A, and protein kinase C (PKC) proteins; B: Statistical evaluation of densitometric data that represent protein levels from the three separate experiments. ^a*P* < 0.05 vs Group III; ^c*P* < 0.05 vs Group V. Data are presented as relative band density ± SD. Group I : Sham-operation; Group II : Bile duct ligation (BDL); Group III : BDL + *Lactobacillus plantarum* (*L. plantarum*); Group IV : BDL + internal biliary drainage (IBD); Group V = BDL + IBD + *L. plantarum*.

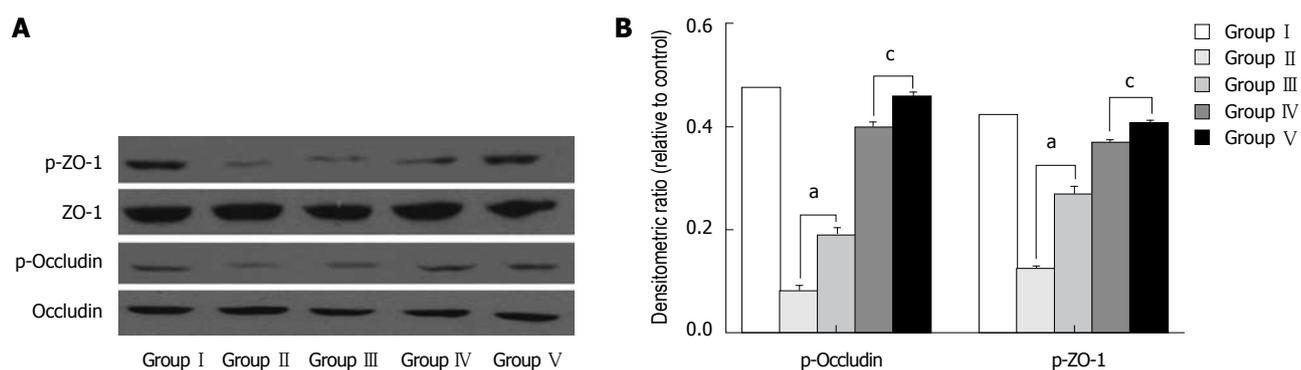


Figure 8 Serine phosphorylation of occludin, zonula occludens-1 in the terminal ileum. Tissue lysates were subjected to immunoprecipitation with the anti-occludin or zonula occludens-1 (ZO-1) antibody, followed by Western blotting analysis with antibodies against phosphoserine. A: p-Occludin and p-ZO-1 protein levels compared with untreated cells; B: Effects of addition of *Lactobacillus plantarum* on the expression of the p-occludin and p-ZO-1 proteins as shown by relative band density. Data in the bar graph represent mean ± SD of the three separate experiments. ^a*P* < 0.05 Group II vs Group III; ^c*P* < 0.05 Group IV vs Group V. Group I : Sham-operation; Group II : Bile duct ligation (BDL); Group III : BDL + *Lactobacillus plantarum* (*L. plantarum*); Group IV : BDL + internal biliary drainage (IBD); Group V : BDL + IBD + *L. plantarum*.

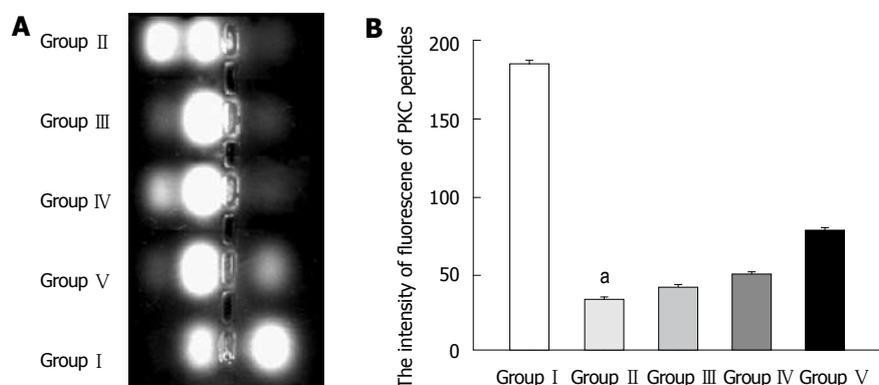


Figure 9 Effect of obstructive jaundice and probiotics on the activity of protein kinase C in the terminal ileum. A: A representative electrophoresis gel from the protein kinase C (PKC) activity assay; B: The averaged figures in each group of terminal ileum from the three separate experiments. ^a*P* < 0.05 vs each of the other four groups. Group I : Sham-operation; Group II : Bile duct ligation (BDL); Group III : BDL + *Lactobacillus plantarum* (*L. plantarum*); Group IV : BDL + internal biliary drainage (IBD); Group V : BDL + IBD + *L. plantarum*.

colitis. These studies support the findings that a number of probiotic agents have the protective effects in gastrointestinal tract as described in the current work.

Previous studies, including experimental models and clinical cases of biliary obstruction, have shown that disruption of intestinal barrier integrity in obstructive jaundice is associated with high intestinal oxidative stress, as evidenced by increased lipid peroxidation and oxidation of proteins, non-protein, and protein thiols^[6,30]. Increased intestinal oxidative stress, a factor in cellular injury, may also play a critical role in regulating important cellular alterations of the intestinal mucosa in obstructive jaundice, such as increased apoptosis and altered TJ expression^[5,31]. The results of this study strongly suggest that obstructive jaundice induces oxidative stress in the intestine.

We also found that apoptotic nuclei were significantly more abundant in markedly atrophic villi in the BDL group. Administration of *L. plantarum* significantly decreased apoptosis of the terminal ileum and improved the histology of the terminal ileum, which also was affected by obstructive jaundice. The protective effect of *L. plantarum* may be related to amelioration of oxidative stress. Previous studies of *L. rhamnosus* GG, a member of the same genus as *L. plantarum*, showed that this bacterium attenuated the H₂O₂-induced disruption of barrier function^[18]. Our studies also revealed that administration of *L. plantarum* significantly reduced the levels of GSSG, MDA and SOD in rats of the BDL + *L. plantarum* group and the BDL + IBD + *L. plantarum* group.

Intestinal epithelial TJs prevent diffusion of potential injurious factors from the gastrointestinal lumen into the tissue. TJs located at the subapical aspect of the lateral membranes contain a large number of membrane-associated proteins, including occludin, JAM and claudins, which are responsible for forming the physical connections between cells that confer the basic barrier properties. Using immunohistochemistry and immunoblotting, previous studies have demonstrated that intestinal mucosal barrier dysfunction in obstructive jaundice is associated with a regional loss of occludin expression in the intestinal epithelium^[4,31]. Similarly, our study showed that levels of TJ-associated proteins such as occludin, ZO-1, claudin-1, claudin-4 and JAM-A were reduced in the intestinal epithelium, especially at the upper part of villi. These data support the conclusion that oral *L. plantarum* administration can enhance the expression of TJ-associated proteins.

A significant body of evidence indicates that PKC is involved in the regulation of the integrity of TJs and AJs. Recent studies have shown differences between Tyr-phosphorylation and Ser/Thr-phosphorylation of occludin. Tyr-phosphorylation of occludin is clearly associated with the disruption of TJs. Ser/Thr-phosphorylation may be required for the assembly of occludin into the TJs. PKC- ζ prevents oxidant-induced iNOS upregulation and protects microtubules and gut barrier integrity^[32]. Thus, PKC- ζ appears to be an endogenous stabilizer of the microtubule cytoskeleton and of intestinal barrier function against oxidative injury^[33].

The probiotic bacterium *L. plantarum* has been shown to improve intestinal barrier function in a range of experimental models of colitis, pancreatitis, liver injury and biliary obstruction^[8,34-37]. Recent studies have shed some light on the mechanisms involved in the beneficial effects of probiotics in the gastrointestinal tract. PKC activity may be involved in epidermal growth factor-mediated protection of the intestinal epithelial barrier function against oxidative stress^[38]. Seth *et al.*^[18] suggested that PKC β I activation may be one of the initial events in the probiotic-mediated protection of TJs and AJs. PKC ϵ may play a role in the downstream events of the signaling pathway involved in this process. TJ-protein phosphorylation mediated by PKC may be related to the mechanism of protection by *L. plantarum* in obstructive jaundice. Previous studies have shown that phosphorylation is a key mechanism for regulating the biological function of TJ proteins. Highly phosphorylated occludin molecules are selectively concentrated at TJs, whereas non- or less phosphorylated occludin molecules are localized in the cytoplasm^[39].

To determine whether PKC mediates the disruption of the intestinal barrier in obstructive jaundice, we examined the phosphorylation status of occludin and ZO-1 using Western blotting analysis with antibodies against phosphoserine. We found that obstructive jaundice decreased p-occludin and p-ZO-1 protein levels compared with sham-operation. Our study also demonstrated that obstructive jaundice resulted in a significant decrease in PKC activity. Co-incubation with *L. plantarum* partly restored PKC activity and increased phosphorylation of serine residues on TJ proteins in both the BDL + *L. plantarum* group and the BDL + IBD + *L. plantarum* group. Phosphorylation of these proteins occurred on Ser residues that have been described as substrates for PKC activity^[40-42]. Our results suggest that the PKC pathway is involved in the process of *L. plantarum*-induced TJ redistribution.

In conclusion, administration of probiotics before and after operation in rats with experimental obstructive jaundice can substantially protect the gut barrier. The protective mechanisms of probiotics are associated with decreased intestinal epithelial cell apoptosis, reduction of oxidative stress, and protection of intestinal mucosal TJs. *L. plantarum* can prevent TJ disruption in biliary obstruction by activating the PKC pathway.

COMMENTS

Background

Biliary tract surgery in patients with obstructive jaundice is associated with a high morbidity and mortality rate, and obstructive jaundice increased gut permeability and bacterial translocation. *Lactobacillus plantarum* (*L. plantarum*) has been shown to have beneficial effects on intestinal barrier function. Protein kinase C (PKC) plays a crucial role in the mediation of intestinal epithelial tight junction (TJ) proteins, and *L. plantarum* may prevent TJ disruption in biliary obstruction by activating the PKC pathway. However, there had been few studies about the mechanism for the protective effect of probiotics on the intestinal barrier in obstructive jaundice. This study focused on the effects of *L. plantarum* on the intestinal mucosal barrier, oxidative stress, epithelial TJ-protein structure

and phosphorylation, especially its impact on the expression and activity of PKC.

Research frontiers

Previous *in vitro* studies have confirmed that probiotics could protect intestinal epithelial cell TJs via a PKC-kinase-dependent mechanism and inhibit epithelial cell apoptosis in cell culture experiments. TJ-protein phosphorylation mediated by PKC may be related to the protective effects of *L. plantarum* in obstructive jaundice.

Innovations and breakthroughs

The administration of *L. plantarum* before and after operation in rats with experimental obstructive jaundice could substantially protect the gut barrier. Protective mechanisms of *L. plantarum* are associated with decreased intestinal epithelial cell apoptosis, reduction of oxidative stress, and protection of intestinal mucosal TJs. *L. plantarum* can prevent TJ disruption in biliary obstruction by activating the PKC pathway.

Applications

By understanding the mechanism and effects of *L. plantarum* on the intestinal mucosal barrier, this study may represent a future strategy in the treatment of patients with obstructive jaundice.

Peer review

This is a very well done experimental study for evaluating the effect of *L. plantarum* on the intestinal mucosal barrier, oxidative stress, epithelial TJ protein structure and phosphorylation, with special regard to its impact on the expression and activity of PKC in experimental obstructive jaundice.

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Diagnostic and therapeutic direct peroral cholangioscopy using an intraductal anchoring balloon

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Abstract

AIM: To report our experience using a recently introduced anchoring balloon for diagnostic and therapeutic direct peroral cholangioscopy (DPOC).

METHODS: Consecutive patients referred for diagnostic or therapeutic peroral cholangioscopy were evaluated in a prospective cohort study. The patients underwent DPOC using an intraductal anchoring balloon, which was recently introduced to allow consistent access to the biliary tree with an ultraslim upper endoscope. The device was later voluntarily withdrawn from the market by the manufacturer.

RESULTS: Fourteen patients underwent DPOC using the anchoring balloon. Biliary access with an ultraslim upper endoscope was accomplished in all 14 patients. In 12 (86%) patients, ductal access required sphincteroplasty with a 10-mm dilating balloon. Intraductal placement of the ultraslim upper endoscope allowed satisfactory visualization of the biliary mucosa to the level of the confluence of the right and left hepatic ducts in 13 of 14 patients (93%). Therapeutic interventions by DPOC were successfully completed in all five attempted cases (intraductal biopsy in one and DPOC guided laser

lithotripsy in four). Adverse events occurred in a patient on immunosuppressive therapy who developed an intrahepatic biloma at the site of the anchoring balloon. This required hospitalization and antibiotics. Repeat endoscopic retrograde cholangiopancreatography 8 wk after the index procedure showed resolution of the biloma.

CONCLUSION: Use of this anchoring balloon allowed consistent access to the biliary tree for performance of diagnostic and therapeutic DPOC distal to the biliary bifurcation.

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Key words: Anchoring balloon; Direct peroral cholangioscopy; Cholangiocarcinoma; Endoscopic retrograde cholangiopancreatography; Choledocholithiasis

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INTRODUCTION

The value of cholangioscopy for diagnosis and treatment of biliary disorders has been well established^[1]. Diagnostic and therapeutic direct peroral cholangioscopy (DPOC) using an ultraslim upper endoscope offers significant advantages over ductoscopy using dedicated cholangioscopes^[2]. The ultraslim endoscope uses a single-operator platform, provides high-definition digital image quality, allows simultaneous irrigation and therapy,

is not fragile, and has a larger working channel enabling enhanced diagnostic sampling and therapeutic interventions. However, DPOC in its current state has significant limitations. Initial free biliary cannulation is cumbersome, time consuming, and associated with a significant failure rate. Even when access is obtained, intraductal maneuverability and stability may be compromised by looping of the endoscope in the stomach or duodenum. To address these technical challenges, a prototype biliary anchoring balloon has been designed. Feasibility of this system in an animal model has been assessed but human reports are lacking^[2].

We performed a study in 14 patients to assess utility of this device for diagnosis and treatment of various biliary disorders prior to voluntary device withdrawal by the manufacturer. We report our experience using this device for ductal access in all 14 patients.

MATERIALS AND METHODS

Study aims and patients

This was a prospective cohort study of consecutive patients referred to our tertiary care center for endoscopic retrograde cholangiopancreatography (ERCP) and peroral cholangioscopy. The primary aim of the study was to assess the ability to gain access to the bile duct with an ultraslim upper endoscope and visualize the extrahepatic biliary mucosa to the level of the confluence of the left and right hepatic ducts. The secondary aim was to examine the feasibility of performing therapeutic procedures through the endoscope while maintaining intraductal access. This study was approved by The Cleveland Clinic Institutional Review Board. The inclusion criteria were presence of biliary pathology and ability of the patients to give informed consent. The exclusion criteria were coagulopathy, suspicion for acute ascending cholangitis, latex allergy, and biliary ductal diameter < 6 mm.

Anchoring balloon

The anchoring balloon used in this study (Cook Medical, Winston-Salem, NC, United States) had five components. A short stylet located at the most proximal end of the device was used to occlude the air channel after the balloon was inflated. This prevented deflation of the balloon after removal of the handle. Immediately distal to the stylet, there was a handle, which could be detached and removed to allow backloading of the device into an ultraslim upper endoscope while maintaining balloon inflation. The device had a 300-cm long, 4-French catheter with a nitinol stiffening core. At the distal end of the catheter, there was a latex balloon that could be inflated to 15 mm and used as an intraductal anchoring point. A radiopaque loop tip was located at the most distal end of the device and allowed positioning of the balloon in the desired duct under fluoroscopic guidance over a guidewire (Figure 1A).

Cholangioscopy procedure

All procedures were performed by an experienced endos-

copist, under monitored anesthesia care sedation with the patients in the prone position. Endoscopic sphincterotomy was or had been performed previously prior to direct peroral cholangioscopy in all patients. Balloon sphincteroplasty to 10 mm was performed as needed. After completion of ERCP, a 0.889-mm guidewire was placed in one of the intrahepatic ducts. The anchoring balloon was then directed into that intrahepatic duct by placing the loop end of the device over the guidewire. Location of the guidewire and the anchoring balloon was verified by fluoroscopy (Figure 1B). The balloon was then inflated with air to anchor it within the duct. A gentle pull on the balloon catheter confirmed ductal anchoring. If the pull on the catheter led to dislodgment of the balloon, it was deflated and the procedure was repeated to reposition the balloon in another intrahepatic duct followed by inflation of the balloon and pulling to confirm anchoring. Once anchoring of the balloon was confirmed, the air channel of the device was covered with the stylet to keep the balloon inflated. The proximal handle was then detached from the catheter and removed. This was followed by removal of the duodenoscope and the guidewire, leaving the inflated balloon and its catheter behind. An ultraslim upper endoscope was backloaded over the catheter and advanced into the bile duct to the level of the confluence of the right and left hepatic ducts (Figure 1C). The anchoring balloon was then removed in most cases. The bile duct was irrigated through the accessory channel of the ultraslim endoscope with sterile saline solution, followed by slow withdrawal of the endoscope, allowing systematic inspection of the biliary tree. Air insufflation was not used, to avoid potential complications. Routine antibiotic prophylaxis was not administered. The ultraslim endoscopes used in this study included GIF-XP160 (outer diameter 5.9 mm), GIF-XP180 (outer diameter 5.5 mm), and GIF-N180 (outer diameter 4.9 mm) (Olympus Corporation, Center Valley, PA United States). All endoscopes had an instrument channel with an inner diameter of 2 mm.

Statistical analysis

Values are presented as mean (range) or frequency (percentage). R version 2.4.1 software (The R Foundation for Statistical Computing, Vienna, Austria) was used to perform all the analyses.

RESULTS

Patients and indications

Fourteen consecutive patients underwent DPOC using the new anchoring balloon. The mean age of the patients was 65 years (range: 30-92 years). Nine (64%) patients were female. The indications for the procedure, DPOC findings and final diagnosis are presented in Table 1.

Biliary access

Biliary access with an ultraslim upper endoscope was accomplished in all patients. In 12 of 14 (86%) patients, biliary access required sphincteroplasty with a 10-mm

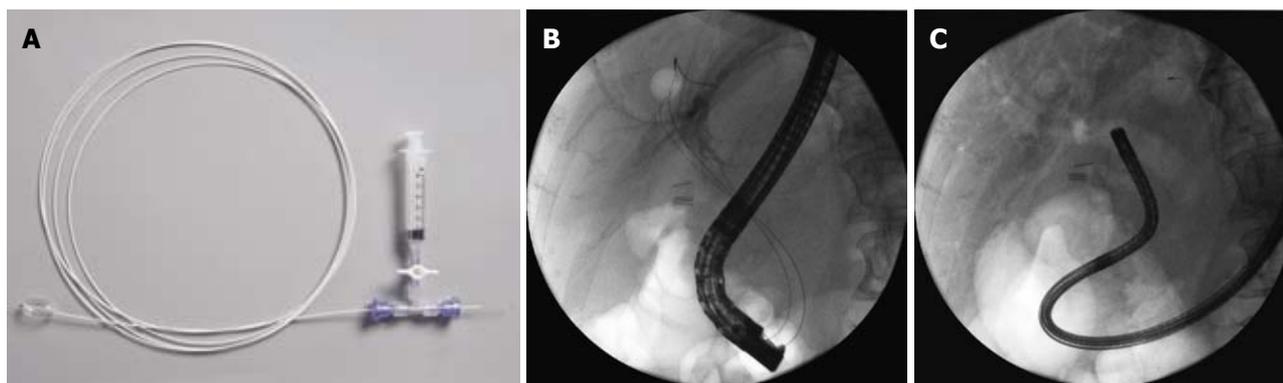


Figure 1 *Ex vivo* and *in vivo* views of the anchoring balloon. A: Overall view of the anchoring balloon; B: Fluoroscopic view of an inflated anchoring balloon that has been passed over a guidewire and anchored in one of the intrahepatic bile ducts; C: Fluoroscopic view of an ultraslim upper endoscope that has been backloaded over the catheter of an inflated anchoring balloon and advanced into the bile duct to the level of the confluence of the right and left hepatic ducts.

Indication	DPOC diagnosis	Final diagnosis	Performance of sphincteroplasty	Largest bile duct diameter (mm)
Obstructive jaundice	Extrinsic stricture	Pancreatic cancer	Yes	18
Obstructive jaundice	Extrinsic stricture	Chronic pancreatitis	No	12
Suspicion for missed stones by ERCP	Stone in the main bile duct	Missed stone by ERCP	Yes	17
Difficult-to-remove bile duct stone	Large CBD stone	DPOC-guided laser lithotripsy followed by stone removal	Yes	11
Biliary stricture in a patient with PSC	Benign stricture	Benign PSC stricture	Yes	10
Biliary filling defect on MRI	Intraductal tumor	Intrahepatic cholangiocarcinoma with intraductal extension	Yes	10
Suspicion for missed stone(s) by ERCP	No stones found		Yes	8
Abnormal LFTs in a post liver transplantation patient	Missed stones during ERCP	Missed stones during ERCP	No	11
Surveillance after biliary polypectomy	Complete removal of the polyp		Yes	9
Difficult-to-remove bile duct stones	Four stones in the main bile duct	DPOC-guided laser lithotripsy followed by stone removal	Yes	13
Suspected polyp in CBD	Stone in CBD	CBD stone	Yes	20
Suspicion for missed stones by ERCP	No stones found		Yes	15
Difficult-to-remove bile duct stone	Large CBD stones	DPOC-guided laser lithotripsy followed by stone removal	Yes	22
Difficult-to-remove bile duct stone	Large stone above anastomotic stricture	DPOC-guided laser lithotripsy followed by stone removal	Yes	16

DPOC: Diagnostic and therapeutic direct peroral cholangioscopy; ERCP: Endoscopic retrograde cholangiopancreatography; CBD: Common bile duct; LFTs: Liver function tests; PSC: Primary sclerosing cholangitis; MRI: Magnetic resonance imaging.

dilating balloon over a guidewire. In 13 of 14 (93%) patients, the bile duct mucosa from the bifurcation to the ampulla could be well visualized. In one patient, the common hepatic duct could not be examined due to a sigmoid-shaped main bile duct that prevented passage of the endoscope proximal to the common bile duct. In this patient, only the mucosa of the common bile duct was inspected.

Biliary intervention

Therapeutic measures were attempted in five patients. Four patients underwent removal of difficult-to-remove bile duct stones and one patient had intraductal biopsy.

The four patients with difficult-to-remove stones had at least one prior unsuccessful attempt at stone extraction by ERCP with sphincterotomy, sphincteroplasty, and mechanical lithotripsy. In all cases, laser lithotripsy through

the ultraslim upper endoscope successfully fragmented the stones, with subsequent removal of the fragments (Figure 2). In one of these cases, however, instability of the ultraslim upper endoscope after removal of the anchoring balloon, required passage of the laser probe (SlimLine GI, Lumenis, Santa Clara, CA, United States) into the bile duct alongside the anchoring balloon, while keeping the balloon inflated in one of the intrahepatic ducts to maintain access. Nonetheless, the procedure was successful with excellent views of the biliary mucosa and performance of laser lithotripsy under direct vision with fragmentation and subsequent removal of the stone fragments.

In one patient, DPOC was performed to evaluate an ill-defined filling defect at the biliary confluence with intrahepatic ductal dilatation seen on magnetic resonance imaging. DPOC successfully visualized the filling defect

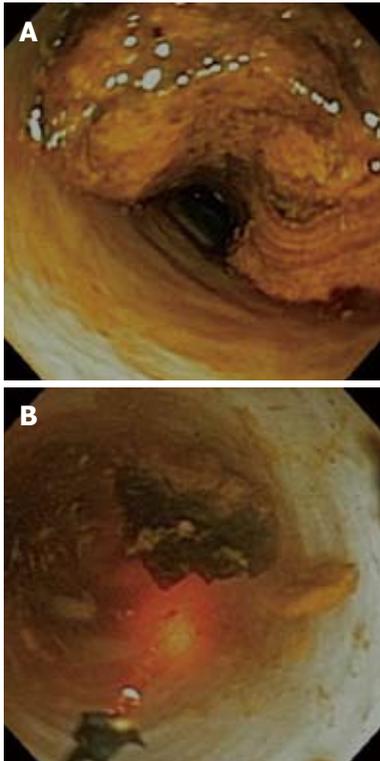


Figure 2 Cholangioscopic view of a large stone. A: The stone could not be removed during prior endoscopic retrograde cholangiopancreatography. A plastic stent was placed and the patient was referred for cholangioscopy-guided laser lithotripsy. Impression of the plastic stent in the stone is clearly visible; B: Cholangioscopic view of the same stone after laser lithotripsy. The laser probe is seen in the left lower corner of the picture.

and showed it to be a tumor projecting into the bile duct lumen (Figure 3). Biopsies of the tumor were obtained through the ultraslim upper endoscope using a regular pediatric forceps (EndoJaw FB-221K; Olympus Corporation, Tokyo, Japan) and showed necrotic tissue. Follow-up imaging studies showed growing intraductal tumor suggesting cholangiocarcinoma.

Adverse events

One patient who had undergone orthotopic liver transplantation 2 years prior to the procedure, and was on immunosuppressive therapy, developed intrahepatic biloma at the site where the balloon was anchored within the duct (Figure 4). She underwent biliary stenting to assure ductal drainage. She also was given an intravenous dose of antibiotics during the procedure and discharged home on oral antibiotics and outpatient follow-up. However, 2 d after discharge she developed fever. She was admitted to the hospital and treated with intravenous antibiotics followed by 2 wk of oral antibiotics after discharge. Repeat ERCP 8 wk after the index procedure showed resolution of the biloma.

DISCUSSION

Despite its many advantages, DPOC is rarely performed in nonacademic settings. The biggest disadvantage of DPOC has been the difficult and time-consuming task



Figure 3 Cholangioscopic image of a tumor in the proximal common hepatic duct. The anchoring balloon catheter is seen in the left lower corner of the picture.



Figure 4 Biloma at the site of the anchored balloon within an intrahepatic bile duct.

of bile duct cannulation with an upper endoscope, often ending in failure. There are several published reports in the endoscopic literature with innovative suggestions on how to achieve this task. Introduction of the endoscope over a guidewire, through a regular overtube, or with the help of a double-balloon overtube are some of the suggestions^[3-6]. However, despite use of these accessories, failure rate remains high^[7].

Different variations of inflatable balloons used as an anchor within the biliary tree have therefore been introduced for easier access^[2,8]. Using such a device, we were able to gain access to the bile duct with an ultraslim upper endoscope in all cases and obtain high-quality images of the biliary mucosa. We were also able to perform necessary interventions such as shock wave lithotripsy or targeted biopsy in all cases in which therapy was attempted. In one of the therapeutic cases, however, the instability of the ultraslim endoscope upon removal of the balloon could only be remedied by keeping the anchoring balloon in position.

Although this device performed well for allowing access to the bile duct for performance of DPOC and assessment and therapy of disorders of the distal biliary system, we found it difficult to maneuver the endoscope and gain access to the ducts proximal to the bifurcation after deflation and removal of the anchoring balloon.

This was mainly due to looping of the endoscope in the stomach or duodenum after removal of the balloon.

The anchoring balloon used in this study was voluntarily withdrawn from the market by the manufacturing company, reportedly because of possible increased risk of air embolism^[9]. The possible increased risk of air embolism is probably due to the ability of the ultraslim upper endoscopes to insufflate the biliary tree with air, while at the same time blocking the escape route of the insufflated air. In this study, we used irrigation with saline rather than air to distend the bile duct and visualize the mucosa of the biliary tree.

One of the patients developed a biloma at the anchoring site of the balloon. Although she had a full recovery with antibiotic therapy, ductal damage at the anchoring site may be another potential adverse event associated with use of anchoring balloons.

One of the main limitations of this study was the small number of patients. However, the primary objective of this study was to assess performance of this device in a limited number of patients. Another limitation was performance of the procedures in a tertiary care referral setting by an endoscopist proficient in all forms of peroral and percutaneous cholangioscopy. This may limit extrapolation of the results to other settings.

In conclusion, our experience suggests that anchoring balloons are effective for consistent access to the bile duct with an ultraslim upper endoscope for performance of diagnostic and therapeutic DPOC distal to the confluence of the right and left hepatic ducts. However, we urge caution with widespread use of anchoring balloons until more information on potential adverse effects is available.

COMMENTS

Background

Direct peroral cholangioscopy (DPOC) in its current state has significant limitations. Initial free biliary cannulation is cumbersome, time consuming, and associated with a significant failure rate. Even when access is obtained, intraductal maneuverability and stability may be compromised by looping of the endoscope in the stomach or duodenum.

Research frontiers

To address the technical challenges associated with DPOC, a prototype biliary anchoring balloon has been designed. Feasibility of this system in an animal model has been assessed but human reports are lacking. This is the first study assessing utility of this prototype anchoring balloon for diagnosis and treatment of various biliary disorders.

Innovations and breakthroughs

Despite its many advantages, DPOC is rarely performed in nonacademic

settings. The biggest disadvantage of DPOC has been the difficult and time-consuming task of bile duct cannulation with an upper endoscope, often ending in failure. There are several published reports in the endoscopic literature with innovative suggestions on how to achieve this task. Introduction of the endoscope over a guidewire, through a regular overtube, or with the help of a double-balloon overtube are some of the suggestions. However, despite use of these accessories, the failure rate remains high. Using an anchoring balloon, the authors were able to gain access to the bile duct with an ultraslim upper endoscope in all cases and obtain high-quality images of the biliary mucosa. They were also able to perform necessary interventions such as shock wave lithotripsy or targeted biopsy in all cases in which therapy was attempted.

Applications

This study suggests that anchoring balloons are effective for consistent access to the bile duct with an ultraslim upper endoscope for performance of diagnostic and therapeutic DPOC distal to the confluence of the right and left hepatic ducts.

Peer review

The authors report clinical experiences of DPOC by using a novel balloon catheter as an assisting accessory. This is a well-written article. However, it is necessary that the authors offer more detailed descriptions and revise several major and minor points.

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Age distribution, polyps and rectal cancer in the Egyptian population-based cancer registry

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Abstract

AIM: To describe the clinical and epidemiologic profiles of the disease and to compare the findings with those generated from the previous hospital-based studies.

METHODS: The Gharbiah cancer registry is the only population-based cancer registry in Egypt since 1998. We analyzed the data of all colorectal cancer patients

included in the registry for the period of 1999–2007. All medical records of the 1364 patients diagnosed in Gharbiah during the study period were retrieved and the following information abstracted: age, residence, diagnosis date, grade, stage, topology, clinical characteristics, and histology variables. Egyptian census data for 1996 and 2006 were used to provide the general population's statistics on age, sex, residence and other related demographic factors. In addition to age- and sex-specific incidence rate analyses, we analyze the data to explore the incidence distribution by rural-urban differences among the 8 districts of the province. We also compared the incidence rates of Gharbiah to the rates of the Surveillance Epidemiology and End Results (SEER) data of the United States.

RESULTS: Over the 9 year-period, 1364 colorectal cancer cases were included. The disease incidence under age 40 years was relatively high ($1.3/10^5$) while the incidence in the age groups 40 and over was very low ($12.0/10^5$, $19.4/10^5$ and $21.2/10^5$ in the age groups 40–59 years, 60–69 years and > 70 years, respectively). The vast majority of tumors (97.2%) had no polyps and 37.2% of the patients presented with primary lesions in the rectum. Colorectal cancer was more common in patients from urban (55%) than rural (45%) areas. Regional differences in colon and rectal cancer incidence in the 8 districts of the study province may reflect different etiologic patterns in this population. The registry data of Egypt shows a slightly higher incidence of colorectal cancer than the United States in subjects under age 40 years. The results also shows significantly lower incidence of colorectal cancer in subjects over age 40 years compared to the same age group in the United States SEER.

CONCLUSION: Low rate of polyps, low incidence in older subjects, and high rate of rectal cancer in Egypt. Future studies should explore clinical and molecular disease patterns.

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Key words: Colorectal cancer; Young-onset; Polyps; Developing countries; Egypt

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INTRODUCTION

Colorectal cancer is a common cancer worldwide. In 2008, Globocan estimated there were 663 000 new cases in men and 571 000 new cases in women^[1,2]. With a combined 608 000 deaths worldwide, colorectal cancer represents the fourth most common cause of cancer-related mortality. There is a wide variability in the incidence rates of colorectal cancer, with most incident cases occurring in developed countries^[1]. Incidence rates of colorectal cancer for men range from 4.1/10⁵ in Karunagappally, India, to 59.1/10⁵ in the Czech Republic. In women, incidence rates range from 3.6/10⁵ in Karunagappally, India, up to 39.5/10⁵ in New Zealand^[2]. Rates for the United States are 34.1/10⁵ for men and 25/10⁵ for women^[1].

Westernization is often associated with higher incidence rates of colorectal cancer. Diet and lifestyle factors are implicated risk factors for the disease. Fruit and vegetable-deficient diet, calorie-dense foods, physical inactivity, obesity, and smoking increase the risk for developing colorectal cancer^[2]. While developing countries historically have a low rate of colorectal cancer, the transition to a more Western diet has been associated with increasing rates of disease^[3,4].

Our previous hospital-based studies in Egypt showed low incidence of colorectal cancer and high proportion of young-onset disease^[5]. In comparing rates of Egyptian colorectal cancer to the Surveillance Epidemiology and End Results Program (SEER) of the United States, Egypt had higher rates up to age 30-34 years, at which point Egyptian rates level out while United States rates increase sharply^[5]. Our studies also revealed low rate of polyps, high proportion of rectal cancers among the colorectal cancer tumors, and lack of molecular characteristics of hereditary non-polyposis colorectal cancer (HNPCC) or young-onset or sporadic colorectal cancer in the United States^[5-7]. Our studies have also shown intense environmental exposures such as organochlorine pesticide levels, however, these environmental and genetic factors were not related to the young or old onset of the disease^[8].

Limitations of the previous studies on colorectal cancer in Egypt included their hospital-based nature and relatively small sample size. Therefore, with the establish-

ment of Egypt's only population-based cancer registry and availability of complete data from 1999-2007, it was intriguing to investigate the epidemiologic and clinical profiles of colorectal cancer and examine the previous findings based on the new population-based registry data.

MATERIALS AND METHODS

The Gharbiah province is in the center of the Nile delta region about 90 km north of Cairo with a population of about 4 million individuals^[9]. It has a male:female ratio of 1.02:1 and age structure approximately equivalent to that of the rest of Egypt^[10].

The Gharbiah population-based cancer registry, the only population-based registry in Egypt was founded in 1998^[9]. It actively collects information on all cancer cases in the province from 3 main cancer hospitals; Tanta Cancer Center, the Gharbiah Cancer Society Hospital, and the Tanta University Hospital. In addition, information on cancer patients is collected from all private clinics and laboratories throughout the province^[9]. The registry receives support and training from the National Cancer Institute (NCI) in Bethesda, Maryland through the Middle East Cancer Consortium (MECC). Technical support, training, and quality control of data are periodically conducted by Rollins School of Public Health, the International Agency for Research on Cancer, and NCI to ensure the high quality of the registry data^[9,11].

All colorectal cancers diagnosed from 1999 to 2007 were included in the study. Medical records of 1364 patients were retrieved and the following information abstracted: age, residence, diagnosis date, grade, stage, topology, clinical characteristics and histology variables. Egyptian census data were used to provide the general population's statistics on age, sex, residence, and other related demographic factors. The 1364 cases included in this study over the 9 years from 1999-2007 average out to about 150 cases a year, which is in keeping with the registry's previous preliminary reports^[9,12,13].

The student's *t*-test was used to determine the significance of differences in mean values of the study variables. A χ^2 test for independence was used to determine the significance of differences in frequency distributions and proportions of variables. The cut-off value for statistical significance was *P* value = 0.05. SAS version 9.2 (SAS Institute, Cary, NC) was used.

RESULTS

The results of the study provide a comprehensive profile of colorectal cancer in this population. This profile sheds some lights on the epidemiologic, clinical, and geographic distribution of colorectal cancer in this population. The results also demonstrate the differences in colorectal cancer incidence between the population-based registry of Gharbiah and the SEER registry of the United States.

A total of 1364 cases of colorectal cancer were included in registry from 1999-2007. Table 1 shows the summary characteristics for the colorectal cancer patients.

Table 1 Characteristics of study population and cancer patterns in Gharbiah, Egypt (1999-2007)

Variable	n (%)
Total cases	1364 (100.0)
Gender	
Male	737 (54.0)
Female	627 (46.0)
Residence	
Urban	752 (55.1)
Rural	612 (44.9)
Year of diagnosis	
1999	135 (9.9)
2000	149 (10.9)
2001	144 (10.6)
2002	154 (11.3)
2003	151 (11.1)
2004	137 (10.0)
2005	161 (11.8)
2006	171 (12.5)
2007	162 (11.9)
District of residence	
Tanta	437 (32.0)
El Mehalla	355 (26.0)
Kafr El Zayat	118 (8.7)
Zefta	98 (7.2)
Samanoud	82 (6.0)
El Santa	103 (7.6)
Kotour	78 (5.7)
Basyoon	93 (6.8)

Slightly more than half the cases were males (54%), giving a male:female ratio of 1.2:1. Patients designated as residing in an urban area constituted 55.1% of all patients. In any given year, between 135 -171 new cases of colorectal cancer were included in the registry. The Tanta district of the Gharbiah province had the most cases (32.0%) with the Kotour district seeing the least (5.7%).

The clinical and histopathologic characteristics of the colorectal cancer patients included in the study are illustrated in Table 2. Age and tumor site distribution of cases showed that 22.0% of all cases were under the age of 40 and 62.8% of cases had primary lesions in the colon with 37.2% having primary lesions in the rectum. The majority of tumors were grade II moderately-differentiated tumors (51.5% of cases) followed by 11.4% grade III. About 25.7% cases had no grade information. The vast majority of patients did not present with polyps (97.2%) though of the 38 patients who had polyps, 76.3% were over the age of 40. Mucinous carcinomas were present in only 23.3% of cases and over two-thirds of those patients (67.9%) were patients over the age of 40 years. Adenocarcinoma was the most common histopathologic type of tumors (87.0%).

Table 3 shows colorectal cancer incidence rates and incidence rate ratios by gender and urban-rural status. For both men and women, living in an urban area significantly increased the risk of developing colorectal cancer (either in the colon or the rectum). The same trend was present looking only at primary lesions in the colon and less pronounced for rectal cancers. Older urban men were more likely to develop rectal cancer while young urban men were more likely to develop colon cancer.

Table 2 Age and clinical characteristics of study population in Gharbiah, Egypt (1999-2007)

Variable	n (%)
Age (yr)	
<40	300 (22.0)
≥40	1064 (78.0)
Basis of diagnosis	
Histology of primary	1210 (88.7)
Histology of metastases	58 (4.3)
Death certificate only	52 (3.8)
Others ¹	44 (3.2)
Tumor site	
Colon	856 (62.8)
Right	347 (40.5)
Left	331 (38.7)
NOS	178 (20.8)
Rectum	508 (37.2)
Grade	
I	96 (7.0)
II	702 (51.5)
III	155 (11.4)
IV	61 (4.5)
Unknown	350 (25.7)
Polyps	
Present	38 (2.8)
< 40 yr	9 (23.7)
≥ 40 yr	29 (76.3)
Not present	1326 (97.2)
Mucinous carcinoma	
Present	318 (23.3)
< 40 yr	102 (32.1)
≥ 40 yr	216 (67.9)
Not present	1046 (76.7)
Histopathology	
Adenocarcinoma	1186 (87.0)
Carcinomas ²	50 (3.7)
Other specified types of cancers	16 (1.2)
Unspecified types of cancers	112 (8.2)

¹Others include clinical only (2 cases), clinical/ultrasound/X-Ray (29 cases), exploratory surgery/autopsy (5 cases), specific biochemistry/immunology. test (1 case), cytology/hematology (5 cases), and unknown (2 cases); ²carcinomas include squamous cell (7 cases) and other carcinomas (43 cases). NOS: Not otherwise specified.

The comparison of the incidence rates and incidence rate ratios of colorectal cancer across the 8 districts are shown in Table 4 for patients under the age of 40 years and those 40 years and older. Compared to the low rates of the Zefta district, living in Tanta and Basyoon districts was significantly associated with increased risk of developing colorectal cancer for both young and old subjects. Living in El Mehalla and Kafr El Zayat was associated with significantly increased risk of developing colorectal cancer for subjects over 40 years only.

Compared to the low rates of colon or rectal cancers in the Zefta district, living in Tanta and Basyoon districts was associated with significantly increased risk of developing both primary colon and rectal cancers. Living in El Mehalla and Kafr El Zayat districts was significantly associated with increased risk of developing colon but not rectal cancer (Table 4).

Table 5 compares age standardized incidence rates by

Table 3 Incidence rates (per 10⁵) and incidence rate ratios (95% confidence interval) by gender and urban-rural status for colorectal cancer patients in Gharbiah (1999-2007)

Age (yr)	Urban incidence		Rural incidence		Urban-rural IRR (95% CI)	
	Male	Female	Male	Female	Male	Female
Total						
5-9	0.17	0.00	0.00	0.00	-	-
10-14	0.00	0.35	0.00	0.16	-	2.23 (0.31, 15.82)
15-19	1.00	0.17	0.78	0.52	1.27 (0.47, 3.45)	0.32 (0.04, 2.59)
20-24	2.70	2.29	0.77	0.77	3.50 (1.57, 7.80)	2.97 (1.25, 7.05)
25-29	3.44	2.27	1.29	1.22	2.67 (1.30, 5.47)	1.86 (0.80, 4.30)
30-34	4.47	4.39	3.40	1.16	1.31 (0.74, 2.34)	3.79 (1.74, 8.28)
35-39	6.56	4.55	3.54	3.81	1.85 (1.07, 3.20)	1.19 (0.64, 2.24)
40-44	12.03	17.22	5.60	3.86	2.15 (1.35, 3.42)	4.46 (2.70, 7.35)
45-49	23.67	16.61	7.11	4.47	3.33 (2.22, 4.99)	3.72 (2.24, 6.16)
50-54	33.30	30.36	11.92	10.10	2.79 (1.93, 4.05)	3.00 (2.05, 4.40)
55-59	32.06	24.93	7.65	9.28	4.19 (2.66, 6.60)	2.69 (1.76, 4.11)
60-64	41.86	29.89	13.24	11.55	3.16 (2.03, 4.92)	2.59 (1.60, 4.18)
65-69	49.14	24.40	12.27	9.04	4.01 (2.34, 6.86)	2.70 (1.47, 4.95)
70-74	45.38	39.93	16.36	7.79	2.77 (1.43, 5.39)	5.13 (2.44, 10.77)
75+	40.95	36.28	16.36	11.99	2.50 (1.21, 5.19)	3.03 (1.52, 6.03)
Colon						
5-9	0.17	0.00	0.00	0.00	-	-
10-14	0.00	0.17	0.00	0.16	-	1.11 (0.10, 12.29)
15-19	0.33	0.17	0.50	0.52	0.67 (0.14, 3.21)	0.32 (0.04, 2.59)
20-24	1.80	2.10	0.39	0.43	4.67 (1.60, 13.67)	4.90 (1.70, 14.11)
25-29	1.72	1.59	0.55	0.51	3.12 (1.08, 8.98)	3.12 (0.99, 9.83)
30-34	2.98	2.32	1.70	0.58	1.75 (0.83, 3.70)	4.01 (1.34, 11.97)
35-39	4.56	2.42	2.32	2.45	1.97 (1.01, 3.83)	0.99 (0.43, 2.28)
40-44	7.78	8.61	3.63	2.18	2.14 (1.20, 3.82)	3.94 (2.00, 7.79)
45-49	14.95	10.79	5.69	2.61	2.63 (1.63, 4.23)	4.14 (2.16, 7.93)
50-54	22.38	15.67	7.48	6.59	2.99 (1.89, 4.75)	2.38 (1.44, 3.91)
55-59	22.81	16.97	5.54	5.95	4.12 (2.41, 7.03)	2.85 (1.69, 4.81)
60-64	28.21	21.59	6.62	5.96	4.26 (2.36, 7.70)	3.62 (1.94, 6.75)
65-69	27.30	14.85	6.43	5.24	4.25 (2.04, 8.86)	2.84 (1.29, 6.25)
70-74	26.27	26.04	12.27	4.67	2.14 (0.95, 4.85)	5.57 (2.16, 14.36)
75+	30.03	32.46	10.52	6.85	2.86 (1.18, 6.89)	4.74 (2.04, 10.98)
Rectum						
5-9	0.00	0.00	0.00	0.00	-	-
10-14	0.00	0.17	0.00	0.00	-	-
15-19	0.66	0.00	0.28	0.00	2.34 (0.58, 9.34)	-
20-24	0.90	0.19	0.39	0.34	2.34 (0.68, 8.07)	0.56 (0.06, 4.99)
25-29	1.72	0.68	0.74	0.71	2.34 (0.88, 6.22)	0.96 (0.25, 3.69)
30-34	1.49	2.06	1.70	0.58	0.88 (0.34, 2.24)	3.57 (1.17, 10.90)
35-39	2.00	2.12	1.22	1.36	1.64 (0.62, 4.30)	1.56 (0.59, 4.10)
40-44	4.25	8.61	1.97	1.68	2.16 (0.98, 4.73)	5.13 (2.44, 10.77)
45-49	8.72	5.81	1.42	1.86	6.13 (2.72, 13.85)	3.12 (1.39, 7.03)
50-54	10.92	14.69	4.44	3.51	2.46 (1.31, 4.61)	4.18 (2.28, 7.67)
55-59	9.25	7.96	2.11	3.33	4.38 (1.86, 10.33)	2.39 (1.15, 4.95)
60-64	13.65	8.30	6.62	5.59	2.06 (1.03, 4.13)	1.49 (0.67, 3.31)
65-69	21.84	9.55	5.84	3.81	3.74 (1.70, 8.24)	2.51 (0.97, 6.50)
70-74	19.11	13.89	4.09	3.12	4.67 (1.41, 15.52)	4.46 (1.34, 14.80)
75+	10.92	3.82	5.84	5.14	1.87 (0.50, 6.96)	0.74 (0.15, 3.68)

IRRs: Incidence rate ratios; 95% CI: 95% confidence intervals.

gender of the Gharbiah registry and the United States SEER data. Incidence rate of colorectal cancer was 5.5/10⁵ in Gharbiah (6.1/10⁵ for males, 4.9/10⁵ for females). These rates were significantly lower than the colorectal cancer incidence rates seen in the United States of 32.0/10⁵ (37.7/10⁵ for males, 27.4/10⁵ for females). While incidence rate of colorectal cancer for those under age 40 years in Gharbiah was slightly higher than the United States incidence rate for the same age group, the incidence rates for subjects 40 years and older in the United

States were significantly higher than the corresponding rates for the same age groups in Egypt.

DISCUSSION

Analysis of the 1364 cases of colorectal cancer collected at the Gharbiah population-based cancer registry from 1999-2007 revealed the following important findings: First, a relatively high incidence of colorectal cancer in young subjects under age 40 years and significantly low

Table 4 Comparison of incidence rates for colorectal cancer patients in the 8 districts of Gharbiah (1999-2007)

District	Young (< 40 yr)		Old (≥ 40 yr)		Colon		Rectum	
	IR	IRR (95% CI)	IR	IRR (95% CI)	IR	IRR (95% CI)	IR	IRR (95% CI)
Tanta	1.52	1.93 (1.23, 3.04)	18.53	2.08 (1.62, 2.67)	3.35	2.25 (1.69, 3.00)	1.98	1.77 (1.26, 2.48)
El Mehalla	0.89	1.13 (0.70, 1.82)	14.85	1.66 (1.29, 2.14)	2.66	1.78 (1.33, 2.38)	1.36	1.21 (0.85, 1.72)
Kafr El Zayat	1.06	1.35 (0.77, 2.35)	12.44	1.39 (1.03, 1.89)	2.17	1.46 (1.03, 2.07)	1.44	1.29 (0.85, 1.95)
Samanoud	0.86	1.09 (0.58, 2.04)	11.40	1.28 (0.92, 1.78)	2.12	1.42 (0.98, 2.07)	1.10	0.98 (0.61, 1.58)
El Santa	0.93	1.18 (0.66, 2.11)	11.28	1.26 (0.92, 1.73)	1.99	1.33 (0.93, 1.91)	1.26	1.13 (0.73, 1.74)
Kotour	1.30	1.65 (0.93, 2.90)	9.56	1.07 (0.75, 1.52)	2.06	1.38 (0.94, 2.02)	1.09	0.97 (0.60, 1.58)
Basyoon	1.63	2.06 (1.18, 3.60)	13.81	1.55 (1.11, 2.15)	2.39	1.60 (1.10, 2.34)	1.97	1.76 (1.15, 2.70)
Zefta	0.79	1.00	8.93	1.00	1.49	1.00	1.12	1.00

IRR: Incidence rate ratio; IR: Incidence rate; 95% CI: 95% confidence interval.

incidence in subjects 40 years and older. Second, high proportion of tumors located in the rectum. Third, a vast majority of tumors (over 97%) did not have polyps. Fourth, living in an urban area was associated with higher rates of colorectal cancer, with variability in rates across the region.

Similar low rates of colorectal cancer in this population in Egypt ($6.9/10^5$ for males and $5.1/10^5$ for females) were reported by the MECC for the short period of 1999-2001^[11]. The low rates were also reported from the Gharbiah cancer registry for the period of 2000-2002, where the age-standardized incidence rates for colorectal cancer was $6.5/10^5$ for males and $4.2/10^5$ for females^[12]. The relatively high rate in subjects under age 40 years was reported by the MECC report in which Egypt had the highest incidence for both genders combined ($1.4/10^5$) and for males ($1.7/10^5$) than the rate in the same age group among Israeli Jewish and Arab populations, Jordanians and Cyprians^[13]. High proportion of young-onset colorectal cancer was also reported in our previous hospital-based studies^[5-7,14] that showed about 1/3 of all Egyptian colorectal cancer patients under age 40 years. It is unclear if the high young-onset rate is due to adoption of a more “westernized” lifestyle and diet, particularly in the younger generation^[6,15] or due to intense environmental exposures with more susceptibility among the younger generations^[15]. While our previous studies showed no familial aggregation among young patients to suggest HNPCC or similar syndromes^[7,16,17], more recent studies of possible mismatch repair gene defects^[18-21] or autosomal recessive inheritance^[22] are warranted in this population, especially in absence of a strong family history and lack of distinct molecular characteristics among young-colorectal cancer patients in Egypt^[7,17].

The high proportion of cancers that are located in the rectum in this study (37.2%) and the low ratio of colon/rectum tumors are characteristic of colorectal cancer in developing countries^[23]. The high proportion of rectal cancer was reported in our previous hospital based studies^[5-7]. However, the proportion of rectal cancer declined from about 50% to 37% perhaps due to the more accurate nature of the population-based studies or the changing life-style with westernization leading to higher proportions of colon than rectal cancers^[23]. How-

Table 5 Age standardized incidence rates by gender in Gharbiah, Egypt and the United States Surveillance Epidemiology and End Results Program

	Gharbiah, Egypt 1999-2007			United States SEER 1999-2001			P value
	Total	Male	Female	Total	Male	Female	
Total cases	1364	737	627	55 480	27 892	27 588	
Total rate	5.5	6.1	4.9	32.0	37.7	27.4	< 0.0001 ¹
< 40 yr	1.3	1.4	1.2	1.2	1.3	1.2	
40-59 yr	12.0	12.8	11.2	37.9	43.3	32.8	
60-69 yr	19.4	22.5	15.8	154.0	185.4	126.4	
> 70 yr	21.2	24.4	18.6	311.3	369.8	270.8	

¹ χ^2 test of total age standardized incidence rates, by age group, Gharbiah, Egypt 1999-2007 vs United States Surveillance Epidemiology and End Results Program (SEER) 1999-2001.

ever, the proportion of primary rectal colorectal cancers in this study is still high compared to Western countries, where only 27.9% of American colorectal cancers are primary rectum^[13]. It is worth noting that recent studies in the United States showed increasing incidence of both young-onset colorectal cancer as well as the proportions of rectal cancers^[24].

The very low rate of polyps reported in this study is unique. The low rate of polyps was also reported in our previous hospital-based studies^[6,7]. It is important to note that pathologists in this population in Egypt report very few polyps in other segments of the resected colon not only in the cancer site. Causes of the very low rate of polyps may be related to diet rich in high fiber, legumes, and green vegetables^[25,26] which is common in this population^[27,28]. Other causes of the low polyp rate may be related to the intake of aspirin or aspirin-like compounds^[29-32] which is also common in this population because of self-medication^[33,34] or other molecular pathways that do not include polyps in the colorectal carcinogenesis in this population.

There are a number of distinctive environmental and possibly genetic factors that may contribute to the variable rates of urban/rural incidence in this population. Intense exposure to pesticides in this predominately agricultural region^[8], industrial pollution^[15,35-37], and high rate of consanguinity and first cousins' marriage^[16,17] may also

lead to this variable cancer incidence and urban/rural rate risk differences.

The higher incidence rate of colorectal cancer in regions of the province may suggest differences in environmental exposures and/or variable access to medical care for colorectal screening or diagnosis. As there is a fairly reliable access to medical care for diagnosis and no screening facilities are available in the province of the registry^[38], differences in risk factors across the region is most likely the cause of the variation in incidence rates. The main occupation in the Gharbiah province is agriculture. Further, pesticide manufacturing in Kafr El Zayat City and textile production in El Mehalla City are also important industries in the province.

This study had the following strengths: (1) the population-based data from the Gharbiah population-based cancer registry and the large sample size give an accurate picture of the state of colorectal cancer in Egypt; (2) the inclusion of key demographic and clinical data allowed for characterization of the clinical profile and suggestions of possible risk factors for colorectal cancer in this population; and (3) previous studies in the same population using hospital-based data were comparable to results from the population-based cancer registry. Weaknesses included inherent nature of population-based cancer registries of limited information on potential risk factors for colorectal cancer, such as diet and lifestyle habits, pesticide exposure, and family history because of lack of interviewing of patients.

In summary this study showed a relatively high incidence of colorectal cancer under age 40 years and a significantly low incidence in the age group of 40 years and older in this population in Egypt. The high proportion of rectal cancers and the vast majority of tumors without polyps are also interesting findings of the study. Regional differences in disease incidence in colon and rectal cancers in the region may reflect different etiologic patterns in this population. Future analytical studies should focus on further understanding of the etiology and pathogenesis of the disease in this population with extensive environmental exposures and possible genetic factors that may modulate the disease risk.

COMMENTS

Background

Previous hospital-based studies in Egypt showed low incidence of colorectal cancer and high proportion of young-onset disease. Egypt has a new reliable resource of a population-based registry in the Gharbiah region of the Nile Delta. The registry data for the period of 1999-2007 was used to examine epidemiologic, clinical and incidence rates of colorectal cancer in this population and to compare that with the results of the United States Surveillance Epidemiology and End Results Program (SEER).

Research frontiers

The vast majority of tumors (97.2%) had no polyps and 37.2% of the patients presented with primary lesions in the rectum. Colorectal cancer was more common in patients from urban (55%) than rural (45%) areas. Regional differences in colon and rectal cancer incidence in the 8 districts of the study province may reflect different etiologic patterns in this population. The registry data of Egypt shows a slightly higher incidence of colorectal cancer than the United States in subjects under age 40 years. The results also shows significantly lower inci-

dence of colorectal cancer in subjects over age 40 years compared to the same age group in the United States SEER.

Innovations and breakthroughs

This study confirms that patients over age 40 years in Egypt have significantly lower incidence of colorectal cancer than subjects in the same age group in the United States. This is the first study on a population-based scale to show the limited proportions of polyps in colorectal cancer patients in Egypt.

Applications

Future clinical and epidemiologic studies should investigate the etiologic factors related to the regional differences of colorectal cancer in this population in Egypt. Studies should also explore clinical and molecular pathways for the district age and polyp distribution of colorectal cancer.

Peer review

The quality of the data set is very important, especially in the population-based cancer registry.

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Faecal pyruvate kinase isoenzyme type M2 for colorectal cancer screening: A meta-analysis

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Abstract

AIM: To present a critical discussion of the efficacy of the faecal pyruvate kinase isoenzyme type M2 (faecal M2-PK) test for colorectal cancer (CRC) screening based on the currently available studies.

METHODS: A literature search in PubMed and Embase was conducted using the following search terms: faecal Tumor M2-PK, faecal Tumour M2-PK, fecal M2-PK, faecal M2-PK, fecal pyruvate kinase, faecal pyruvate kinase, pyruvate kinase stool and M2-PK stool.

RESULTS: Stool samples from 704 patients with CRC and from 11 412 healthy subjects have been investigated for faecal M2-PK concentrations in seventeen independent studies. The mean faecal M2-PK sensitivity was 80.3%; the specificity was 95.2%. Four studies compared faecal M2-PK head-to-head with guaiac-based faecal occult blood test (gFOBT). Faecal M2-PK demonstrated a sensitivity of 81.1%, whereas the gFOBT detected only 36.9% of the CRCs. Eight inde-

pendent studies investigated the sensitivity of faecal M2-PK for adenoma ($n = 554$), with the following sensitivities: adenoma < 1 cm in diameter: 25%; adenoma > 1 cm: 44%; adenoma of unspecified diameter: 51%. In a direct comparison with gFOBT of adenoma > 1 cm in diameter, 47% tested positive with the faecal M2-PK test, whereas the gFOBT detected only 27%.

CONCLUSION: We recommend faecal M2-PK as a routine test for CRC screening. Faecal M2-PK closes a gap in clinical practice because it detects bleeding and non-bleeding tumors and adenoma with high sensitivity and specificity.

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Key words: Faecal pyruvate kinase isoenzyme type M2; Colorectal cancer screening; Colorectal cancer; Stool; Faecal occult blood; Adenoma; Polyps

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INTRODUCTION

Colorectal cancer (CRC) is the most frequent malignant disease in Europe according to an estimation of cancer incidence and mortality by the International Agency for Research on Cancer in Lyon, France^[1]. In 2008, 436 000

persons were diagnosed with CRC, followed by breast cancer with 421 000 cases, lung cancer with 391 000 cases and prostate cancer with 382 000 cases. Approximately 212 000 patients died due to CRC that year, which makes it the second most common death from cancer (after lung cancer with approximately 342 000 deaths in 2008)^[1]. Worldwide, in the developed countries about 1.167 million new cases of CRC and about 603 000 deaths due to CRC were estimated for 2007^[2].

However, due to the long process of carcinogenesis in CRC (adenoma-carcinoma sequence), CRC has an overall good prognosis when diagnosed at an early stage. For that reason different CRC screening programs have been developed and are offered in various European countries.

The gold standard for early detection of colorectal neoplasia is colonoscopy. A great advantage of colonoscopy is that adenomas, the potential precursors of carcinogenesis, can be simultaneously detected and removed. However, the acceptance of screening colonoscopy among patients is low. For example, in Germany only 2.7% of insured people exercise their right to a colonoscopy even though it is reimbursed for people over 55 years old^[3]. The most common *in-vitro* diagnostic method for CRC screening is the detection of occult blood in the stool using the guaiac-based faecal occult blood test (gFOBT). This test is based on the peroxidase activity of haemoglobin, which induces an oxidation and blue colouration of guaiac in the presence of hydrogen peroxide. Red meat and a number of vegetables may result in false positive results whereas vitamin C may result in false negative staining. Therefore, dietary restriction is recommended for three days prior to and during testing. A widespread criticism of gFOBT is its low sensitivity for adenomas and carcinomas (13%-50%)^[4-8]. The immunological faecal occult blood tests (iFOBTs) specifically quantify human haemoglobin with antibodies. Comparative evaluations of immunochemical faecal occult blood tests from different manufacturers have revealed great variations in their respective sensitivities for colorectal adenoma detection^[9,10].

The faecal pyruvate kinase isoenzyme type M2 (faecal M2-PK) test recognises a key enzyme controlling the metabolism of cells with a high proliferation rate, such as tumour cells, and thereby detects specific alterations in intestinal cells, such as polyps and CRC, as well as high-risk patients with acute or chronic inflammatory bowel diseases (IBD) (i.e., ulcerative colitis, Crohn's disease).

M2-PK is a special isoenzyme of pyruvate kinase, a key enzyme within glycolysis which catalyzes the ATP-producing conversion of phosphoenolpyruvate (PEP) to pyruvate. Depending upon the metabolic functions of the tissues, different isoenzymes of pyruvate kinase are expressed. During tumour formation the tissue-specific isoenzymes disappear and the pyruvate kinase isoenzyme type M2 is expressed^[11]. In contrast to all other pyruvate kinase isoenzymes (type L, M1 and R) which consist of four subunits, the M2 pyruvate kinase isoenzyme may occur in a highly active tetrameric form as well as in a dimeric form with low activity. The dimeric form is nearly

inactive and favours the channelling of glucose carbons into synthetic processes, such as nucleic acid, amino acid and fatty acid synthesis. The tetrameric form is highly active and favours the energy-regenerating conversion of PEP to pyruvate and lactate (the Warburg effect). In tumour cells, M2-PK is mainly found to be in the dimeric form and has therefore been termed "Tumour M2-PK". The dimerisation of M2-PK is induced by interaction with different oncoproteins, including pp60v-src-kinase, oncogenic fibroblast growth factor1 and human papilloma virus 16 E7^[11].

The dimeric form of M2-PK is released from tumours into the blood and can be quantified by a sandwich enzyme-linked immunosorbent assay (ELISA; ScheBo Biotech AG, Giessen, Germany). About 40 studies have been published on M2-PK concentrations in blood since 1997. These demonstrate a significant increase in M2-PK and correlation with staging for the following tumours: melanoma, thyroid, breast, lung, kidney, oesophageal, gastric, pancreatic, colorectal, ovarian, cervical and renal cell cancer^[12-19]. The long-term determination of M2-PK in EDTA-plasma is used as a tool for follow-up studies to monitor failure, relapse or success during therapy. In CRC and adenoma M2-PK is also released into the patients' faeces. A sandwich ELISA and a lateral flow rapid test (for doctor's office, point-of-care and laboratory use), both based upon two monoclonal antibodies which specifically recognise the dimeric form of M2-PK, are commercially available for the quantification of M2-PK in stool. The potential of the faecal M2-PK test for CRC screening has been evaluated in at least 17 different independent international studies. The objectives of this review were to obtain an overview of the currently available studies with faecal M2-PK and to present a critical discussion of the efficacy of the faecal M2-PK test for CRC screening.

MATERIALS AND METHODS

Search procedure for studies

In order to find the most relevant studies about faecal M2-PK and CRC screening, a literature search in PubMed and Embase was conducted using the following search terms: fecal tumor M2-PK, faecal tumour M2-PK, fecal M2-PK, faecal M2-PK, fecal pyruvate kinase, faecal pyruvate kinase, pyruvate kinase stool, M2-PK stool. In June 2011 this search revealed 34 publications dealing with faecal M2-PK^[7,8,10,18,20-49] (Table 1). The ScheBo faecal M2-PK test was used in 33 publications, whereas one publication used another antibody combination and was therefore excluded. The following were also omitted from the meta-analysis: seven publications which summarized results from previous papers as reviews; three author-replies to questions about an existing published paper; one publication written in Bulgarian; two publications which investigated neither sensitivity nor specificity; seven publications that only referred to IBD (which was outside the scope of our review) (Table 1). The remaining 13 publications were included in the meta-analysis^[7,8,10,30,31,33,35,37,41,44-46,49]. In

Table 1 Results of the literature search	
Results	Reference
All papers dealing with faecal M2-PK found in a literature search of Pubmed and Embase	[7, 8, 10,18, 20-49]
Additional published studies known to the authors	[50-53]
Excluded papers - reasons for exclusion	
Unique combination of antibodies	[47]
Reviews	[18, 24, 26, 28, 34, 38, 42]
Author replies or comments	[27, 32, 40]
Paper in Bulgarian language	[29]
No sensitivities or specificities calculated	[21, 48]
Studies referred to IBD	[20, 22, 23, 25, 36, 39, 43]
Included papers	
Studies found in Pubmed and Embase	[7, 8, 10, 30, 31, 33, 35, 37, 41, 44-46, 49]
Published studies known to the authors	[50-53]

IBD: Inflammatory bowel diseases; faecal M2-PK: Faecal pyruvate kinase isoenzyme type M2.

Table 2 Overview of included studies		
Reference	Country of study	Conflict of interest regarding faecal M2-PK
Shastri <i>et al</i> ^[27] , 2006	Germany	None declared
Koss <i>et al</i> ^[8] , 2008	United Kingdom	None declared
Möslein <i>et al</i> ^[10] , 2010	Germany	None declared
Haug <i>et al</i> ^[30] , 2008	Germany	None declared
Shastri <i>et al</i> ^[51] , 2008	Germany	Coauthor Stein: Conference speaker for ScheBo Biotech AG
Haug <i>et al</i> ^[33] , 2007	Germany	None declared
Mulder <i>et al</i> ^[35] , 2007	The Netherlands	None declared
Ewald <i>et al</i> ^[37] , 2007	Germany	None declared
Tonus <i>et al</i> ^[41] , 2006	Germany	Non declared
Vogel <i>et al</i> ^[44] , 2005	Germany	Tests performed by ScheBo Biotech AG
Naumann <i>et al</i> ^[45] , 2004	Germany	None declared
Hardt <i>et al</i> ^[46] , 2004	Germany	None declared
Tonus <i>et al</i> ^[49] , 2009	Germany	None declared
Kloer <i>et al</i> ^[50] , 2005	Germany	None declared
McLoughlin <i>et al</i> ^[51] , 2005	Ireland	None declared
Bellutti <i>et al</i> ^[52] , 2005	Germany	None declared
Schmidt <i>et al</i> ^[53] , 2009	Germany	None declared

Faecal M2-PK: Faecal pyruvate kinase isoenzyme type M2.

addition, three posters from conferences^[50-52] and a German doctoral thesis^[53] known to the authors have been added to the list of relevant studies (Table 1). Hence, 17 published studies in total have been incorporated into the meta-analysis (Tables 1 and 2). For our meta-analysis the sensitivities for CRC and adenoma, positivity rates, as well as the specificities published within the individual papers were summarized in individual tables, together with the number of cases which underlie the calculated sensitivities and specificities. mean ± SD was calculated for the sensitivities and specificities of the combined data from the different studies using the Statistics package of SigmaPlot Version 11.0. The sensitivities for CRC and adenoma in all studies are based upon colonoscopy results.

Table 3 Published sensitivities of the faecal pyruvate kinase isoenzyme type M2 test for colorectal cancer	
Reference	n (%)
Hardt <i>et al</i> ^[46] , 2004	60 (73)
Naumann <i>et al</i> ^[45] , 2004	27 (85.2)
Kloer <i>et al</i> ^[50] , 2005	147 (79.6)
McLoughlin <i>et al</i> ^[51] , 2005	35 (97)
Vogel <i>et al</i> ^[44] , 2005	22 (77)
Shastri <i>et al</i> ^[7] , 2006	74 (81.1)
Tonus <i>et al</i> ^[41] , 2006	54 (78)
Haug <i>et al</i> ^[33] , 2007	65 (68)
Mulder <i>et al</i> ^[35] , 2007	52 (85)
Koss <i>et al</i> ^[8] , 2008	32 (81)
Shastri <i>et al</i> ^[51] , 2008	55 (78.2)
Schmidt <i>et al</i> ^[53] , 2009	81 (80.3)
Sum	704
mean ± SD	80.3 ± 7.1

n: Number of colorectal cancer samples; %: Sensitivity.

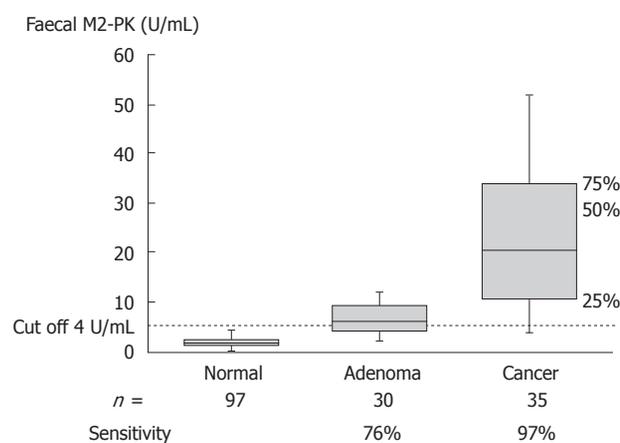


Figure 1 Faecal pyruvate kinase isoenzyme type M2 in healthy controls, patients with colorectal adenoma and colorectal cancer^[51]. Faecal M2-PK: Faecal pyruvate kinase isoenzyme type M2.

Calculated specificities are either based on colonoscopy results or are authors' estimates derived from published prevalence data of CRC and adenoma in screening populations. In the absence of colonoscopies or estimated specificities, only the percentages of test-negative individuals were included in the tables.

Faecal M2-PK test

In all seventeen studies included in our meta-analysis, the M2-PK stool test from ScheBo Biotech AG in Giessen, Germany was used. This test is a sandwich ELISA based on two monoclonal antibodies which specifically recognise the dimeric form of M2-PK.

In accordance with the manufacturer's protocol all studies included a cut-off value of 4 U/mL. One study also included a lower cut-off value (3.33 U/mL^[8]) and another also incorporated additional higher cut off values (5 U/mL and 6 U/mL^[45]) to calculate the resultant sensitivities and specificities. To ensure comparability only those results obtained with the cut-off value of 4 U/mL are included in the meta-analysis.

Table 4 Correlation of faecal pyruvate kinase isoenzyme type M2 sensitivity with tumor node metastasis and Dukes classification *n* (%)

Reference	Tumor node metastasis classificatoin				Dukes classification			
	T1	T2	T3	T4	Dukes A	Dukes B	Dukes C	Dukes D
Kloer <i>et al</i> ^[50] , 2005	9 (55.5)	18 (61.1)	49 (81.6)	12 (83.3)	23 (52.2)	24 (76.0)	26 (80.8)	17 (82.4)
Tonus <i>et al</i> ^[41] , 2006	5 (60)	11 (64)	25 (89)	4 (100)	5 (60.0)	17 (76.0)	9 (89)	10 (90.0)
Haug <i>et al</i> ^[33] , 2007	6 (67)	16 (44)	34 (71)	4 (100)	12 (67.0)	18 (61.0)	12 (67.0)	6 (100.0)
Schmidt <i>et al</i> ^[53] , 2009	8 (57)	20 (84)	42 (79)	11 (91)				
Hardt <i>et al</i> ^[46] , 2004	7 (57)	11 (64)	33 (78)	9 (78)				
Sum	35	76	183	40	40	59	47	33
mean ± SD	59 ± 5	63 ± 14	80 ± 7	90 ± 10	60 ± 7	71 ± 9	79 ± 11	91 ± 9

n: Number of samples tested; %: Sensitivity.

Table 5 Head-to-head comparison of the sensitivity for colorectal cancer of faecal pyruvate kinase isoenzyme type M2 and guaiac-based faecal occult blood test *n* (%)

Reference	CRC M2-PK	CRC gFOBT
Naumann <i>et al</i> ^[45] , 2004	27 (85.2)	27 (62.9)
Vogel <i>et al</i> ^[44] , 2005	22 (77)	22 (27)
Shastri <i>et al</i> ^[7] , 2006	74 (81.1)	74 (36.5)
Koss <i>et al</i> ^[8] , 2008	32 (81)	32 (21)
Sum	155	155
mean ± SD	81.1 ± 3.3	36.9 ± 18.5

n: Number of samples tested; %: Sensitivity; CRC: Colorectal cancer; gFOBT: Guaiac-based faecal occult blood test; M2-PK: Pyruvate kinase isoenzyme type M2.

Table 6 Sensitivity of faecal pyruvate kinase isoenzyme type M2 for adenoma *n* (%)

Reference	Adenoma without diameter	Adenoma < 1 cm ø	Adenoma > 1 cm ø
Naumann <i>et al</i> ^[45] , 2004		11 (27.3)	13 (61.5)
McLoughlin <i>et al</i> ^[51] , 2005	30 (76)		
Vogel <i>et al</i> ^[44] , 2005	21 (48)		
Shastri <i>et al</i> ^[7] , 2006		21 (28.6)	10 (20.0)
Mulder <i>et al</i> ^[35] , 2007	47 (28)		
Koss <i>et al</i> ^[8] , 2008		5 (20)	5 (60)
Shastri <i>et al</i> ^[31] , 2008		48 (29.2)	21 (57.1)
Haug <i>et al</i> ^[30] , 2008		254 (22.1)	68 (23.5)
Sum	98	339	117
mean ± SD	51 ± 24	25 ± 4	44 ± 21

n: Number of samples tested; %: Sensitivity; ø: Diameter.

RESULTS

Sensitivity of faecal M2-PK for colorectal carcinoma

Sensitivity of the faecal M2-PK test for CRC was investigated and calculated in twelve independent studies (Table 3 and Figure 1), which found sensitivities of faecal M2-PK for detection of CRC between 68% and 97%. The mean sensitivity of all twelve studies is 80.3% ± 7.1%. These twelve studies measured faecal M2-PK concentrations in a total of 704 stool samples of patients with CRC, whereby 559 tested positive. Five studies considered the tumor node metastases and/or Dukes classification and showed a close correlation between the sensitivity of the faecal M2-PK test and staging (Table 4). The mean sensitivities ranged from 59% for T1 to 90% for T4 and from 60% for Dukes A to 91% for Dukes D. gFOBT studies from various countries showed much lower sensitivities for CRC which ranged between 13% and 50%^[4-6]. The higher sensitivity of faecal M2-PK compared to gFOBT was confirmed in four studies which measured faecal M2-PK and gFOBT head-to-head in the same patients (Table 5). Combining all four studies, 155 samples from patients with CRC were tested for faecal M2-PK and gFOBT. M2-PK correctly detected 81.1% whereas the gFOBT detected only 36.9%.

Sensitivity of faecal M2-PK for adenoma

More than 90% of colorectal carcinomas evolve from adenoma *via* the adenoma-carcinoma sequence within 10 to 15 years. Therefore, the early detection and removal

of adenoma is an important aspect in the prevention of CRC. The sensitivity of faecal M2-PK for adenoma was investigated in eight studies and ranged between 20% and 76%, whereby a clear dependency with the diameter of the adenoma is described (Table 6). In total, 339 adenomas with a diameter < 1 cm and 117 adenomas with a diameter > 1 cm were investigated. Twenty-five percent of the adenomas < 1 cm in diameter tested positive with the faecal M2-PK test and 44% of the adenomas > 1 cm were correctly detected. Three studies included a total of 98 stool samples from patients with adenoma of unclassified diameter. Faecal M2-PK concentrations above the cut-off were found in 51% of the samples. In direct comparisons of faecal M2-PK with gFOBT, 25% of patients with polyps < 1 cm tested positive with the M2-PK test whereas only 9% were identified by the gFOBT (Table 7). Forty-seven percent of adenomas > 1 cm in diameter tested positive with the M2-PK test whereas the gFOBT detected only 27% (Table 7). One study with adenomas of unclassified diameter revealed a sensitivity of 48% for M2-PK in comparison to 9% for gFOBT. Möslein *et al*^[10] combined adenomas > 1 cm in diameter and CRC to form a group with 55 cases of “advanced neoplasia”. The resultant sensitivity of faecal M2-PK for advanced neoplasia was 27.3% whereas the sensitivity of gFOBT was only 9.1%. This study also included a head-to-head comparison of four iFOBTs from different manufacturers using the same 55 samples of patients with advanced neoplasia. With sensitivities of 7.3%, 8.5%, 18.9% and

Table 7 Head-to-head comparison of sensitivity for adenoma of faecal pyruvate kinase isoenzyme type M2 and guaiac-based faecal occult blood test *n* (%)

Reference	Adenoma < 1 cm \emptyset M2-PK	Adenoma < 1 cm \emptyset gFOBT	Adenoma > 1 cm \emptyset M2-PK	Adenoma > 1 cm \emptyset gFOBT	Adenoma w/o \emptyset M2-PK	Adenoma w/o \emptyset gFOBT
Naumann <i>et al</i> ^[45] , 2004	11 (27.3)	11 (18.2)	13 (61.5)	13 (30.8)		
Vogel <i>et al</i> ^[44] , 2005					21 (48)	21 (9)
Shastri <i>et al</i> ^[7] , 2006	21 (28.6)	21 (9.5)	10 (20.0)	10 (30.0)		
Koss <i>et al</i> ^[8] , 2008	5 (20.0)	5 (0.0)	5 (60.0)	5 (20.0)		
Sum	37	37	28	28	21	21
mean \pm SD	25 \pm 5	9 \pm 9	47 \pm 24	27 \pm 6		

n: Number of samples tested; %: Sensitivity; w/o \emptyset : Without measurement of diameter; \emptyset : Diameter; gFOBT: Guaiac-based faecal occult blood test; M2-PK: Pyruvate kinase isoenzyme type M2.

Table 8 Measurements of faecal pyruvate kinase isoenzyme type M2 in stool samples of healthy individuals

Reference	No. of healthy participants	Test-negative participants (%)	Colonoscopy (yes/no)	Specificity (%)
Belluti <i>et al</i> ^[52] , 2005	2787	91.6	No	97.4 (e)
McLoughlin <i>et al</i> ^[51] , 2005	97	98	Yes	98
Tonus <i>et al</i> ^[41] , 2006	42	93	Yes	93
Ewald <i>et al</i> ^[37] , 2007	1906	90.4	No	
Haug <i>et al</i> ^[33] , 2007	917	78.6	No	
Koss <i>et al</i> ^[8] , 2008	13	100.0	Yes	100.0
Tonus <i>et al</i> ^[49] , 2009	4854	91.2	No	93.4 (e)
Möslein <i>et al</i> ^[10] , 2010	796	89.5	Yes	89.5
Sum	11 412			
mean \pm SD		91.5 \pm 6.4		95.2 \pm 3.9

e: Estimated specificities calculated by authors based on the sensitivity of faecal pyruvate kinase isoenzyme type M2 for colorectal cancer (CRC) and advanced neoplasia, and the prevalence of CRC and advanced adenoma.

20%, respectively, all four iFOBTs were less sensitive than faecal M2-PK.

Specificity of faecal M2-PK for colorectal carcinoma

The specificity of an *in-vitro* diagnostic test reflects the proportion of correctly identified negatives. Consequently, the composition of the control group has a profound effect on the specificity. By its very definition, screening is used in a population to detect a disease in individuals without signs or symptoms of that disease. Therefore, symptoms in the gastrointestinal tract, such as pain, visible blood in the stool or known inflammation are not appropriate for inclusion into the control group of a CRC screening study. In total, seventeen publications calculated specificities for the M2-PK stool test. Nine of these studies included patients from hospitals (clinical settings instead of screening settings) with positive gFOBTs and with inflammation and/or other symptoms in the gastrointestinal tract into the control group and hence these studies have been discounted from our evaluation of the specificity of faecal M2-PK^[7,30,31,35,44-46,50,53]. Eight studies, comprising 11 412 samples in total, had control groups which conformed to the correct composition for screening studies (Table 8, Figures 2 and 3). Ninety one point five percent tested negative which means that about 9% of those tested had a faecal M2-PK value above the cut-off value of 4 U/mL. Colonoscopies were performed in four studies^[8,10,51,41] (Table 8) and revealed specificities of 98% (*n* = 97), 93% (*n* = 42), 100% (*n* = 13) and 89.5%

(*n* = 796). In study 49 with 4854 participants, the authors calculated an estimated specificity of 93.4% based on a prevalence of CRC of 2%. Based on a prevalence of 0.5% for CRC and 18% for advanced adenoma, the authors of study 52 with 2787 participants calculated an estimated specificity for colorectal neoplasia of 97.4%. The screening in study 49 with 4854 participants describes a continuous increase in the percentage of faecal M2-PK positive volunteers with age from 30 years old upwards (Figure 3).

DISCUSSION

With a sensitivity of about 80% for CRC and 44% for adenoma > 1 cm, faecal M2-PK outclasses the gFOBT which has sensitivity between 13% and 50% for CRC (Tables 3-7, and literature^[4-6]). The superiority of faecal M2-PK may be due to the fact that M2-PK is a metabolic biomarker which is characteristic for the metabolic state of tumour cells and their precursors, whereas detection of bowel cancer using the gFOBT is restricted to bleeding tumours and adenoma. Therefore, faecal M2-PK has the advantage that it detects both bleeding as well as non-bleeding tumours and adenoma and will close a gap in clinical practice. Conversely, faecal M2-PK does not have false positive results due to various non-cancerous sources of bleeding, e.g., haemorrhoids and fissures. Screening studies involving a total of more than 11 000 healthy subjects have demonstrated a mean specificity of 95.2% for the detection of CRC/advanced neoplasia with faecal M2-

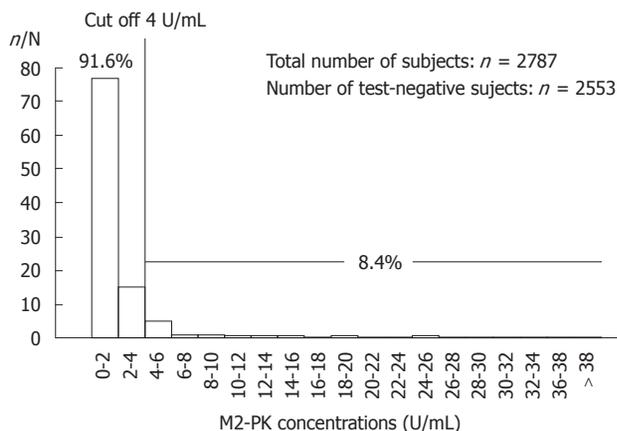


Figure 2 Distribution of faecal pyruvate kinase isoenzyme type M2 concentrations in a screening collective of 2787 participants aged from 45 to 65 years^[52]. n: Number of test negative; N: Total number of subjects; M2-PK: Pyruvate kinase isoenzyme type M2.

PK. The specificities were 100%, 98%, 93% and 89.5%, respectively, in studies which incorporated colonoscopies; 97.4% and 93.4% in studies with estimated specificities; and 90.4% and 78.6% in studies without colonoscopies (Table 8). This demonstrates that specificities were higher in studies with confirmatory colonoscopies in comparison to studies without colonoscopies. Whilst gFOBT specificities $\geq 94\%$ are reported in the literature^[5,6], the authors of a meta-analysis of over 440 000 subjects from six independent studies concluded that more than 80% of the positive gFOBT results are actually false positives^[54]. In most studies the calculated specificities are based on the results of colonoscopy. Colonoscopy is the gold standard for early detection of CRC and polyps and has the advantage that polyps, the potential precursors of carcinogenesis, can be simultaneously detected and removed. However, recent studies have revealed that colonoscopies may have false negative results, e.g., due to suboptimal bowel preparation. For example, a systematic review which summarized six studies totaling 465 patients who had undergone two colonoscopies on the same day revealed a pooled miss rate of 22% for polyps of any size^[55].

IBD may also be a cause of increased faecal M2-PK levels and hence detection of previously undiagnosed patients by faecal M2-PK is another advantage of the test, whereas those patients with known IBD are subject to their own endoscopic monitoring program and are not categorized as suitable for inclusion in a non-invasive CRC screening program.

The cost of one faecal M2-PK ELISA test is about 15-25 US\$. In comparison, based on 2004 data from privately insured beneficiaries, costs were estimated to be about 557 US\$ (range: 150-1112 US\$) for a colonoscopy, 174 US\$ (range: 54-392 US\$) for a flexible sigmoidoscopy and 7 US\$ (range: 2-16 US\$) for a guaiac faecal occult blood test^[56].

In conclusion, faecal M2-PK, either as an ELISA or as a lateral flow rapid test, is a cost-effective and easy-to-perform routine test. In contrast to the gFOBT, only one

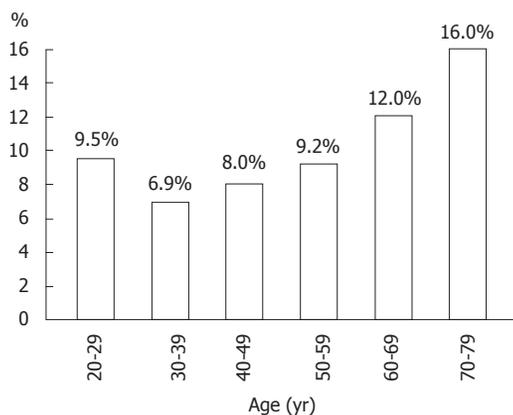


Figure 3 Percentage of faecal pyruvate kinase isoenzyme type M2-positive volunteers by age group (from Tonus *et al.*^[49]).

small stool sample (from a single stool passage), which may be collected with a convenient stool sample device, is necessary and no dietary restrictions are needed. Faecal M2-PK is an appropriately sensitive tool to pre-select those patients who require colonoscopy for further diagnostic confirmation or exclusion of CRC. Based on the current data we recommend the use of faecal M2-PK as a routine *in-vitro* diagnostic test for CRC screening.

COMMENTS

Background

Colorectal cancer (CRC) is the most frequent malignant disease worldwide. The gold standard for early detection of colorectal neoplasia is colonoscopy. However, the acceptance of screening colonoscopy by potential screenees is low. Faecal pyruvate kinase isoenzyme type M2 (faecal M2-PK) is an *in-vitro* diagnostic test which recognizes a specific metabolic characteristic of proliferating cells in 4 mg stool samples. The simplicity of sample collection can encourage participation in CRC screening programs.

Research frontiers

The sensitivity and specificity of faecal M2-PK for CRC screening has been investigated in numerous publications. Here the paper presents a critical discussion of the efficacy of faecal M2-PK for CRC screening based on the accumulated data from currently available studies.

Innovations and breakthroughs

The most established *in-vitro* diagnostic test for CRC screening is the guaiac-based faecal occult blood test (gFOBT). In contrast to the FOBTs, faecal M2-PK detects bleeding and non-bleeding tumors. With a sensitivity of about 80% for CRC and 44% for adenoma > 1 cm, faecal M2-PK outclasses the gFOBT which has a sensitivity between 13% and 50% for CRC.

Applications

This meta-analysis summarizes the results of 17 published studies evaluating the faecal M2-PK test for CRC screening. The data will help to critically assess the efficiency of the faecal M2-PK test in comparison to other *in-vitro* diagnostic tests for CRC screening.

Peer review

This is a meta-analysis about screening CRC with fecal MK-pyruvate kinase.

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Clinical trial: *Lactobacillus plantarum* 299v (DSM 9843) improves symptoms of irritable bowel syndrome

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Abstract

AIM: To assess the symptomatic efficacy of *Lactobacillus plantarum* 299v (*L. plantarum* 299v) (DSM 9843) for the relief of abdominal symptoms in a large subset of irritable bowel syndrome (IBS) patients fulfilling the Rome III criteria.

METHODS: In this double blind, placebo-controlled, parallel-designed study, subjects were randomized to daily receive either one capsule of *L. plantarum* 299v (DSM 9843) or placebo for 4 wk. Frequency and intensity of abdominal pain, bloating and feeling of incomplete rectal emptying were assessed weekly on a visual analogue scale while stool frequency was calculated.

RESULTS: Two hundred and fourteen IBS patients were recruited. After 4 wk, both pain severity (0.68 + 0.53 vs 0.92 + 0.57, $P < 0.05$) and daily frequency (1.01 + 0.77 vs 1.71 + 0.93, $P < 0.05$) were lower with

L. plantarum 299v (DSM 9843) than with placebo. Similar results were obtained for bloating. At week 4, 78.1 % of the patients scored the *L. plantarum* 299v (DSM 9843) symptomatic effect as excellent or good vs only 8.1 % for placebo ($P < 0.01$).

CONCLUSION: A 4-wk treatment with *L. plantarum* 299v (DSM 9843) provided effective symptom relief, particularly of abdominal pain and bloating, in IBS patients fulfilling the Rome III criteria.

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Key words: Irritable bowel syndrome; Probiotics; *Lactobacillus plantarum* 299v; Clinical trial; Abdominal pain

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INTRODUCTION

Irritable bowel syndrome (IBS) is one of the most frequent digestive tract disorders encountered by general practitioners and gastroenterologists. IBS is a functional bowel disorder characterized by chronic and relapsing abdominal pain or discomfort associated with altered bowel habits. The primary aim of any treatment is the relief of abdominal pain which can significantly impair the patient's quality of life. According to published guidelines, the main treatment options for abdominal pain include antispasmodics or anti-depressants at low dose while anti-

diarrheal or laxative drugs are given to improve transit disturbances^[1,2]. However, in many cases, all these options remain disappointing for the relief of abdominal pain. The therapeutic efficacy in IBS is probably impacted by the heterogeneous pathogenesis of the disease which includes altered intestinal motility, visceral hypersensitivity, abnormal brain-gut interactions, food intolerance, altered intestinal permeability and post infectious and/or inflammatory changes^[3].

Recently, the deleterious role of qualitative or quantitative alterations of gut microbiota at the onset of symptoms has been emphasized. Therefore, a rationale exists to discuss the therapeutic use of probiotics, which are live microorganisms conferring health benefits to the host when ingested in adequate amounts^[4]. Clinical evidence regarding the efficacy of some probiotic strains to improve IBS symptoms has recently emerged^[5,6], although the mechanism of action of probiotics on IBS symptoms is not completely understood. Some probiotics bind to small and large bowel epithelium and may produce substances with antibiotic properties, while others compete for attachment and thereby reduce invasion by pathogenic organisms^[7]. Probiotics also modulate gastrointestinal luminal immunity by changing the cytokine and cellular milieu from a pro-inflammatory to anti-inflammatory state^[8]. They may also convert undigested carbohydrates into short chain fatty acids, which act as nutrients for colonocytes and affect gut motility^[4].

Lactobacillus plantarum 299v (*L. plantarum* 299v) (DSM 9843) is a probiotic strain able to reside in the human colonic mucosa *in vivo* due to a specific mechanism of mannose adhesion^[7]. *L. plantarum* 299v (DSM 9843) also increases the amount of carboxylic acid, particularly acetic and propionic acids, in the stools of healthy volunteers^[9]. The strain has shown antibacterial activity against several potential pathogenic agents such as *Listeria monocytogenes*, *Escherichia coli*, *Yersinia enterocolitica*, *Enterobacter cloacae* and *Enterococcus faecalis*^[10]. *L. plantarum* 299v (DSM 9843) also has beneficial immunomodulatory activity *via* an increased interleukin-10 synthesis and secretion in macrophages and T-cells derived from the inflamed colon. And recently, an experimental study reported that *L. plantarum* 299v (DSM 9843) increased the transcription and excretion of the mucins MUC2 and MUC3 in goblet cells^[11,12].

Three single-centre studies have tested the clinical efficacy of *L. plantarum* 299v (DSM 9843) in IBS patients^[13-15]. Two trials have demonstrated significant benefits in comparison with placebo on improvement of flatulence scores^[13] and a reduction of abdominal pain^[14] while the results of the third trial, based on only 12 patients, were not conclusive. The aim of the present randomized, double-blind, placebo controlled clinical trial was to assess the symptomatic efficacy of *L. plantarum* 299v (DSM 9843) in a larger subset of IBS patients fulfilling the Rome III criteria.

MATERIALS AND METHODS

Patients

Participants ($n = 214$) were recruited by general practitio-

ners in four clinical centres in India: one in Mumbai, two in Chennai and one in Bangalore. Subjects between 18-70 years of age with IBS according to the Rome III criteria were eligible for inclusion. All subjects had a colonic examination at baseline to exclude any organic disease while an intestinal infection was excluded by stool cultures in any patient in whom this diagnosis was suspected. Subjects with severe chronic medical disease including colorectal and other gastrointestinal diseases were excluded. Pregnant and breast-feeding women and patients with dietary habits which might interfere with the assessment of the study product or patients with known allergy to the study product components were also excluded. Throughout the study, the subjects were not allowed to consume any other probiotic and were encouraged not to change their usual dietary and physical exercise habits.

Study design

This study was designed as a multicentre double blind, placebo-controlled study with parallel groups to assess the beneficial effects of a daily consumption of *L. plantarum* 299v (DSM 9843) on IBS symptoms. Treatment duration was 4 wk with 3 follow-up visits at weekly intervals. The study protocol was conducted in accordance with the Declaration of Helsinki and approved by the local Ethics Committee. All volunteers gave written informed consent prior to participation in the study.

Study products

The test product was a probiotic preparation containing a mixture of freeze-dried lactic acid bacteria and excipients. The lactic acid bacteria strain was *L. plantarum* 299v (DSM 9843). It is deposited at the DSM collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) under number DSM 9843. The test product contained 10 billion colony-forming units (cfu) per capsule in a potato starch and magnesium stearate base. The control product contained potato starch (97%) and magnesium stearate (3%). Both the test and control products had a similar appearance, texture and taste. Both products were specifically prepared for the study and provided by the Rosell-Lallemand Institute (Blagnac, France).

Assessments and study endpoints

The primary endpoint was the improvement of the frequency of abdominal pain episodes. Secondary endpoints were changes in severity of abdominal pain, changes in frequency and severity of abdominal bloating and in feeling of incomplete rectal emptying. Both frequency of abdominal pain and feeling of incomplete rectal emptying were assessed weekly using a four-point scale ranging from 1 (only occasional symptom) to 4 (daily symptom). Symptom severity (abdominal pain, abdominal bloating and feeling of incomplete rectal emptying) was rated on a visual analogue scale (VAS 1-10) and converted to a 4 point scale ranging from 0 (No pain, VAS = 0) to 3 (Severe, VAS = 8 to 10).

The daily number of stools and bloating episodes were calculated and registered at each visit. At the end

Table 1 Baseline characteristics of the subjects between the two groups (mean ± SD)

	<i>L. plantarum</i> 299v (DSM 9843) (n = 108)	Placebo (n = 106)	P
Age (yr)	36.53 ± 12.08	38.40 ± 13.13	NS
Men/women	70/38	81/25	NS
IBS duration (yr)	3.4	4.6	NS
Abdominal pain frequency	2.1 ± 1.01	1.98 ± 0.91	NS
Abdominal pain severity	1.24 ± 0.60	1.20 ± 0.63	NS
Bloating severity	1.07 ± 0.62	1.14 ± 0.64	NS
Stool frequency	3.94 ± 1.51	3.69 ± 1.34	NS
Pure vegetarians (%)	30.5	20.2	NS
Daily yoghurt intake (%)	46.7	42.1	NS

No significant differences were found between the groups for all the variables tested. NS: No significant; *L. plantarum* 299v: *Lactobacillus plantarum* 299v.

of the 4-wk treatment period, both the patient’s and the practitioner’s opinion about the overall efficacy of the treatment were recorded using a 4-point scale, from “poor” to “excellent”.

Regarding safety assessment, blood samples were taken at baseline and week 4 in each patient for the assessment of blood cell counts, glycaemia, blood urea nitrogen and liver function tests. Physical examinations and verification of any adverse events were performed at each visit.

Sample size and randomization

The sample-size calculation was based on the main outcome, the frequency of abdominal pain episodes. The trial sample size required to detect a significant difference of 20% between the two groups with an 80% power and 5% statistical significance level was calculated to be at least seventy-nine patients per group. Taking into account that all subjects who withdrew prematurely were not replaced, 214 subjects were randomised according to a computer-generated randomization list in the ratio 1:1. For each site, randomization charts were provided to investigators keeping a 1:1 ratio. All investigators, patients and monitors were blinded throughout the study. To ensure allocation concealment, packaging and labelling were performed by a third party, and the randomization code was kept in a secure place during the study.

Statistical analysis

All the analyses of efficacy were performed with full analysis set (FAS) population. The FAS population corresponds to all randomised subjects who took at least one dose of the study drug and who had at least one post-baseline efficacy assessment. Overall assessment of symptoms were analysed using a repeated-measures analysis of variance (ANOVA) with time, treatment group, interaction time x product and baseline score as fixed factors for each period.

RESULTS

The flow chart of the study is given in Figure 1. A total

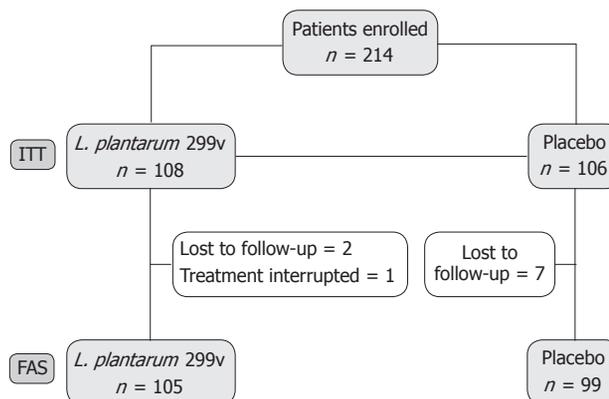


Figure 1 Flow-chart of the study. *L. plantarum* 299v: *Lactobacillus plantarum* 299v; FAS: Full analysis set; ITT: Intention to treat.

of 214 patients were randomized and 108 subjects assigned to receive *L. plantarum* 299v (DSM 9843) group and 106 patients the placebo. Among these 214 subjects, 10 were excluded, either because they did not complete the entire 4-wk double-blind period, or because they did not provide any available data about the treatment period. A majority of patients were IBS-D patients, 63.89% and 60.3% in *L. plantarum* 299v (DSM 9843) and placebo groups, respectively. Baseline characteristics of the two groups are given in Table 1.

Frequency of digestive symptoms

The mean changes over this 4-wk period of the frequency of each digestive symptom are shown in Figure 2. The decrease of abdominal pain frequency was significantly higher in the *L. plantarum* 299v (DSM 9843) group than in the placebo group at weeks 3 and 4. At the end of week 4 the mean frequency was reduced significantly by 51.9% in the *L. plantarum* 299v group in comparison with the 13.6% reduction in the placebo group. Overall reductions in stool frequency, bloating and feeling of incomplete emptying frequency were also significantly greater in the *L. plantarum* 299v (DSM 9843) group when compared with the placebo group over the 4-wk period ($P < 0.05$). The effects of both treatments on stool frequency are shown in Figure 3. A significant reduction of the daily number of stools was observed with *L. plantarum* 299v (DSM 9843) after the second week of treatment.

Severity of digestive symptoms

The change in mean severity of abdominal pain over the 4-wk period was analysed on the VAS. At the end of the 4th week, the mean score was reduced by 45.2% in the *L. plantarum* 299v (DSM 9843) group and reduced by only 23.3% in the placebo group (Figure 2A). The weekly analysis of this score showed significantly lower scores at weeks 2, 3 and 4 in the *L. plantarum* 299v (DSM 9843) group in comparison with placebo. The decrease of the mean scores of severity of abdominal bloating and feeling of incomplete emptying were also statistically higher in the *L. plantarum* 299v (DSM 9843) group when compared to the placebo group at weeks 3 and 4 (Figure 2B and C).

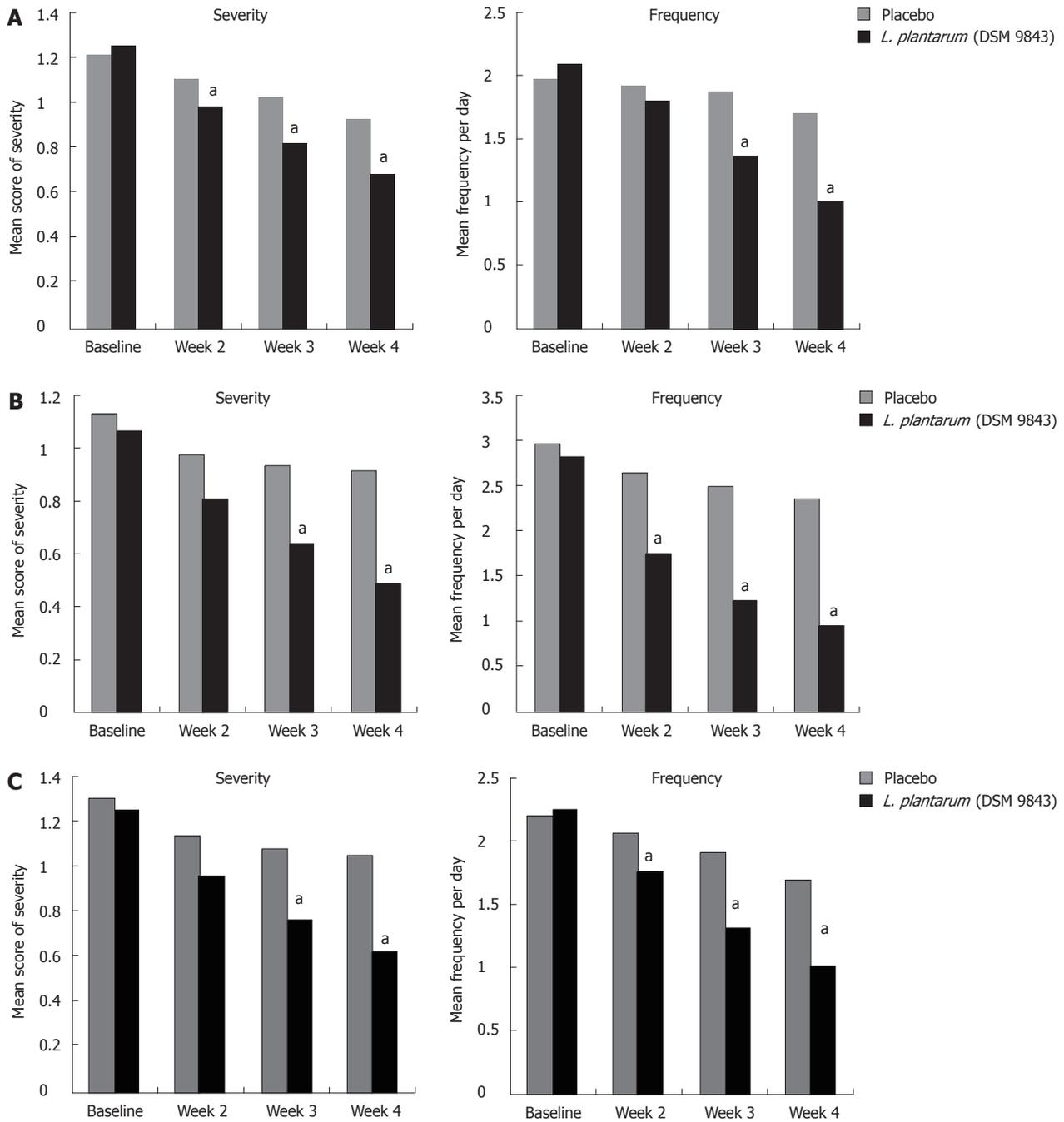


Figure 2 Changes in frequency and severity of symptoms in both groups. A: Abdominal pain; B: Bloating; C: Feeling of incomplete evacuation. *L. plantarum* (DSM 9843): *Lactobacillus plantarum* 299v. ^a*P* < 0.05 vs baseline group.

Overall assessment

The percentage of patients who considered the efficacy of the treatment they received as good or excellent was significantly higher in the *L. plantarum* 299v (DSM 9843) group than in the placebo group (78.1% vs 8.1%) (Figure 4). Similar results were observed when the efficacy was estimated by the investigators (82.8% vs 11.1%) (Figure 4).

Comparative efficacy according to dietary habits

Yoghurt consumption did not affect the results and did not induce any difference between the two arms of treatment (data not shown). The frequency of abdominal pain

was also not different between the two arms when the vegetarian or non vegetarian status was considered. However, the severity of the abdominal pain with *L. plantarum* 299v (DSM 9843) was lower in the vegetarians than in the non-vegetarians at weeks 2, 3 and 4 (*P* < 0.05).

Safety

No significant side-effect was reported in any group during the 4 wk of treatment. The only adverse event reported was a transient vertigo onset by one of the patients who received *L. plantarum* 299v (DSM 9843). No change in blood parameters was detected throughout the study.

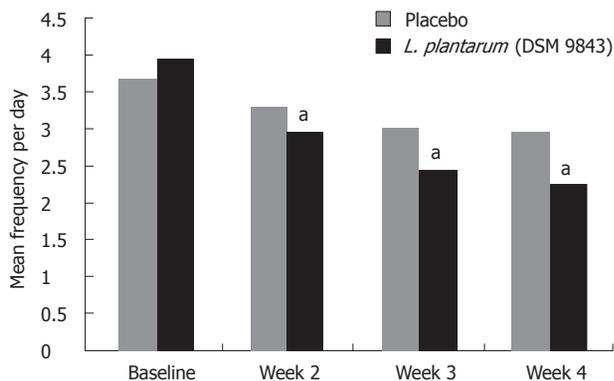


Figure 3 Changes in stool frequency in both groups. *L. plantarum* (DSM 9843): *Lactobacillus plantarum* 299v. ^a*P* < 0.05 vs baseline group.

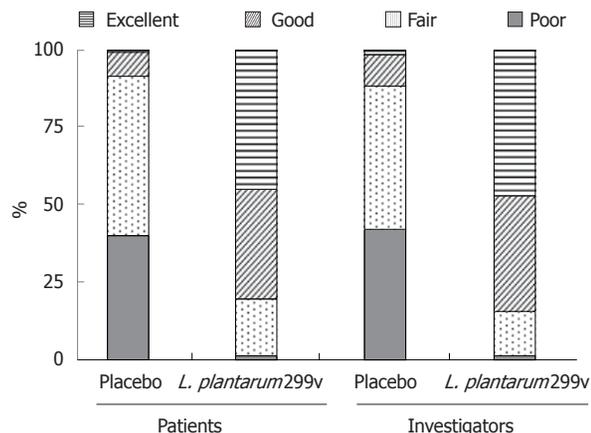


Figure 4 Overall assessment of the treatment efficacy by the patients and by the investigators.

DISCUSSION

The present placebo-controlled trial demonstrated that in an Indian population *L. plantarum* 299v (DSM 9843) is a probiotic strain able to relieve IBS symptoms, particularly abdominal pain and bloating, in IBS patients fulfilling the Rome III criteria. Abdominal pain was chosen as the primary end point because it is the major symptom leading to the seeking of medical advice by IBS patients. This trial was designed for a group of IBS patients of any subtype, complaining of moderate IBS symptoms and recruited by general practitioners. Several trials with probiotics have involved mainly IBS-D patients but microbiological studies have emphasized that qualitative changes of the microbiota exist in all IBS sub-types^[16]. Therefore, we considered that any IBS patient, whatever the subtype, could be eligible to participate. In the present study, the majority of recruited participants were males as compared to previous trials where approximately two-thirds of study subjects were females. The female predominance in IBS patients reported in the West has not been observed in Asian populations, particularly in India. Two major recent community studies reported higher prevalence of IBS in the male population. In Mumbai, male prevalence was 7.9% vs female prevalence of 6.9%, and in a pan-Indian study male prevalence was 4.3% vs female prevalence of 4.0%^[17]. However, other population surveys in the Indian subcontinent have reported an IBS prevalence of 8.5% using the Rome I criteria and demonstrated a female predominance similar to Western countries^[18]. The notable gender difference between the population of this study and that of previously published trials can also be explained by the fact that, in the Indian subcontinent but not in other parts of Asia, men seem to have a greater access to healthcare^[19]. However, data about the consultation behaviour of the community groups are not all in agreement. In the recent large survey conducted by the Indian Society of Gastroenterology Task Force (3000 IBS patients and 4500 community subjects in 18 centres), 33% of men and 38% of women had consulted a doctor in the preceding 12 mo^[20]. Eating behaviours of the patients enrolled in this trial were also somewhat different from that of Western IBS patients

with a high percentage of pure vegetarians and with daily yoghurt consumption in almost half of the cases. Due to the possible interactions between nutrients and bacteria, we cannot exclude that this regimen might have influenced the therapeutic results even if eating behaviours were not different between the two groups. We have even observed that *L. plantarum* results on abdominal pain intensity were better in vegetarian than in non vegetarian IBS patients. This suggests that the symptomatic effect of the strain could be, at least partly, related to interactions between the luminal content and *L. plantarum* or that the strain affects the luminal metabolism of nutrients. However, the design of our study does not allow us to conclude that this is indeed the case.

This trial was performed according to the Rome III guidelines on design of trials for functional GI disorders^[21] in order to demonstrate statistical superiority to placebo with a double-blind, placebo-controlled parallel design and outcome measures including both the effect of the treatment on the main symptom, i.e., abdominal pain, and a global assessment of the treatment efficacy to obtain adequate relief. Several clinical trials testing the symptomatic efficacy of probiotics in IBS were longer than this trial. However, a duration of treatment of 4 weeks follows not only the Rome III guidelines but is also the recommendation of international agencies^[22]. One potential weakness of this study was the choice of a four-point Likert scale to analyze the frequency of symptoms instead of a score such as the IBS symptom severity scale that has been shown to be responsive to treatment effect^[23].

We enrolled patients with moderate abdominal pain. Some studies have suggested that the achievement of a satisfactory relief end-point was significantly influenced by baseline symptom severity^[23,24]. However, the concern that baseline severity compromises the achievement of an end point, such as satisfactory relief, does not appear to affect the current design of clinical trials. For example, trials with 5-HT₃ antagonists^[25] or antidepressant at low dose^[26] or even with a non pharmacological approach^[27] have not confirmed the impact of baseline severity on the

achievement of an adequate relief as a trial end point^[17,28].

In accordance with previous findings in many trials, IBS patients who received placebo exhibited a significant improvement with time. However this improvement was lower than in the *L. plantarum* group and the overall number of patients in the placebo group who considered themselves as improved was low. Furthermore, in the present study, the placebo results were lower than that calculated in a recent meta-analysis of 73 randomized controlled trials (RCTs) reporting a pooled placebo response of 37.5%. But the same meta-analysis of the factors affecting placebo response rate outlined that rates were significantly higher in European RCTs^[29]. The percentage of patients who considered the efficacy of the treatment as good or excellent was very high (78.1%) in the *L. plantarum* 299v (DSM 9843) group and low in the placebo group (8.1%). This result cannot be explained only by the greater effects of *L. plantarum* 299v (DSM 9843) *vs* placebo on each IBS symptom. This satisfaction rate could also be explained by a possible efficacy of the strain on upper abdominal symptoms that are very frequent in Indian IBS patients. Indeed, the Indian Society of Gastroenterology Task Force have outlined that 49% of Indian IBS patients reported epigastric pain, and that 70% complained of upper abdominal fullness or bloating rather than pain^[20].

Three studies using *L. plantarum* 299v (DSM 9843) have been published prior to this trial. In the first study, Nobaek *et al.*^[13] enrolled 60 IBS patients and compared *L. plantarum* 299v to placebo to determine whether endogenous colonic flora could be altered by probiotic consumption. Multiple secondary symptom-based end-points were also evaluated. The active treatment period lasted 4 wk after a 2-wk observation period. Compared with placebo, a statistically significant decrease in flatulence was observed during the second half of the treatment period but only 52/60 patients were included in the analysis of this secondary endpoint. In another study, Niedzielin *et al.*^[14] enrolled 40 IBS patients and assessed abdominal pain and global IBS symptoms as primary and secondary outcomes, respectively. At 4 wk, 20/20 patients in the *L. plantarum* 299v group compared to 11/20 in the control group had complete resolution of their pain ($P = 0.0012$). Moreover, 19/20 patients in the *L. plantarum* 299v group compared to 3/20 patients in the control group also experienced improvement in their global IBS symptoms ($P < 0.0001$). In both trials, no adverse effects were identified. The final study, performed by Sen *et al.*^[15], showed no significant improvement but it was a pseudo-randomized study with only 12 patients with a cross-over design and evaluated changes of a composite score of IBS symptoms. At 8 wk, no significant differences were identified between groups^[15]. Given the significant differences in the enrolled populations, study designs, outcome variables, and statistical analyses, it is difficult to make comparisons across the studies and all three previous studies suffered from multiple design flaws.

In conclusion, the present study shows the potential benefit of a particular strain *L. plantarum* 299v (DSM

9843), in the management of IBS. Further studies are warranted in order to identify the mechanism of the probiotic's potential beneficial effect.

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COMMENTS

Background

Lactobacillus plantarum 299v (*L. plantarum* 299v) (DSM 9843) is a probiotic strain able to reside in the human colonic mucosa *in vivo*, with an antibacterial activity against several potential pathogenic agents and an immunomodulatory activity *via* an increased interleukin-10 synthesis and secretion in colonic macrophages and T-cells.

Research frontiers

Recent studies have highlighted disturbances of the relationship between the complex community of the gut microbiota and their host in irritable bowel syndrome. The potential to correct this using probiotics has been suggested but the effective strains need to be determined.

Innovations and breakthroughs

After a treatment of 4 wk, the relief or improvement of irritable bowel syndrome (IBS) symptoms was greater with the *L. plantarum* 299v group than with placebo ($P < 0.05$) leading to greater patient satisfaction.

Applications

L. plantarum 299v (DSM 9843) is a suitable candidate for the relief of moderate symptoms in any IBS patient.

Peer review

Overall this is a well written paper reporting a trial of reasonable methodology. It should be published if authors can revise it in a satisfactory manner.

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Incidental gallbladder cancer during laparoscopic cholecystectomy: Managing an unexpected finding

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Abstract

AIM: To evaluate the impact of incidental gallbladder cancer on surgical experience.

METHODS: Between 1998 and 2008 all cases of cholecystectomy at two divisions of general surgery, one university based and one at a public hospital, were retrospectively reviewed. Gallbladder pathology was diagnosed by history, physical examination, and laboratory and imaging studies [ultrasonography and computed tomography (CT)]. Patients with gallbladder cancer (GBC) were further analyzed for demographic data, and type of operation, surgical morbidity and mortality,

histopathological classification, and survival. Incidental GBC was compared with suspected or preoperatively diagnosed GBC. The primary endpoint was disease-free survival (DFS). The secondary endpoint was the difference in DFS between patients previously treated with laparoscopic cholecystectomy and those who had oncological resection as first intervention.

RESULTS: Nineteen patients (11 women and eight men) were found to have GBC. The male to female ratio was 1:1.4 and the mean age was 68 years (range: 45-82 years). Preoperative diagnosis was made in 10 cases, and eight were diagnosed postoperatively. One was suspected intraoperatively and confirmed by frozen sections. The ratio between incidental and nonincidental cases was 9/19. The tumor node metastasis stage was: pTis (1), pT1a (2), pT1b (4), pT2 (6), pT3 (4), pT4 (2); five cases with stage I a (T1 a-b); two with stage I b (T2 N0); one with stage II a (T3 N0); six with stage II b (T1-T3 N1); two with stage III (T4 Nx Nx); and one with stage IV (Tx Nx Mx). Eighty-eight percent of the incidental cases were discovered at an early stage (\leq II). Preoperative diagnosis of the 19 patients with GBC was: GBC with liver invasion diagnosed by preoperative CT (nine cases), gallbladder abscess perforated into hepatic parenchyma and involving the transversal mesocolon and hepatic hilum (one case), porcelain gallbladder (one case), gallbladder adenoma (one case), and chronic cholelithiasis (eight cases). Every case, except one, with a T1b or more advanced invasion underwent IVb + V wedge liver resection and pericholecystic/hepatoduodenal lymphadenectomy. One patient with stage T1b GBC refused further surgery. Cases with Tis and T1a involvement were treated with cholecystectomy alone. One incidental case was diagnosed by intraoperative frozen section and treated with cholecystectomy alone. Six of the nine patients with incidental diagnosis reached 5-year DFS. One patient reached 38 mo survival despite a port-site recurrence 2 years after original surgery. Cases with non in-

cidental diagnosis were more locally advanced and only two patients experienced 5-year DFS.

CONCLUSION: Laparoscopic cholecystectomy does not affect survival if implemented properly. Reoperation should have two objectives: R0 resection and clearance of the lymph nodes.

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Key words: Incidental gallbladder cancer; Laparoscopic cholecystectomy; Lymph nodes; Hepatic resection; Management; Outcome

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INTRODUCTION

The widespread use of laparoscopic techniques has led to an increase in referrals for cholecystectomy. As a consequence, the incidental finding of gallbladder cancer (GBC) at an earlier stage has altered the management and the outcome of the disease. However, GBC remains a lethal disease associated with a dismal prognosis. Controversies exist on the optimal treatment of this unexpected finding during routine laparoscopic cholecystectomy. The management is difficult because no guidelines have been established and some authors have reported worse overall prognosis when the patient was not adequately treated during the first operation. If GBC is suspected preoperatively, open cholecystectomy must be performed to enable a complete evaluation of the disease extent and to allow radical resection, if necessary.

Simple cholecystectomy may be adequate treatment only for the earlier stages: T_{is} and T_{1a}. Reoperation is recommended in cases of T₂ tumors and more advanced stages of disease. On the contrary, controversies still exist on the need for more radical resection for T_{1b} GBC. During reoperation it is also unclear what the appropriate extent of hepatic resection is, and whether hepatic resection can prevent liver recurrence.

We report our 10 years experience (19 cases) in the treatment of GBC, and we present a systematic review to evaluate the role of extended surgery in the treatment of the incidental GBC. A Medline search was performed using the keywords "Incidental gallbladder cancer", "laparoscopic cholecystectomy", "lymph nodes dissection" and "hepatic resection".

Reviewing the literature, we focused on the following key points, which are still considered controversial in the management of GBC: (1) How laparoscopy has modified the presentation, the outcome, and the management of the patients with gallbladder cancer? (2) What is an appropriate extent of hepatic resection during reoperation, and can hepatic resection prevent liver recurrence? (3) What is the optimal extent of lymph node dissection? (4) When is resection of the common bile duct necessary? (5) Which type of surgical strategy should be used according to depth invasion? (6) Does laparoscopic cholecystectomy worsen prognosis? (7) Are port-site metastases a real problem? and (8) When is additional radical resection not indicated?

MATERIALS AND METHODS

Ethics

This work was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. All patients provided informed consent.

Data collection

From 1998 to 2008, in the Department of General Surgery of Catania University Hospital and in the General Surgery Unit of Taormina Hospital, 1490 patients underwent cholecystectomy. Within this group of patients, all the cases of GBC were retrospectively reviewed. Patients' demographic data, as well as type of operation, surgical morbidity and mortality, histopathological classification, and survival data were collected in a database for further analysis. The diagnosis of gallbladder pathology was made by history, physical examination, and laboratory and imaging studies [ultrasonography and computed tomography (CT)].

Disease-free survival analysis

The patients were divided in two groups: incidental diagnosis of gallbladder carcinoma, and known or suspected diagnosis preoperatively. The primary endpoint of the study was disease-free survival (DFS) at different stages of diagnosis. The secondary endpoint was the difference in DFS between patients previously treated with laparoscopic cholecystectomy and patients who had oncological resection as their first intervention. The results are reported in percentages and means.

RESULTS

GBC was diagnosed in 19 patients, 11 women and eight men. The male to female ratio was 1:1.4 and the mean age was 68 years (range: 45-82 years).

According to tumor node metastasis staging of the 6th edition of the American Joint Committee on Cancer (AJCC), our patients were divided into: pT_{is} (1), pT_{1a} (2), pT_{1b} (4), pT₂ (6), pT₃ (4), pT₄ (2); five cases with stage I a (T₁ a-b); two with stage I b (T₂ N₀); one with stage II a (T₃ N₀); six with stage II b (T₁-T₃ N₁); two

Table 1 Patient characteristics with gallbladder cancer *n* (%)

	IGBC	NIGBC
No. of patients (<i>n</i> = 19)	9	10
Polyposis lesions	7 (77.8)	5 (50)
Nonpolyposis lesions	1 (11.1)	5 (50)
Histopathological grade		
G1	3 (33.3)	1 (10)
G2	6 (66.7)	3 (30)
G3	0	6 (60)
Lymphatic invasion		
+	2 (22.2)	4 (40)
-	7 (77.8)	6 (60)
Vessel invasion		
+	1 (11.1)	1 (10)
-	8 (88.9)	9 (90)
Perineural invasion		
+	1 (11.1)	3 (30)
-	8 (88.9)	7 (70)
Stage		
0	1 (11.1)	0
I A	4 (44.4)	2 (20)
I B	1 (11.1)	1 (10)
II A	0	1 (10)
II B	2 (22.2)	4 (40)
III	0	1 (10)
IV	1 (11.1)	1 (10)

IGBC: Incidental gallbladder cancer; NIGBC: Nonincidental gallbladder cancer. +: Positive; -: Negative.

with stage III (T4 Nx Nx); and one with Stage IV (Tx Nx Mx). Eighty-eight percent of the incidental cases were discovered at an early stage (\leq II). A preoperative diagnosis was possible only in 10 cases; eight were diagnosed postoperatively during the pathological examination; and one was suspected intraoperatively and then confirmed by frozen sections. The ratio between incidental and nonincidental cases was 9/19, with eight cases discovered after laparoscopic cholecystectomy. The preoperative diagnosis of the 19 patients with GBC was: GBC with liver invasion diagnosed by preoperative CT (nine cases); gallbladder abscess perforated into hepatic parenchyma and involving the transversal mesocolon and hepatic hilum (one case); porcelain gallbladder (one case); gallbladder adenoma (one case); and chronic cholecystolithiasis (eight cases).

Pathological characteristics of the tumors were: one *in situ* cancer; three well-differentiated polypoid adenocarcinoma (G1); one well-differentiated nonpolypoid adenocarcinoma of the gallbladder fundus (G1); seven moderately differentiated polypoid adenocarcinoma (G2-G3); one moderately differentiated nonpolypoid adenocarcinoma (G2); and one and five polypoid and nonpolypoid poorly differentiated GBC (G3), respectively (Table 1).

Every case, except one, with a T1b or more advanced invasion underwent IVb + V wedge liver resection and pericholedochic/hepatoduodenal lymphadenectomy. One patient with stage T1b refused further surgery. Cases with Tis and T1a involvement were treated with cholecystectomy alone. One incidental case was diagnosed by intraoperative frozen section and treated with cholecystectomy alone. Six of the nine patients with in-

cidental diagnosis reached 5-year DFS. Surprisingly, one patient reached 38 mo survival despite a port-site recurrence 2 years after the original surgery requiring further resection. Cases with nonincidental diagnosis were more locally advanced and only two patients experienced 5 years DFS (Tables 2 and 3).

DISCUSSION

How laparoscopy has modified presentation, outcome and management of patients with GBC

Presentation and outcome: The widespread use of laparoscopic cholecystectomy has led to discovery of this deadly disease at an earlier stage, altering the management and the outcome of these patients. GBC is an incidental finding in 0.25%-3% of patients and almost half of these cases are occasionally discovered during or after laparoscopic cholecystectomy for benign disease, such as gallstones and their complications (47% in the series of Memorial Sloan-Kettering Cancer Centre, 50% in the series of Johns Hopkins)^[1,2]. The earlier discovery results in an earlier pathological stage, and consequently, increased long-term survival^[2-4]. Patients with incidental GBC had a significant increase in survival when compared with those who had a preoperative diagnosis (overall 5-year survival 15% *vs* 33%)^[2]. Therefore, the general surgeon should be prepared to deal with GBC suspected or diagnosed incidentally, following a well-established treatment algorithm^[5-8]. It is paramount not to violate oncological principles during the first operation, if a two-stage approach is necessary. For this reason, the surgeon during video-laparoscopic cholecystectomy should always follow these simple rules: (1) perform a thorough preoperative diagnosis; (2) when in doubt, give up the laparoscopy to open access; (3) try to preserve the integrity of the gallbladder, handling it as little as possible; (4) close possible breaches of the wall with clips or endoloops; (5) always use the endobag for the removal of the gallbladder; (6) carefully inspect the gallbladder once extracted; (7) if in doubt, perform a histological examination impromptu; and (8) desufflate the pneumoperitoneum with the trocars *in situ*. During cholecystectomy, accidental opening of the gallbladder is described in 25%-30% of the cases, which clearly have a worse prognosis^[3,9].

Management: The approach to incidental GBC is still controversial because of the difficulty of comparing data deriving from nonuniform case studies. Particularly discordant are the data deriving from western cancer registries with respect to the Japanese ones^[3,4,10-13]. The only constant element seems to be that the prognosis strongly depends on the stage and on the possibility of achieving R0 oncological resection^[3,4]. When incidental GBC is diagnosed afterwards by the pathologist, it is essential to restage the patients carefully by CT, magnetic resonance imaging and positron emission tomography, with a targeted study of the liver bed, peritoneum and of orifices of the trocars^[14,15]. Moreover, a reassessment of

Table 2 Patient characteristics: Demographic data, histopathological classification, tumor node metastasis staging

Patient	Gender	Age (yr)	Incidental	TNM 6th edition	Cystic duct	Resection	Size (mm)	Grade	Lymphatic	Vessel	Perineural	5-yr survival
1	M	63	No	pT2 N1 Mx	R0	R0	10 (NP)	G3	No	R0	No	Alive, 15 mo
2	F	82	No	pT4 N2 M1	R0	R1	45 (NP)	G3	Yes	Yes	Yes	Dead, 3 mo
3	F	60	No	pT3 N1 Mx	R1	R1	60 (P)	G3	No	No	Yes	Dead, 6 mo
4	F	72	No	pT3 N1 Mx	R0	R1	32 (NP)	G3	Yes	No	No	Dead, 8 mo
5	M	76	No	pT4 N1 Mx	R0	R1	49 (NP)	G3	Yes	No	Yes	Dead, 7 mo
6	M	81	No	pT3 N0 Mx	R0	R1	44 (NP)	G3	No	No	No	Dead, 9 mo
7	F	77	No	pT2 N0 Mx	R0	R0	20 (P)	G2	No	No	No	Dead, 24 mo
8	F	45	No	PT1a N0 Mx	R0	R0	25 (P)	G1	No	No	No	Alive, no recurrence at 5 yr
9	F	81	No	PT3 N1 Mx	R0	R1	24 (P)	G2	Yes	No	No	Dead, 28 mo
10	F	66	No	pT1b N0 Mx	R0	R0	7 (P)	G2	No	No	No	Alive, no recurrence at 5 yr
11	M	69	Yes	pT1b N0 Mx	R0	R0	15 (NP)	G1	No	No	No	Alive, 38 mo (disease recurrence)
12	M	65	Yes	PT1a Nx Mx	R0	R0	18 (P)	G1	No	No	No	Alive, no recurrence at 6 yr
13	F	72	Yes	pT2 N0 Mx	R0	R0	10 (NP)	G2	No	No	No	Alive, no recurrence at 5 yr
14	M	55	Yes	pT2 N0 M1	R1	R1	30 (P)	G2-3	No	Yes	Yes	Dead, 8 mo
15	F	78	Yes	pT2 N1 Mx	R0	R0	14 (P)	G2-3	Yes	No	No	Dead, 26 mo
16	F	57	Yes	pT1b N0 Mx	R0	R0	30 (P)	G2-3	No	No	No	Alive, no recurrence at 5 yr
17	M	71	Yes	pT2 N1 Mx	R0	R0	20 (P)	G2-3	Yes	No	No	Dead, 23 mo
18	F	61	Yes	pTis Nx Mx	R0	R0	12 (P)	G1	No	No	No	Alive, no recurrence at 5 yr
19	M	69	Yes	pT1b N0 Mx	R0	R0	5 (P)	G2	No	No	No	Alive, no recurrence at 5 yr

TNM: Tumor node metastasis; M: Male; F: Female; NP: Non polypoid; P: Polypoid.

the histological examination has to be performed, with a possible second opinion. This is important in order to: (1) confirm the pT; (2) specify the exact site of the tumor (hepatic side, bottom, infundibulum); (3) have a thorough evaluation of the cystic duct; and (4) evaluate whether the cystic lymph node is included in the histological examination. Today reoperation for incidental GBC should have two fundamental objectives: R0 resection of the liver parenchyma with the other adjacent structures, and clearance of the locoregional lymph nodes^[7,8].

What is an appropriate extent of hepatic resection during reoperation and can hepatic resection prevent liver recurrence?

Hepatic resection for GBC must have two main aims: resect the tumor that has directly invaded the liver from the gallbladder bed, and prevent micrometastases that may recur around the gallbladder bed^[3]. However, it remains unclear what an appropriate extent of hepatic resection is, and whether hepatic resection can prevent liver recurrence. Generally, operative procedures for incidental GBC include: extended cholecystectomy or Glenn resection (i.e., cholecystectomy plus partial resection of liver segments 4 and 5, approximately 2-3 cm from the gallbladder bed); anatomic resection of liver segment 5 and lower part of segment 4 when GC invades the liver bed to a depth of 2 cm or more; right hepatectomy when GC invaded the right Glisson capsule^[3,7,8].

As noted in the literature, the preference today is for

parenchyma-sparing operations, such as no anatomical wedge resection^[1,3]. Araida *et al*^[16] showed in a multi-center retrospective study, that there was no significant differences in survival and in recurrence rates of liver metastasis between patients that underwent resection of the gallbladder bed, anatomical segmentectomy 4b + 5 and hepatectomy for pT2 and pT3 GBC. He also proved that there were no particular preferences of recurrent liver metastasis for segment 4a + 5. Similarly, other authors have reported that there was no association between major hepatectomy and long-term survival, and that there was an increased association between major hepatic surgery and perioperative morbidity^[1,3].

In order to support this, Pawlik *et al*^[5] have proved that patients who had undergone major hepatic resection (anatomical segmentectomy of 4a + 5 or hemihepatectomy) had a similar risk of specific death as patients who underwent hepatic wedge resection, on both univariate and multivariate analyses. Rather than the type of hepatic resection, the most important factor that determines the final outcome is to obtain R0 resection. In fact, R1/R2 margin status is associated with decreased long-term survival^[17].

In conclusion, for gallbladder cancer without hepatoduodenal ligament invasion and without any locoregional liver involvement, the wedge resection of the gallbladder bed (3 cm) is preferable to hepatectomy^[3,5,17]. With regard to GBC that has invaded the gallbladder bed, in order to obtain negative histological margins, the preferred approach is nonanatomical resection of hepatic parenchyma, with a distal clearance of at least 2 cm^[3,5,17].

Table 3 Patient characteristics: Type of operation and survival data

Patient	Gender	Age (yr)	Incidental	TNM 6th edition	Cystic duct	Resection	Surgery	5-yr survival
1	M	63	No	pT2 N1 Mx	R0	R0	Wedge res. (IVb + V) + lymphadenectomy (I stage)	Alive, 15 mo
2	F	82	No	pT4 N2 M1	R0	R1	Wedge res. (IVb + V) + lymphadenectomy (I stage) + CBD res.	Dead, 3 mo
3	F	60	No	pT3 N1 Mx	R1	R1	Wedge res. (IVb + V) + lymphadenectomy (I stage)	Dead, 6 mo
4	F	72	No	pT3 N1 Mx	R0	R1	Wedge res. (IVb + V) + lymphadenectomy (I stage)	Dead, 8 mo
5	M	76	No	pT4 N1 Mx	R0	R1	Wedge res. (IVb + V) + lymphadenectomy (I stage)	Dead, 7 mo
6	M	81	No	pT3 N0 Mx	R0	R1	Wedge res. (IVb + V) + lymphadenectomy (I stage)	Dead, 9 mo
7	F	77	No	pT2 N0 Mx	R0	R0	Wedge res. (IVb + V) + lymphadenectomy (I stage)	Dead, 24 mo
8	F	45	No	PT1a N0 Mx	R0	R0	Cholecystectomy, no further surgery	Alive, no recurrence at 5 yr
9	F	81	No	PT3 N1 Mx	R0	R1	Wedge res. (IVb + V) + lymphadenectomy (I stage)	Dead, 28 mo
10	F	66	No	pT1b N0 Mx	R0	R0	Wedge res. (IVb + V) + lymphadenectomy (I stage)	Alive, no recurrence at 5 yr
11	M	69	Yes	pT1b N0 Mx	R0	R0	LC (stage) - wedge res. (IVb + V) + lymphadenectomy (II stage) + PS exc	Alive, 38 mo (disease recurrence)
12	M	65	Yes	PT1a Nx Mx	R0	R0	Cholecystectomy	Alive, no recurrence at 6 yr
13	F	72	Yes	pT2 N0 Mx	R0	R0	LC (stage) - wedge res. (IVb + V) + lymphadenectomy (II stage) + PS exc	Alive, no recurrence at 5 yr
14	M	55	Yes	pT2 N0 M1	R1	R1	LC (stage) - wedge res. (IVb + V) + lymphadenectomy (II stage) + CBD and PS exc	Dead, 8 mo
15	F	78	Yes	pT2 N1 Mx	R0	R0	Cholecystectomy, refused further surgery	Dead, 26 mo
16	F	57	Yes	pT1b N0 Mx	R0	R0	LC (stage) - wedge res. (IVb + V) + lymphadenectomy (II stage) + PS exc	Alive, no recurrence at 5 yr
17	M	71	Yes	pT2 N1 Mx	R0	R0	LC (stage) - wedge res. (IVb + V) + lymphadenectomy (II stage) + PS exc	Dead, 23 mo
18	F	61	Yes	pTis Nx Mx	R0	R0	LC	Alive, no recurrence at 5 yr
19	M	69	Yes	pT1b N0 Mx	R0	R0	Cholecystectomy, refused further surgery	Alive, no recurrence at 5 yr

TNM: Tumor node metastasis; M: Male; F: Female; CBD: Common bile duct; PS: Port site; LC: Laparoscopic cholecystectomy; res: Resection of segments; exc: Excision.

Optimal extent of lymph node dissection?

In GBC, besides radical R0 resection, another main aim of surgery is to obtain complete clearance of the locoregional lymph nodes. GBC spreads through different pathways: direct locoregional invasion to lymphatic, vascular and neural invasion. The most common route of dissemination is lymphatic diffusion. This is facilitated by lymphatic channels in both the muscular and subserosal layers of the gallbladder. In addition, neoplastic cells, even without evident transmural invasion, often spread superficially to the other lymph nodes along the bile ducts^[18-20]. The lymph nodes involved in the locoregional spread of GC can be divided into three: (1) cystic, pericholedochal and hilar lymph nodes; (2) lymph nodes around the portal vein, the common hepatic artery and periduodenal and peripancreatic lymph nodes; and (3) celiac, superior mesenteric artery and the para-aortic lymph nodes^[18-20].

Although the cystic and pericholedochal lymph nodes are the first key station, the pathways of lymph node involvement from the first site of diffusion to the hepatoduodenal ligament (cystic, pericholedochal and hilar lymph nodes) tend to be highly variable^[21]. In fact, GBC can spread directly to the third level of lymph nodes, along the perivascular soft tissue (celiac, superior mesenteric artery and the para-aortic lymph nodes), according to the three pathways of lymphatic drainage proposed

by Ito *et al*^[21]: cholecysto-retropancreatic pathway (main pathway), cholecysto-celiac and cholecysto-mesenteric pathways (accessory pathways). The incidence of occult lymphatic metastasis discovered during reoperation for incidental GBC can vary from 0% to 85% in relation to the depth of organ invasion (pT).

In fact, the reported incidence of occult lymphatic metastasis by stage is as follows: for T1a 0%-2.5%, for T1b 15%-25%, for T2 30%-50%, for T3 45%-75%, and for T4 > 85%^[5,18,22-26] (Table 4). Similarly to other cancers, lymphadenectomy not only provides important staging information, but more importantly, may decrease the risk of locoregional recurrence. In fact, after tumor resection, the level of lymph node metastasis correlates with overall prognosis within the same pT stage category^[25,26]. Miyakawa *et al*^[27] reported 5-year survival of 60.3% for pN0 patients, 30.0% for pN1, 16.8% for pN2, and 5.9% for pN3. Hence, little controversy exists on the optimal management of T1a GBC. In fact, cholecystectomy alone is sufficient^[3]. On the contrary, controversy still exists on the need for more radical resection in T1b GBC^[24,26]. Moreover, different authors have advocated that not all T1b stages are the same, and treatment should be individualized. In fact, due to the strong correlation between lymphatic invasion and lymph node metastasis, Shibata *et al*^[20] have advocated the use of

Table 4 Residual disease in the lymph nodes after re-resection for each pT (%)

Pathological T	Ogura <i>et al.</i> ^[22]	Tsukada <i>et al.</i> ^[18]	Foster <i>et al.</i> ^[23]	Pawlik <i>et al.</i> ^[5]	You <i>et al.</i> ^[24]	Liang <i>et al.</i> ^[25]	Erich <i>et al.</i> ^[26]
Tis							
T1a	2.5	0			0	0	0
T1b	15.5	0			3.8	0	24.4
T1 (tot)	18	0		12.5	3.8	0	24.4
T2	44.3	46	33	31.3		29.2	44.9
T3	72		75	45.5		58.7	63.7
T4						85.36	

lymphatic invasion as guidance for additional radical resection. However, this remains controversial because the absence of lymph node invasion does not exclude other recurrence such as liver metastases, peritoneal carcinomatosis or recurrence at the port sites, or expression of other forms of diffusion of GBC^[28,29].

Based on our review, we believe that resection of the gallbladder bed with regional lymph node dissection is the best choice for treatment of T1b GBC. In Western countries, lymphadenectomy is usually confined to the hepatoduodenal ligament around the hilar area (N1 lymph nodes: cystic, pericholedochal and hilar lymph nodes). Extended radical lymphadenectomy of N2 lymph nodes (including lymph nodes around the portal vein, common hepatic artery, and periduodenal and peripancreatic lymph nodes) is not routinely advocated^[3,4]. Currently, according to the 7th edition of AJCC staging, N2 involvement is considered as M1 metastasis, and represent a potential contraindication to additional radical surgery^[30].

When is resection of the common bile duct necessary?

Resection of the common bile duct performed at the time of the hepatic resection and lymphadenectomy is controversial^[31-33]. GC has a strong tendency to invade the hepatoduodenal ligament in the form of perineural invasion or lymph node metastasis, therefore, *en bloc* resection of the regional lymph nodes together with excision of the connective tissue around the portal and hepatic artery should be performed, whenever lymph node dissection of the hepatoduodenal ligament is entertained^[34-36]. Dissection of the hepatoduodenal ligament implies a risk of inducing ischemic damage to the common bile duct, therefore, Shimizu *et al.*^[10] proposed routine resection of the extrahepatic bile duct to facilitate lymphadenectomy, avoiding common bile duct ischemia, and increasing the number of lymph nodes harvested. However, these benefits have not been confirmed in other studies^[32,33]. Pawlik *et al.*^[5] showed that the median number of lymph nodes harvested at the time of lymphadenectomy was the same ($n = 3$), regardless of whether the common bile duct was or was not resected concomitantly with lymph node dissection ($P = 0.35$). Araida *et al.*^[31] found that, in patients with advanced GBC, who did not have direct invasion of the hepatoduodenal ligament and/or of the cystic duct, bile duct resection did not result in any differences in terms of recurrence and overall survival, but it only exposes patients to the potential complications of the bilioenteric anastomosis.

In conclusion, bile duct resection should be performed only when the patients have a positive involvement of the cystic duct margins, discovered either on the pathological review of the initial cholecystectomy or through biopsy of the cystic duct at the time of the second operation^[3,32,33]. In fact, microscopic involvement of the cystic duct margin is associated with a residual and/or additional disease in the common bile duct in over one-third of the cases^[32,33].

Type of treatment according to depth invasion

Contrary to other gastrointestinal carcinomas, the depth of invasion of GBC dictates the extent of surgical resection. In cases of carcinoma *in situ* or tumor invading the mucosa (Tis and T1a), simple cholecystectomy with negative surgical margins can be considered as curative surgery^[3,4,23,37].

The 5-year survival after simple cholecystectomy is between 99% and 100%^[23,37]. When the muscularis layer is involved (T1b), a 20%-50% local-regional recurrence can be expected after simple cholecystectomy^[3,6,37] (Table 5). At the time of reoperation, it has been shown that there is a 10% incidence of residual disease in the liver bed associated with a 15%-25% incidence of residual metastatic lymph node involvement^[22,24,26]. The 5-year survival after simple cholecystectomy is between 40% and 50%^[6,23,36-39]. Therefore, the recommended procedure is cholecystectomy associated with resection of at least 3 cm of liver parenchyma (wedge resection), plus adequate lymphadenectomy (Glenn's resection)^[37-39]. When the tumor extends beyond the serosa and invades the liver or an organ or an adjacent structure (T3), there is a 36% incidence of residual disease at the liver level and 45%-75% incidence of lymph node dissemination^[5,22,25,26]. The goal of surgical intervention is to obtain R0 resection, hence, mandatory steps include extended lymphadenectomy and extended hepatic resection, associated with resection of other organs and structures, when necessary^[35]. T3 patients are at high risk of peritoneal metastases, therefore, explorative laparoscopy should be considered in order to avoid unnecessary laparotomy. The 5-year survival after simple cholecystectomy is 0%-15%, and reaches 25%-65% after extended resection^[5,23,36] (Table 5).

Does laparoscopic cholecystectomy worsen prognosis?

More cases of GBC are incidentally diagnosed during laparoscopic cholecystectomy, thus, the question arises whether laparoscopic cholecystectomy worsens the prog-

Table 5 Five-year survival according to both stage of gallbladder cancer and type of surgery (%)

Author	T1a LC	T1b LC	T2 LC	T2 extended resection
Fong <i>et al</i> ^[36]			19	61
Waghlikar <i>et al</i> ^[37]	100	41.67		
Fong <i>et al</i> ^[38]			20	60
Foster <i>et al</i> ^[23]	100	50	38	78
Chijiwa <i>et al</i> ^[39]			17	75

LC: Laparoscopic cholecystectomy.

nosis of these patients. Drouard *et al*^[40] first described the development of port-site metastases in 1991, and additional proof came in 1994^[41]. This contributed to the loss of interest in approaching malignancy laparoscopically. Furthermore, excessive manipulation of the organ and perforation can cause intraperitoneal spread of malignant cells, resulting in a worse long-term survival^[42]. In fact, the incidence of port-site recurrence increased from 9% in patients without intraoperative perforation to 40% in those in whom perforation could be demonstrated^[43]. Other studies proved that pneumoperitoneum significantly increased tumor cell implantation at trocar sites, and tumor growth in the peritoneum^[44-46]. However, laparoscopic cholecystectomy, if correctly performed, did not influence the long-term prognosis of early stage tumors (T1a, T1b, T2)^[7,8]. Also, radical re-resection, performed several months after laparoscopic cholecystectomy, has similar results to radical resection in one stage, and long-term survival can be achieved in tumors with infiltration of the liver in patients who have previously undergone noncurative surgery^[1,7,8,23]. Survival is strictly related to the depth of parietal invasion of the tumor, but there is no significant difference between patients with incidental GBC discovered during or after cholecystectomy ($P = 0.235$)^[7]. The real problem is to have a clear understanding of how to deal with this eventuality.

Are port-site metastasis a real problem?

Port-site metastasis is the most common form of parietal recurrence (Table 6). It has been reported at all stages of gallbladder carcinoma and at any of the trocar sites. It generally presents after latency, ranging from a few months to 3-4 years. Many factors can contribute to port-site metastasis. One of the most important is intraoperative spillage of bile from gallbladder wall perforation, which has been described in 30% of laparoscopic cholecystectomy cases, and it has been linked to port-site metastasis^[43,44,47,48]. Intraoperative manipulation of the tumor, in the form of tension, dissection and isolation, often leads to the disintegration of a certain proportion of cancer cells, as confirmed by the presence of granular cells in 40% of laparoscopic instruments^[49,50]. The increased intraperitoneal pressure induced by the CO₂ pneumoperitoneum can spread and redistribute cancer cells within the peritoneal cavity and in damaged surfaces. Finally, evidence exists on the immunosuppressive action of CO₂ which would favor the implantation of tumor

Table 6 Metastasis at port-site and at subcostal laparotomy (%)

Author	Metastasis at port-site	Metastasis at subcostal laparotomy
Z'graggen <i>et al</i> ^[43]	14	
Wu <i>et al</i> ^[44]	16	6.5
Paolucci <i>et al</i> ^[47]	17.1	
Paolucci <i>et al</i> ^[48]	14	12

cells^[50]. The median survival after port-site metastasis is approximately 1 year, and it is mandatory to perform resection at the time of reintervention in patients previously treated with laparoscopic cholecystectomy^[3,7,8].

Contraindications to additional radical resection

With the primary goal of surgery in mind (R0 resection), the only contraindication to additional surgery is the inability to obtain radical R0 resection. In particular, the presence of peritoneal metastasis, distant metastasis, locally advanced GBC with N2 or M1 (according to the 7th edition of AJCC staging), lymph node invasion along the hepatic artery, portal vein and celiac and mesenteric vessels are all considered contraindications to radical resection^[35,51-53]. On the other hand, the presence of peripancreatic (head only) lymph node disease is not a contraindication to surgical excision, and radical lymphadenectomy and pancreaticoduodenectomy can be carried out together with liver resection^[35,53]. Also, the depth of liver involvement and multiorgan locoregional involvement do not represent a contraindication for additional radical resection^[51,52]. Combined pancreaticoduodenectomy, right hemicolectomy and major hepatectomy are effective treatment for GBC with direct invasion of the adjacent organs (stomach, duodenum, pancreas, colon and liver), but only if potentially curative resection (R0) is feasible. In these cases of multiorgan resection for GBC, given radical R0 resection, the long-term survival will depend on bile duct involvement^[35,51-53]. In fact, stromal invasion of the extrahepatic bile ducts is sometimes a prelude to hepatoduodenal ligament involvement, and is also associated with a higher rate of metastases to para-aortic nodes with a high incidence of residual tumor and poor outcome after surgery^[32].

In conclusion, incidental carcinoma of the gallbladder, as our experience confirms, generally is diagnosed at an earlier stage and carries a better prognosis than non-incidentally found cancer. Laparoscopic cholecystectomy does not affect survival if implemented with proper technique. Simple cholecystectomy may be an adequate treatment only for earlier stage GBC: Tis and T1a. All other stages, starting from T1b should be treated with lymphadenectomy and resection of at least 2-3 cm of liver parenchyma around the liver bed, provided that no residual microscopic cancer (R0) remains. Resection of the main bile ducts could be necessary in hilum-type cancers with positive margins of the cystic duct. More extensive liver resection or performance of multiorgan resection can be pursued in order to achieve R0 resection.

COMMENTS

Background

Gallbladder carcinoma remains a rare, but highly aggressive disease. Its dismal prognosis is associated with the advanced stage of the disease at the time of diagnosis.

Research frontiers

Controversy exists about the optimal management of the disease. In particular, the debate involves the extent of surgical resection of the liver and surrounding organs, the need for resection of the main bile duct, the extent of lymph node removal, and the potential for negative effects of previous laparoscopic cholecystectomy. It is also unclear when surgery is not indicated at all.

Innovations and breakthroughs

Previous studies have proved how simple cholecystectomy is sufficient treatment for early stages of gallbladder carcinoma. Also, it seems that, at more advanced stages, it is paramount to obtain complete gross oncological resection (R0) without the need for anatomical hepatic resection. In order to minimize port-site metastasis, the laparoscopic approach to apparently benign gallbladder disease has to follow specific principles: minimal manipulation of the gallbladder; avoidance of rupture of the gallbladder and bile spillage; extraction of the specimen with a protective bag to avoid contact with the skin; and evacuation of the intraperitoneally insufflated gas via the cannulae.

Applications

The authors conclude that gallbladder cancer can be adequately cured when the diagnosis is early and the treatment is standardized by stage. Incidental carcinoma of the gallbladder is generally diagnosed at an earlier stage and carries a better prognosis than nonincidentally found cancer. Laparoscopic cholecystectomy does not affect survival if implemented with proper technique. Simple cholecystectomy may be adequate treatment only for the earlier stages. All other stages should be treated with lymphadenectomy and resection of at least 2-3 cm of liver parenchyma around the liver bed, provided that no residual microscopic cancer remains. Resection of the main bile ducts could be necessary in cancers with positive margins of the cystic duct. More extensive liver resection or performance of multiorgan resection can be pursued in order to achieve complete resection of the tumor. This research can certainly guide surgeons that encounter this rare entity unexpectedly. In fact, as long as the appropriate referrals are made, the prognosis does not worsen. This can also increase awareness of this rare, but potentially lethal disease.

Terminology

R0 is the surgical removal of all the grossly apparent tumor cells. Port-site metastasis refers to implantation of tumor cells at the skin incisions utilized to place the laparoscopic trocars.

Peer review

The authors present solid experience with a rare disease. Their clinical analysis, along with a thorough review of the literature, provides a clear algorithm to approach the disease at different stages.

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Matrix metalloproteinases in the restorative proctocolectomy pouch of pediatric ulcerative colitis

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Abstract

AIM: To investigate matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in pouch mucosa of pediatric onset ulcerative colitis (UC).

METHODS: In this cross-sectional study, 28 patients with pediatric onset UC underwent ileal pouch biopsy 13 years (median) after proctocolectomy. Expression of MMPs-3, -7, -8, -9, -12 and -26 and TIMPs-1, -2 and -3 in samples was examined using immunohistochemical methods, and another biopsy was used to evaluate the grade of histological inflammation. Two investigators independently graded the immunohistochemical specimens in a semiquantitative fashion, using a scale marking staining intensity as follows: 0 = less than 20 positive cells; 1 = 20-50 positive cells; 2 = 50-200 positive cells; 3 = over 20 positive cells. Fecal calprotectin and blood inflammatory markers [serum C-reactive protein (CRP) and erythrocyte sedimentation rate] were determined during a follow-up visit to examine correlations between these markers and the expression of MMPs and TIMPs.

RESULTS: Of the 28 patients with pediatric onset UC, nine had not experienced pouchitis, whereas thirteen reported a single episode, and six had recurrent pouchitis (≥ 4 episodes). At the time of the study, six patients required metronidazole. In all of the others, the most recent episode of pouchitis had occurred over one month earlier, and none were on antibiotics. Only four samples depicted no sign of inflammation, and these were all from patients who had not had pouchitis. Two samples were too small to determine the grade of inflammation, but both had suffered pouchitis, the other recurrent. No sample depicted signs of colonic metaplasia. Most pouch samples showed expression of epithelial (e) and stromal (s) MMP-3 (e, $n = 22$; s, $n = 20$), MMP-7 (e, $n = 28$; s, $n = 27$), MMP-12 (e, $n = 20$; s, $n = 24$), TIMP-2 (e, $n = 23$; s, $n = 23$) and MMP-3 (e, $n = 23$; s, $n = 28$) but MMP-8 (e, $n = 0$; s, $n = 1$), MMP-9 (e, $n = 0$; s, $n = 9$) and MMP-26 (e, $n = 0$; s, $n = 3$) and TIMP-1 ($n = 0$, both) were lacking. In samples with low grade of inflammatory activity, the epithelial MMP-3 and MMP-7 expression was increased ($r = -0.614$ and $r = -0.472$, respectively, $P < 0.05$ in both). MMPs and

TIMPs did not correlate with the markers of inflammation, fecal calprotectin, erythrocyte sedimentation rate, or CRP, with the exception of patients with low fecal calprotectin (< 100 µg/g) in whom a higher expression of epithelial MMP-7 was found no differences in MMP- or TIMP-profiles were seen in patients with a history of pouchitis compared to ones with no such episodes. Anastomosis with either straight ileoanal anastomosis or ileoanal anastomosis with J-pouch did depict differences in MMP- or TIMP-expression.

CONCLUSION: The expression of MMPs pediatric UC pouch in the long-term shares characteristics with inflammatory bowel disease, but inflammation cannot be classified as a reactivation of the disease.

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Key words: Children; Matrix metalloproteinase 3; Tissue inhibitor of matrix metalloproteinase 3; Matrix metalloproteinase 7; Pouchitis; Ulcerative colitis

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INTRODUCTION

In children, ulcerative colitis (UC) appears to present more aggressively than in adults^[1,2]. The number of pediatric UC cases requiring colectomy within the first decade after diagnosis may reach 24%^[1]. Restorative proctocolectomy with ileoanal anastomosis (IAA) and an ileal pouch-anal anastomosis is the surgical treatment of choice in UC^[2].

In proctocolectomized patients, pouchitis is a common complication, and over two thirds of children undergoing proctocolectomy will suffer at least one episode of pouchitis during the following decade^[2]. Yet, studies on pouchitis in children are limited. Pouchitis is an idiopathic inflammatory condition of the ileal reservoir occurring frequently after colectomy with IAA^[3]. The etiology of pouchitis is poorly understood, but it is associated with risk factors such as extensive colonic disease preoperatively and young age at proctocolectomy^[4]. The incidence of pouchitis is higher in UC than in familial adenomatous polyposis^[5], and pouchitis has been consid-

ered a recurrent form of colitis^[5,6]. Ileal pouch mucosa may acquire colonic characteristics^[7,8], and colonic metaplasia is more pronounced in patients with pouchitis^[9]. This mucosal transformation could have a role in the pathogenesis of pouchitis, possibly allowing the recurrence of inflammatory bowel disease (IBD) and may represent a novel manifestation IBD^[7-9].

Matrix metalloproteinases (MMPs) are a family of 24 zinc-dependent enzymes implicated in mucosal damage in IBD^[10]. MMPs take part in normal mucosal defense, and are capable of degrading extracellular matrix and basement membrane proteins in tissue remodeling processes both in normal and in pathological conditions^[10]. In IBD, MMP-9 is the most abundantly expressed MMP, and it shows increased activity in inflamed UC mucosa^[11]. Other MMPs have also been linked to inflammation severity in IBD, such as MMP-7^[12]. Our group has demonstrated enhanced MMPs-1, -3 and -7 expression in IBD, and shown MMP-10 expression in IBD granulation tissue^[13,14]. In pediatric IBD, we have described increased expression of epithelial MMP-10 and stromal tissue inhibitor (TIMP)-3^[15].

The pathogenesis of pouchitis is unknown, and it is debated whether or not the inflammation is similar to that found in UC, or whether it is inflammation of a novel kind. The management of pouchitis by antibiotics and probiotics is widely accepted. Yet, according to a recent review, antibiotics are not significantly better than a placebo, compared to the high dose probiotics. By altering the endogenous flora and the expression of inflammation parameters, as well as competing with pathogens for receptor binding, nutrients, and growth factors, the high dose probiotics are effective in treatment of pouchitis^[16]. The occurring inflammation and acquisition of colonic characteristics^[7,8] inspires an interest in the MMP and TIMP-profile of pouch. We are aware of only two studies of MMP expression in pouchitis in adults^[17,18]. Expression of MMP-1 and MMP-2 in pouchitis is different from normal ileal mucosa, but is similar to expression in active UC colitis^[17]. We examined the MMP and TIMP profiles of pouch patients who had undergone proctocolectomy in their childhood or adolescence, and to find clues to the type of inflammation appearing in the pouch. It is important to determine whether this inflammation is IBD-like or of a novel kind, to appropriately prevent and manage pouchitis in the future.

MATERIALS AND METHODS

Patients and setting

Between 1985 and 2005, 81 pediatric patients with UC underwent proctocolectomy with ileal anastomosis at Tampere University Hospital or Children's Hospital, Helsinki. Of these colectomized patients, one died of an unrelated cause and one emigrated. Thus, 79 patients were traced from the database of the Population Register Centre and contacted by mail during 2006. Thirty-five patients agreed to participate for a follow up visit described elsewhere

Table 1 Patient characteristics

Patient characteristics	Median (range)
Number of patients (male)	28 (9)
Age (yr); median (range)	
At diagnosis	12 (1-15)
At operation	13 (2-19)
At the time of study	25 (8-41)
Duration from surgery to study	13 (4-22)
Clinical inflammation markers; mean (range)	
Fecal calprotectin ($\mu\text{g/g}$)	371 (12-2859)
ESR (mm/h)	14 (2-69)
CRP (mg/L)	3 (0-19)

ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein.

Table 2 Medications at the time of study

Intestinal inflammation	<i>n</i>
5-ASA	1
5-ASA + antibiotics	1
Antibiotics	5
Bowel function	
To decrease bowel motility	10
To increase bulk mass	1
Analgesics	1
Lactic acid bacteria	1
Other chronic illnesses	
Cholangitis	1
Mood stabilizing agents	2
Antibiotics for acne	1
Gastro-esophageal reflux	1
Cardiac arrhythmias	1
Infertility	1
Asthma	1
Hyperthyroidism	1

5-ASA: 5-Aminosalicylic acid formulations.

in detail^[2], but seven had been rediagnosed as Crohn's disease (CD). The 28 UC cases were included in this study (Table 1). All proctocolectomies included transanal mucosectomy and a hand-sewn anastomosis. Twenty-one patients had an IAA with J-pouch, and 7 had straight ileoanal anastomosis (SIAA). Patients filled out a questionnaire surveying the current diagnosis, complications, medical therapy and pouchitis. To map the latter two, we asked about medications to control stool frequency, and the history and treatment of pouchitis. During the outpatient visit, biopsy of the pulled through ileum, and stool and blood samples were obtained. The Ethical Committee of the Helsinki University Hospital approved the and the participants (or their guardians) gave informed consent.

Fifteen UC patients had other chronic illnesses: sclerosing cholangitis, active hepatitis, gastroesophageal reflux, gallstones, rheumatoid arthritis, sacroiliac-joint arthritis, knee arthritis, hyperthyroidism, cardiac arrhythmias, psychiatric illness, endometriosis, fibromyalgia, osteoporosis, asthma, atopic skin and contact allergy. Specific medications at the time of the study are shown in Table 2. Post-operatively, 18 (64%) UC patients had received antibiotics

as treatment for pouchitis, and of these, 7 (25%) also had received corticosteroids.

Blood inflammatory markers and fecal calprotectin

Inflammation biomarkers in blood [erythrocyte sedimentation rate, erythrocyte sedimentation rate (ESR), and serum concentration of C-reactive protein (CRP)] and fecal calprotectin (quantitative enzyme immunoassay, PhiCal test, Calpro AS, Oslo, Norway) were routinely determined^[18]. For immunohistochemistry, biopsies from pouch were used. The histological grade of inflammation in the samples was assessed according to the scoring system for pathological changes in the ileal reservoir mucosa^[7,19].

Immunohistochemistry

One biopsy was analyzed for the grade of histological inflammation, the other biopsy was used for MMP immunohistochemistry. Immunohistochemistry was performed using streptavidin-biotin-peroxidase complex technique (DakoCytomation, StreptABComplex/HRP Duet, Mouse/Rabbit, Glostrup, Denmark; and Elite goat Vectastain ABC kit, Vector laboratories, Burlingame, CA, United States) or the antibody-polymer detection technique (ImmPRESS universal reagent, Anti-Mouse/Rabbit IG, Vector laboratories Burlingame, CA, United States). Diaminobenzidine or NovaRED (Vector Laboratories) were used as chromogenic substrates and Mayer's hematoxylin as counterstain. Monoclonal antibodies were used to stain for MMP-7 (1:600, MAB3315, Millipore, Temecula, CA, United States)^[15], MMP-8 (1:100, IM38, Calbiochem, La Jolla, CA, United States)^[15], MMP-9 (1:100, MS-569-P1, Lab Vision Corporation Neomarkers, Fremont, CA, United States)^[15], TIMP-1 (1:50, IM63, Calbiochem)^[15], TIMP-2 (1:600, IM56, Calbiochem) and TIMP-3 (1:300, IM43L, Calbiochem)^[15]. Polyclonal antibodies were used for MMP-3 (1:50, ab32607, Abcam Ltd, Cambridge, United Kingdom), MMP-12 (1:80, sc-12361, Santa Cruz Biotechnology, CA, United States)^[15] and MMP-26 (1:120, a gift from Professor Isaka K, Tokyo Medical University)^[15]. MMP-8 and MMP-9 were pretreated with 1% trypsin solution for 30 min at 37 °C. MMP-3, MMP-7, MMP-26, TIMP-1, TIMP-2 and TIMP-3 were pretreated in a 95 °C water bath for 30 min (Dako retrieval solution pH 6; DakoCytomation). MMP-12 required no pre-treatment. The incubation conditions for the antibodies were: 4 °C overnight for MMPs-7, -9, -12 and TIMPs-1, -2, -3; 1 h 45 min at 37 °C for MMP-8 and 1 h at room temperature for MMP-3 and MMP-26. For negative controls, parallel sections of the same samples were processed using preimmune sera or normal rabbit or mouse immunoglobulin. As positive controls, we used formalin fixed, paraffin embedded sections of hailey-hailey, pyoderma gangrenosum and dermatitis herpetiformis (MMP-3), adenocarcinoma (MMP-7), squamous cell cancer (MMP-8), chronic wounds (MMP-9 and TIMP-2 and -3), foreign body reaction (MMP-12), endometrium (MMP-26), and pyoderma gangrenosum (TIMP-1). Immunohistochemical specimens were graded independently by two different investigators (Mäkitalo L, Piekkala M) in a semiquantitative fashion under a light-

Table 3 Expression profiles of matrix metalloproteinases and tissue inhibitor of matrix metalloproteinase in ulcerative colitis samples

	Number of positive samples	mean \pm SEM ¹
MMP-3		
Epithelium	22	0.93 \pm 0.114
Stroma	20	1.25 \pm 0.183
MMP-7		
Epithelium	28	1.36 \pm 0.092
Stroma	27	1.89 \pm 0.149
MMP-8		
Epithelium	0	0.00 \pm 0.000
Stroma	1	0.04 \pm 0.036
MMP-9		
Epithelium	0	0.00 \pm 0.000
Stroma	9	0.32 \pm 0.090
MMP-12		
Epithelium	20	0.75 \pm 0.098
Stroma	24	1.46 \pm 0.141
MMP-26		
Epithelium	0	0.00 \pm 0.000
Stroma	3	0.11 \pm 0.060
TIMP-1		
Epithelium	0	0.00 \pm 0.000
Stroma	0	0.00 \pm 0.000
TIMP-2		
Epithelium	23	0.89 \pm 0.094
Stroma	23	1.75 \pm 0.203
TIMP-3		
Epithelium	23	1.04 \pm 0.120
Stroma	28	2.39 \pm 0.130
Histology		
Neutrophils		0.85 \pm 0.154
Lymphocytes		1.07 \pm 0.185
Eosinophils		1.04 \pm 0.167
Villus atrophy		1.19 \pm 0.227
Grade		1.95 \pm 0.203

¹mean calculated from values: 0 = less than 20 positive cells; 1 = 20-50 positive cells; 2 = 50-200 positive cells; 3 = over 200 positive cells; Grade: Grade of inflammation; MMPs: Matrix metalloproteinases; TIMP: Tissue inhibitor of matrix metalloproteinase.

field microscope at $\times 100$ magnification using a scale marking staining intensity as follows: 0 = less than 20 positive cells; 1 = 20-50 positive cells; 2 = 50-200 positive cells; 3 = over 200 positive cells^[15,20]. The identity of the cell types producing each MMP or TIMP was confirmed together with an experienced pathologist (Karikoski R).

Statistical analysis

Non-parametric Mann-Whitney's test, Spearman's correlation test and independent samples t test were performed with the SPSS 17.0 for Windows (Chicago, IL) to investigate the significance of results. A $P < 0.05$ was considered significant.

RESULTS

Of the 28 patients with pediatric onset UC, nine (32.1%) had not experienced pouchitis, whereas 13 (46.4%) reported a single episode, and six (21.4%) had recurrent pouchitis (≥ 4 episodes)^[19]. Six patients were on metronidazole medication at the time of the study (Table 2)

one of them also on a 5-aminosalicylic acid formulation and showed inflammation of grades 1 to 3^[7,20]. In all the others the last episode of pouchitis had occurred more than one month earlier, in most cases several months earlier and they were not on antibiotics. Only four samples (14%) depicted no signs of inflammation, and these were all from patients who had not experienced pouchitis. Of the 19 patients who had experienced pouchitis, 7 showed mild inflammation, 3 depicted moderate and 7 severe inflammation in the biopsy. Two samples were too small to determine the grade of inflammation, but both had suffered pouchitis, the other recurrent. No sample depicted signs of colonic metaplasia.

Expression of MMP-3 was seen in the majority of the samples in the epithelium and in stroma in plasma cells, macrophages and eosinophils (Table 3 and Figure 1A, B). Also, MMP-3 positive endothelium was observed (Figure 1B1, B4). Epithelial MMP-7 was present in all samples, and stromal MMP-7 in 27 samples in plasma cells, macrophages and eosinophils, as well as endothelium (Table 3 and Figure 1C, D). Conversely, stromal MMP-9 was present in 9 samples in macrophages, plasma cells and eosinophils, and in intraepithelial neutrophils, but no positive enterocytes could be found (Table 3 and Figure 2D, E). MMP-12 was found in the majority of the samples in the epithelium and in stroma in macrophages, plasma cells, and eosinophils and intraepithelial neutrophils (Table 3 and Figure 2F, G1). Stromal cells showed positivity for MMP-26 in 3 samples in neutrophils and plasma cells but epithelial cells were negative for this MMP (Table 3 and Figure 2B). MMP-26 positive neutrophils were present in blood vessels (Figure 2B3). TIMP-2 was positive in the majority of samples in the epithelium and in stromal cells including plasma cells, eosinophils and macrophages (Table 3 and Figure 1E, F). TIMP-3 was found in enterocytes in 23 (82%) samples and in all samples in plasma cells and macrophages in stroma (mean 2.39) (Table 3 and Figure 1G). Endothelial cells were also positive for TIMP-3 (Figure 1G1, G2). Expression of MMP-8 and TIMP-1 was generally absent, although a few neutrophils in stroma were positive for MMP-8 and cryptal cells showed positivity for TIMP-1 (Figure 2A, C).

No differences in MMP- or TIMP-profiles were seen when comparing samples from patients that had experienced pouchitis (single or several episodes) ($n = 19$) to ones that had not ($n = 9$). The same was true when performing comparisons related to the frequency of pouchitis (data not shown). When comparing patients with SIAA to those with SIAA with J-pouch, no differences in MMP- and TIMP-expression profiles or the frequency of pouchitis episodes could be found.

MMPs and TIMPs did not generally correlate with inflammation markers fecal calprotectin, ESR and CRP. However, patients considered have no active inflammation (fecal calprotectin $< 100 \mu\text{g/g}$) showed higher expression of epithelial MMP-7 (Figure 1C) in their pouch biopsies compared to samples from those with active inflammation (fecal calprotectin $\geq 100 \mu\text{g/g}$)^[21] (Figure 1D1; means 1.75 *vs* 1.14, $P = 0.020$, respectively).

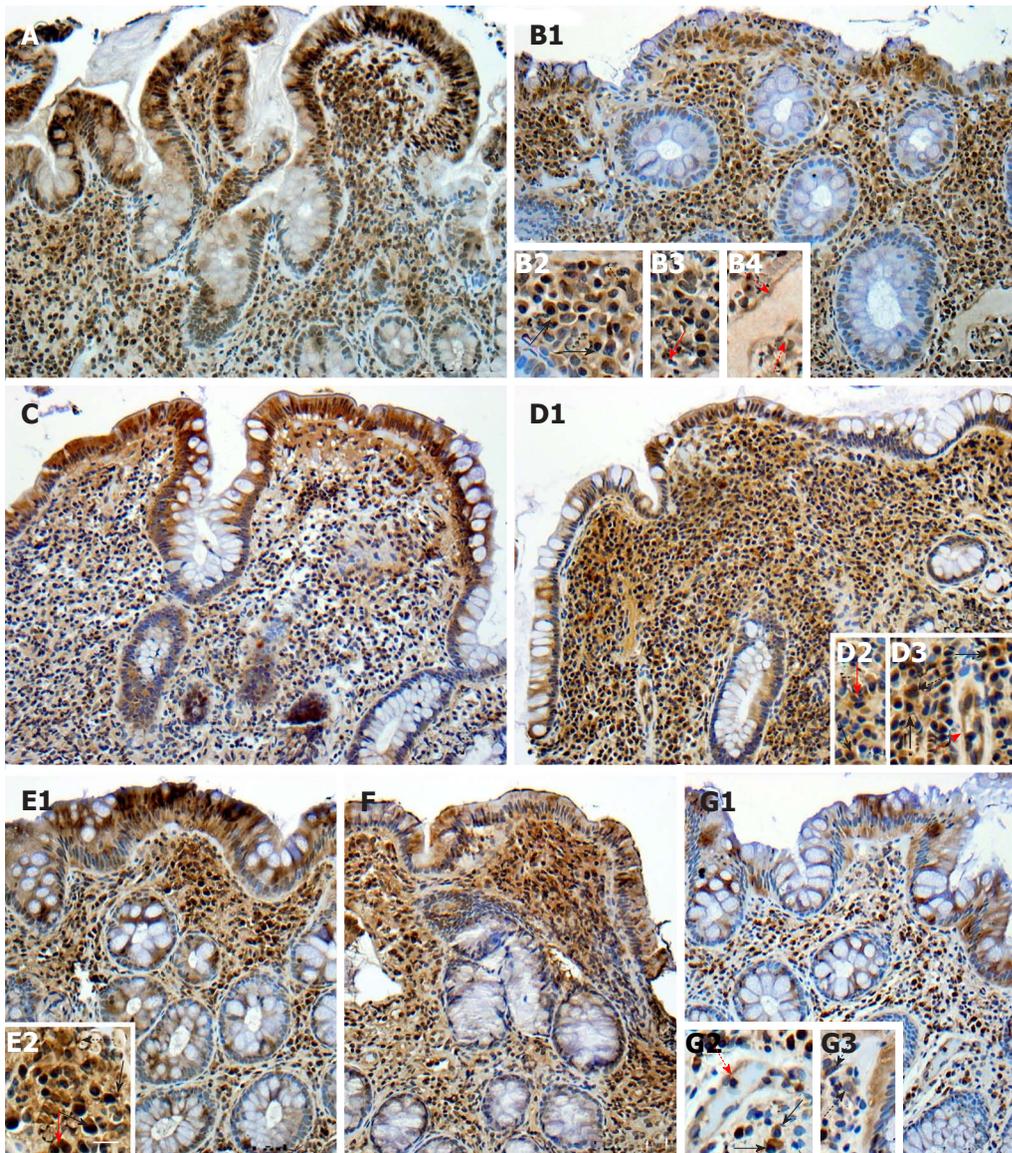


Figure 1 Matrix metalloproteinases-3, matrix metalloproteinases-7 and tissue inhibitor of matrix metalloproteinase-2 in lower (A, C, E1, respectively) and higher grade of inflammation and calprotectin levels (B1, D1, F, respectively), tissue inhibitor of matrix metalloproteinase-3 in pouch (G1). Black solid arrows plasma cells, black dotted arrows macrophages, red solid arrows eosinophils and red dotted arrows endothelium. Scale bars: 15 μm (A, B1, C, D1, E1, F, G1); 7.5 μm (B2-B4, D2, D3, E2, G2, G3). Stainings were performed using diaminobenzidine or NovaRED as chromogenic substrates and Mayer's hematoxylin as counterstain. Images were obtained using a light-field microscope, and edited using Adobe Photoshop 7.0 (Adobe Systems Incorporated).

The grade of inflammation in histological assessment^[7] correlated negatively with epithelial MMP-3 (Table 4) ($r = -0.614, P = 0.002$) (Figure 1A, B1) and MMP-7 ($r = -0.472, P = 0.027$) (Figure 1C, D1).

DISCUSSION

In pediatric patients, these MMPs and TIMPs have not been studied in pouches, and in adults, reports of only MMPs 1-3 and MMP-9 exist^[17,18]. This study reports for the first time expression of MMPs-7, -8, -12, TIMP-2, and TIMP-3 in ileal reservoir mucosa. In long-term, the MMP- and TIMP-profiles in pouch demonstrate similarities with IBD, but profiles in pouch cannot be said to replicate those in UC colon.

In adult inflamed and non-inflamed pouches of UC

patients, MMP-3 activity is considered weak^[18]. Here, MMP-3 was present in all but few samples in epithelium and stroma. In pediatric IBD, MMP-3 is elevated in endoscopically abnormal colon compared to healthy colon^[22]. MMP-3 plays an important role in T-cell- and tumor necrosis factor (TNF)- α -mediated gut injury^[23,24], and IBD gut plasma cells produce more MMP-3 than those of healthy controls^[25]. Here, expression of epithelial MMP-3 associated with the degree of inflammation, complying with animal studies where lack of MMP-3 associated with impaired wound healing^[26]. The present findings suggest that MMP-3 is an important MMP in pouch mucosa, resembling IBD. However, its relationship to the development of pouchitis is unclear. Similar to MMP-3, we found TIMP-3 in a large number of cells in stroma, expression paralleling reports in IBD colon^[15,20].

Table 4 Matrix metalloproteinases and tissue inhibitor of matrix metalloproteinase that correlated with inflammation indicators

Inflammation indicators	Correlation coefficients values				
	MMP-3	MMP-7	MMP-12		TIMP-2
	Epithelium	Epithelium	Epithelium	Stroma	Epithelium
Fecal calprotectin	NS	NS	-0.350	NS	NS
C-reactive protein	NS	NS	NS	-0.422	NS
Grade of inflammation	-0.614	-0.472	NS	NS	-0.420

NS: Not significant; MMPs: Matrix metalloproteinases; TIMP: Tissue inhibitor of matrix metalloproteinase. Correlations were calculated using Spearman's correlation test.

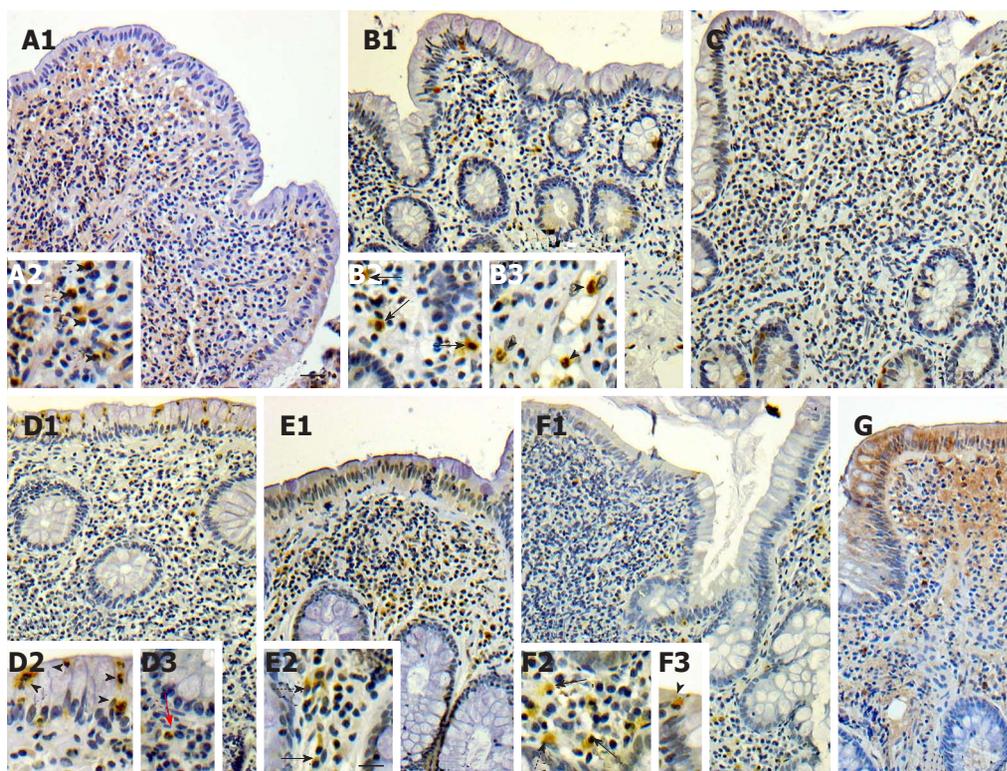


Figure 2 matrix metalloproteinases-8 (A1), matrix metalloproteinases-9 (D1, E1), matrix metalloproteinases-12 (F1, G), matrix metalloproteinases-26 (B1) and tissue inhibitor of matrix metalloproteinase-1 (C) in pouch. Inset g' added to figure from another sample, not shown in here in lesser magnification. Arrowheads depict neutrophils, black solid arrows plasma cells, black dotted arrows macrophages, red solid arrows eosinophils. Scale bars: 15 μ m (A1, B1, C, D1, E1, F1, G); 7.5 μ m (A2, B2, B3, D2, D3, E2, F2, F3). Stainings were performed using diaminobenzidine or NovaRED as chromogenic substrates and Mayer's hematoxylin as counterstain. Images were obtained using a light-field microscope, and edited using Adobe Photoshop 7.0 (Adobe Systems Incorporated).

Our group has noted that in intestinal ulcerations in IBD, TIMP-3 is expressed in a greater number of stromal cells compared to normal intestine^[14]. TIMP-3 inhibits TNF- α ^[27], an important cytokine in IBD inflammation^[28], and elevated TIMP-3 protein is likely to associate with elevated TNF- α -levels as seen in a human colonic cell line^[29]. Also, abundant TIMP-3 expression in stroma in pouch resembles that found in pediatric UC^[15], possibly preventing destructive activity as suggested for other autoimmune diseases^[30].

Of the other MMPs, expression of MMP-7, MMP-8, and MMP-9 was not IBD-like. Unlike MMP-8 and MMP-9, stromal MMP-7 was present in notable numbers of cells in almost all samples. We recently examined MMP-7 expression in pediatric IBD colon, and found weaker expression in epithelium in UC samples^[15]. Here, MMP-7 was found

in a smaller number of epithelial cells in samples with higher grade of histological inflammation and fecal calprotectin levels. This contradicts earlier results in UC^[12,31]. However, MMP-7 contributes to intestinal wound closure but over-expression may delay epithelial wound healing^[32]. Epithelial expression in pouch thus may be due to a regenerative process. MMP-7 has also been suggested to aid the growth of myofibroblasts and their function^[33] and stromal MMP-7 may take part in maintaining mucosal homeostasis. In normal colon, MMP-8 is undetectable^[10] but it may be involved in ulcer formation^[34] as elevated levels are found in ulcer bases of both UC and CD^[10]. We are not aware of previous studies of MMP-8 in pouch mucosa, but in our study, MMP-8 was generally absent from pouches. MMP-9 is important in IBD-related inflammation, and has also been shown to correlate

with disease activity^[10]. We found expression of MMP-9 in only a few samples, not suggestive of IBD-like inflammation.

In our study, MMP-12 was found in almost all pouch samples, although it is not expressed in normal adult ileum^[14,35]. While its role in IBD is unknown, this MMP may take part in macrophage migration and can activate TNF- α ^[10,36]. MMP-12 has been proposed to be a final step in tissue injury in celiac disease^[37] and might have a pathological role in pouch mucosa. Its expression suggests that pouch mucosa is dissimilar to normal ileal mucosa, even in the absence of inflammation.

In the present study, expression of MMP-26 was rare. We have proposed that immunosuppressive drugs modulate disease activity in CD by downregulating MMP-26^[20]. It is found in the colon of pediatric IBD patients^[15], but also in healthy ileum^[38], and expression is not IBD-specific. TIMP-1 is present in greater amount in inflamed adult IBD than in healthy mucosa^[31], whereas in pediatric IBD, we have reported weak TIMP-1 expression^[15]. We found no expression of TIMP-1 in the pouch, but epithelial and stromal TIMP-2 was found in most samples. Structural features of TIMP-2 suggest that it is expressed in constant amount^[39], and it has been reported to be present in normal-appearing IBD mucosa, with no significant increase in inflamed mucosa^[40]. Expression of MMP-26, TIMP-1 and TIMP-2 were inconclusive, neither supporting of contradicting IBD-etiology.

Taken together, earlier studies on MMPs and TIMPs in pouch are scarce, but in adults elevated MMP-1 and MMP-2 have been found in pouchitis mucosa^[17]. Due to small sizes of the biopsy and limited stainable slides, these MMPs were not studied here. The presence of MMP-3 and TIMP-3 in our samples is comparable to findings in IBD. In line with this, MMP-12 is detected in active IBD but not in normal ileum^[14,35,41], and here, expression of MMP-12 in pouch suggests altered mucosa. The lack of proper MMP-8 and MMP-9 expression in the pouch, however, contradicts IBD-related inflammation, as does the expression profile of MMP-7.

The results here are based on immunohistochemistry only, a limitation of this study, and further research using other techniques, e.g., Western blotting and polymerase chain reaction, would be necessary to verify the findings. We could not include a side-by-side analysis of samples from active UC and normal ileum and colon. Parallel analysis of ileal and colonic samples is challenging, as the follow up lasted until early adulthood. It is possible, that the use of medication in some cases may affect the expression of MMPs and TIMPs. It has been put forth, that probiotic use - that seems to elevate interleukin-10 in the gut-might downregulate the expression of MMP activity in the pouch mucosa and alter the expression of other proinflammatory cytokines^[18], but this finding needs more systematic studies. However, due to small sample size, we focused on comparing the expression patterns with established markers depicting the activity of inflammation. Interestingly, we found no differences in MMP or TIMP expression depending on the frequency of

pouchitis, but most samples depicted inflammation with concurrent elevated calprotectin. It seems reasonable that since there was no variation in MMP- or TIMP-profile according to the frequency of pouchitis, that inflammation markers depict very little correlation with MMPs. While even a longer follow-up may be necessary to further investigate the nature of pouch mucosa, the strength of the study is the long follow-up of the patients with pediatric onset disease, up to two decades after surgery.

In conclusion, in long-term, MMP expression in pouch shares characteristics with UC, but inflammation cannot be classified as a reactivation *per se*. Expression of MMP-3 and TIMP-3 in pouch resembles that of IBD, but the lack of MMP-9 contradicts an IBD-like pathomechanism. To the best of our knowledge, this study is the first to show that MMPs-7, -12, and TIMP-2 are also important in pouch mucosa. Further studies on the role of MMP profiles in etiopathology and the development of pouchitis in pediatric onset UC are warranted.

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COMMENTS

Background

Approximately 24% of pediatric ulcerative colitis (UC) patients undergo colectomy with ileal pouch anal anastomosis within the first decade after diagnosis. Pouchitis is the most common complication occurring in approximately two thirds of the patients at least once during the following decade. The etiology of pouchitis is unknown and it is debated whether the inflammation is a novel kind or similar to that of found in UC. Matrix metalloproteinases (MMPs) have been linked to both physiological and pathological event in inflammatory bowel disease (IBD) mucosa.

Research frontiers

In pediatric IBD MMP 10 and tissue inhibitor of matrix metalloproteinase (TIMP) 3 have been linked to the colonic inflammation. Most abundantly expressed MMP in IBD-like inflammation is MMP-9 and the MMP-7 is linked to the severity of inflammation. There are no reports of MMP expression in pediatric pouch. Thus, the authors studied the expression of MMPs-3, -7, -8, -9 and -12 and TIMPs-1, -2, and -3 from the biopsies of pediatric UC patients' pouches to find MMPs and TIMPs specific to the pediatric pouchitis.

Innovations and breakthroughs

The expression of MMPs and TIMPs in pediatric pouches has not been studied previously and adult reports of only MMPs-1, -3 and -9 exist. This study, for first time, reports the epithelial and stromal expression of MMP-3, -7 -12, TIMP-2, and -3 and the lack of MMPs-8, -9, -26, and TIMP-1 expression in pediatric ileal reservoirs. The presence of MMPs-3, -12 and TIMP-3 share some similarities with IBD, but the lack of MMP-9 contradicts the IBD-like inflammation.

Applications

By understanding the MMP- and TIMP-profile of pouch mucosa, this study takes part in characterizing molecular environment in pouch, which may in turn aid in finding specific biomolecular targets for treatment of pouchitis.

Terminology

UC is chronic disease of the mucosa of the colon and the rectum characterized by bloody diarrhea, abdominal pain, and weight loss. Pouchitis is the inflammation of the ileal reservoir that is made after colectomy to avoid permanent ostomy. MMPs are a family of 24 zinc-dependent enzymes that are capable to degrade nearly all extracellular matrix and basement membrane proteins and take part to both normal and pathological tissue remodeling. TIMPs inhibit the action

of MMPs but the actions of TIMPs are not restricted to the MMP-inhibition, they also modulate other cellular processes.

Peer review

This is a study on matrix metalloproteinases and their tissue inhibitors in pouch mucosa of pediatric onset UC. This is a good study and the topic is so important because pouchitis is often seen in those who have undergone ileal pouch-anal anastomosis.

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Overexpression of the M2 isoform of pyruvate kinase is an adverse prognostic factor for signet ring cell gastric cancer

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Abstract

AIM: To investigate M2 isoform of pyruvate kinase (PKM2) expression in gastric cancers and evaluate its potential as a prognostic biomarker and an anticancer target.

METHODS: All tissue samples were derived from gastric cancer patients underwent curative gastrectomy as a primary treatment. Clinical and pathological information were obtained from the medical records. Gene ex-

pression microarray data from 60 cancer and 19 non-cancer gastric tissues were analyzed to evaluate the expression level of PKM2 mRNA. Tissue microarrays were constructed from 368 gastric cancer patients. Immunohistochemistry was used to measure PKM2 expression and PKM2 positivity of cancer was determined by proportion of PKM2-positive tumor cells and staining intensity. Association between PKM2 expression and the clinicopathological factors was evaluated and the correlation between PKM2 and cancer prognosis was evaluated.

RESULTS: PKM2 mRNA levels were increased more than 2-fold in primary gastric cancers compared to adjacent normal tissues from the same patients (log transformed expression level: 7.6 ± 0.65 vs 6.3 ± 0.51 , $P < 0.001$). Moreover, differentiated type cancers had significantly higher PKM2 mRNA compared to undifferentiated type cancers (log transformed expression level: 7.8 ± 0.70 vs 6.7 ± 0.71 , $P < 0.001$). PKM2 protein was mainly localized in the cytoplasm of primary cancer cells and detected in 144 of 368 (39.1%) human gastric cancer cases. PKM2 expression was not related with stage ($P = 0.811$), but strongly correlated with gastric cancer differentiation ($P < 0.001$). Differentiated type cancers expressed more PKM2 protein than did the undifferentiated ones. Well differentiated adenocarcinoma showed 63.6% PKM2-positive cells; in contrast, signet-ring cell cancers showed only 17.7% PKM2-positive cells. Importantly, PKM2 expression was correlated with shorter overall survival ($P < 0.05$) independent of stage only in signet-ring cell cancers.

CONCLUSION: PKM2 expression might be an adverse prognostic factor for signet-ring cell carcinomas. Its function and potential as a prognostic marker should be further verified in gastric cancer.

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Key words: Gastric cancer; M2 isoform of pyruvate kinase; Biomarker; Signet ring cell carcinoma; Prognosis

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INTRODUCTION

Gastric cancer is the second leading cause of cancer-related deaths worldwide^[1]. Although surgery is the standard curative treatment for gastric cancer, relapses occur in many patients even after adjuvant therapy. Gastric cancer patients with the same stage of the disease present different clinical courses and have different prognosis^[2]. This heterogeneity of gastric cancer is present at the molecular level and has a genetic predisposition to it^[3-6]. Therefore, it is important to identify new molecular markers to predict patients' outcomes and personalize treatments according to the individual biology of each cancer.

Cancer cells take up glucose at higher rates than do normal cells but produce energy mainly by glycolysis, rather than by mitochondrial oxidation of pyruvate^[7]. This process, called aerobic glycolysis or the Warburg effect, is very important for tumor growth^[8]. Glycolysis increases lactate production resulting in acidification of the extracellular environment, which is believed to facilitate cell invasion and metastasis^[9]. The M2 isoform of pyruvate kinase (PKM2) was identified as a driver of aerobic glycolysis, and has been shown to be the isoform preferentially overexpressed in tumor cells^[10]. Other isoenzymes of pyruvate kinase (pyruvate kinase type M1, pyruvate kinase type L, pyruvate kinase type R) are expressed depending upon the metabolic responsibilities of the various non-cancerous cells and tissues^[10].

Several studies have shown that PKM2 is selectively stained in cancer cells in immunohistochemical assay^[11,12]. It has been suggested that plasma PKM2 could be a valuable tumor marker for diagnosis or monitoring of lung, pancreas, kidney, breast, tongue, and gastrointestinal cancers^[11-17]. However, little is known about the biological function of PKM2 in cancer and its potential as an anti-cancer target. Previous studies reported that PKM2 protein level was increased in both the tumors and plasma of gastric cancer patients^[17], and that it positively correlated with cisplatin sensitivity in gastric cancer cell lines^[18]. However, clinical and prognostic implications of PKM2 as a marker for gastric cancer are still unclear. Therefore, we decided to analyze whether PKM2 expression is correlated with cancer progression and prognosis in human gastric cancer patients.

MATERIALS AND METHODS

Gene expression microarray data analysis

The previously generated gene expression data from 60 gastric cancer patients is available in the NCBI's GEO public database (microarray data accession number, GSE13861)^[2]. All patients underwent curative gastrectomy as a primary treatment in 2005 at Severance Hospital, Yonsei University College of Medicine, Seoul, South Korea. Clinical and pathologic data were obtained by review of the Severance Hospital medical records. The gene expression data of 60 cancer and 19 non-cancer gastric tissues from 60 gastric cancer patients were analyzed. Class comparison using two sample *t* test (significance $P < 0.001$, 10 000 random permutation) identified gastric cancer specific genes.

Patients and tissues

We selected primary gastric adenocarcinoma patients who had undergone curative gastrectomy as the primary treatment between 1999 and 2007 at Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, South Korea. Patients were followed up more than 36 mo after surgery or presented recurrence or death within 36 mo after surgery. We obtained paraffin-embedded tissues and clinical data from patients. The demographic details of the cases analyzed are described in Table 1. Clinical and pathological information were obtained from the medical records. Tumors were staged according to the 7th edition of the American Joint Committee Guidelines on cancer staging issued in 2010. Tumor histology was classified as differentiated (well and moderately differentiated adenocarcinoma) and undifferentiated (poorly differentiated adenocarcinoma and signet ring cell carcinoma) type. The median follow-up duration was 70.6 mo (range: 3.6-144.6 mo). A total of 125 (34%) patients did not receive any adjuvant chemotherapy, and most of their cancers were classified as stage I. No radiation was given to any of the patients. The study was approved by the Investigational Review Board of Gangnam Severance Hospital.

Tissue microarray construction and immunohistochemistry

The paraffin-embedded tissue microarray blocks of gastric cancer tissue specimens obtained from 368 patients were used. Each block had a 3-mm core of gastric cancer tissue. Immunohistochemistry was performed on 4 μ m-thick tissue microarray tissue sections on an Enzyme-conjugated polymer backbone: Dextran (EnVision Detection kit, DAKO Cytomation, Glostrup, Denmark) according to the manufacturer's instructions after microwave-based antigen retrieval. Antibody to PKM2 1:500, Cell Signaling, Cambridge, MA, United States) was applied to the sections, which were incubated for 2 h at room temperature. The sections were incubated with secondary antibody (HRP-Rabbit/Mouse) for 15 min at room temperature, and developed using a Nova-RED substrate kit (VECTOR Laboratory, Burlingame,

Table 1 Correlation between the M2 isoform of pyruvate kinase expression and clinicopathologic characteristics of gastric cancer patients *n* (%)

Characteristics	Total (<i>n</i> = 368)	M2 isoform of pyruvate kinase expression		<i>P</i>
		Negative (<i>n</i> = 224)	Positive (<i>n</i> = 144)	
Median follow-up (70.6 mo)				
Relapse	143 (39.0)			
Death	138 (37.7)			
Adjuvant chemotherapy	243 (66.0)			
Age (yr)				0.027
≤ 60	230	150 (65.2)	80 (34.8)	
> 60	138	74 (53.6)	64 (46.4)	
Gender				0.263
Male	222	130 (58.6)	92 (41.4)	
Female	146	94 (64.4)	52 (35.6)	
AJCC 7th stage				0.811
I	105	67 (63.8)	38 (36.2)	
II	89	51 (57.3)	38 (42.7)	
III	172	105 (61.0)	67 (39.0)	
IV	2	1 (50.0)	1 (50.0)	
T stage				0.009
T1	94	62 (65.9)	32 (34.1)	
T2	42	30 (71.4)	12 (28.6)	
T3	87	40 (45.9)	47 (54.1)	
T4	145	92 (63.4)	53 (36.6)	
N stage				0.086
N0	131	80 (61.1)	51 (38.9)	
N1	62	33 (53.2)	29 (46.8)	
N2	69	37 (53.6)	32 (46.4)	
N3	106	74 (69.8)	32 (30.2)	
Histology				< 0.001
Well differentiated adenocarcinoma	22	8 (36.4)	14 (63.6)	
Moderately differentiated adenocarcinoma	96	39 (40.6)	57 (59.4)	
Poorly differentiated adenocarcinoma	143	91 (63.6)	52 (36.4)	
Signet ring cell carcinoma	79	65 (82.3)	14 (17.7)	

AJCC: American Joint Committee on Cancer; T: Tumor; N: Node.

CA, United States) and counterstained with Harris hematoxylin. The slides were photographed using a Zeiss microscope. The degree of immunostaining was scored independently by 2 observers based on the proportion of positively stained tumor cells and the intensity of staining. Tumor cell proportion was classified as follows: 0%, 10%-25%, 25%-50%, and > 50% PKM2-positive tumor cells. Staining intensity was classified as none, weak and strong staining.

We measured PKM2 expression in non-cancer gastric epithelial cells and malignant lesions. Tumors with more than 25% PKM2-positive cells were considered tumors with positive PKM2 expression, and those with less than 25% PKM2-positive cells were considered negative for PKM2 expression.

Statistical analysis

The correlation between the immunohistochemical expression scores and patient survival after surgery was estimated using the Kaplan-Meier method, followed by univariate comparison between the groups using the log-rank test. To adjust for potential confounding variables and to single out independent predictors of survival, a multivariate analysis of survival was done using the Cox's proportional hazard model using a forward stepwise mode. Statistical analyses were done with GraphPad Prism 5

(GraphPad Software, San Diego, CA) and PASW Statistics 18.0 (SPSS Inc., Chicago, IL). Association between PKM2 expression and the clinicopathological factors was tested using the χ^2 test. Two-tailed *P* values of 0.05 or less were considered statistically significant.

RESULTS

Upregulation of PKM2 mRNA in primary gastric cancers

From sixty gastric cancer patients, 60 gastric cancer tissues and 19 non-cancer adjacent gastric tissues were used for gene expression microarray analysis. PKM2 was identified as one of 3360 gastric cancer-specific genes by class comparison using the 2-sample *t* test (Data not shown). PKM2 mRNA levels were increased > 2-fold in human primary gastric cancers compared to normal adjacent tissues from the same patients (log transformed expression level: 7.6 ± 0.65 vs 6.3 ± 0.51 , $P < 0.001$, Figure 1A). Among cancer types, differentiated type cancers displayed > 2-fold increase in PKM2 levels compared to undifferentiated type cancers (log transformed expression level: 7.8 ± 0.70 vs 6.7 ± 0.71 , $P < 0.001$, Figure 1B).

Overexpression of PKM2 in primary gastric cancer

To examine whether PKM2 protein upregulation was linked to the clinical characteristics of gastric cancers,

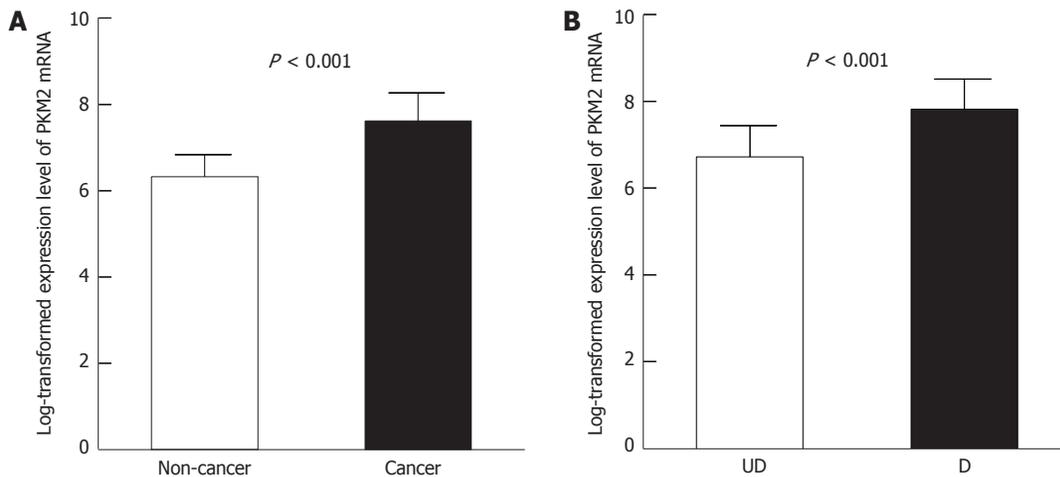


Figure 1 Expression level of the M2 isoform of pyruvate kinase mRNA by gene expression microarray. A: The M2 isoform of pyruvate kinase (PKM2) up-regulation in the 18 primary gastric cancers compared to gastric adjacent noncancerous tissues paired from the same patient ($P < 0.001$); B: PKM2 up-regulation in the 22 differentiated type (D) gastric cancers compared to 27 undifferentiated type (UD) gastric cancers ($P < 0.001$). The column and bar represent mean and standard deviation, respectively.

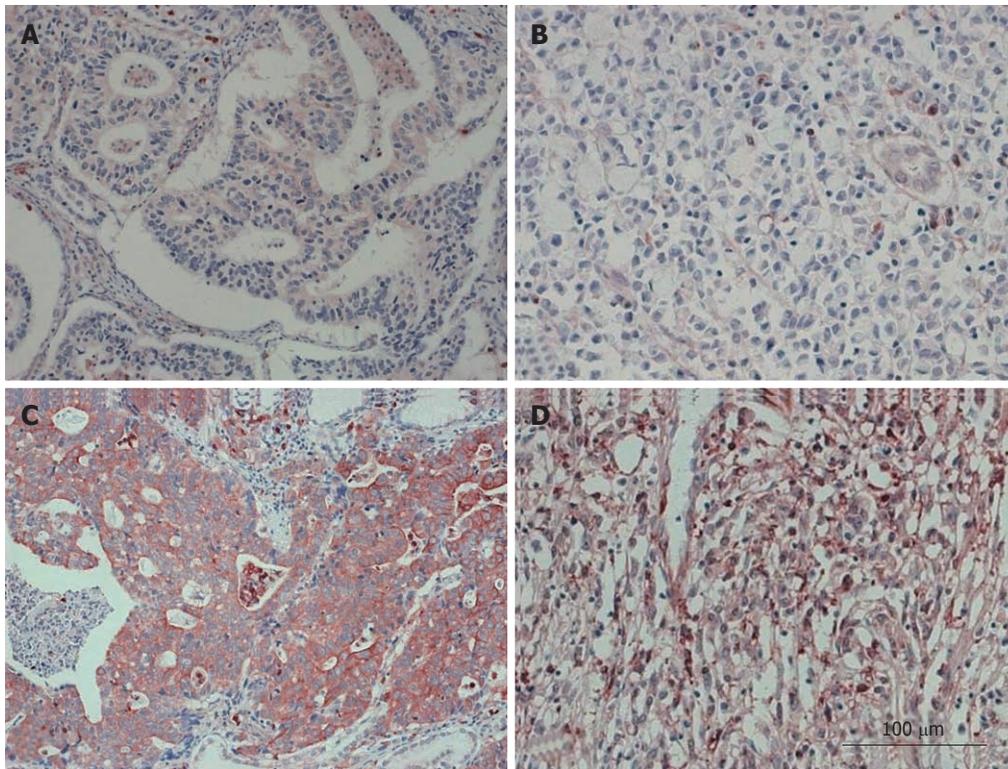


Figure 2 Representative images from immunohistochemistry assays of 368 archived gastric cancer cases at 20×10 magnification. A: Moderately differentiated adenocarcinoma and negative for the M2 isoform of pyruvate kinase (PKM2) expression; B: Signet ring cell carcinoma and negative for PKM2 expression; C: Moderately differentiated adenocarcinoma and positive for PKM2 expression; D: Signet ring cell carcinoma and positive for PKM2 expression.

the following samples were subjected to immunohistochemistry with a human PKM2 antibody: 368 paraffin-embedded, archived gastric cancer tissue samples, including 194 cases of stages I / II and 174 cases of stage III/IV tumors. The results are summarized in Table 1. PKM2 protein was detected in 144 of 368 (39.1%) human gastric cancer cases. Strong cytoplasmic staining of PKM2 was detected in 42 (11.4%) tumors and weak staining was detected in 102 (27.7%) tumors. As shown in Figure 2, PKM2 was mainly localized in the cytoplasm

of primary cancer cells. Diffuse and/or intense cytoplasmic staining was noted in only cancer cells. In contrast, PKM2 was either undetectable or only marginally detectable in the normal epithelial body gland of noncancerous tissues in the adjacent section regions (Figure 2).

Relationship between PKM2 expression and the clinical features of gastric cancers

As shown in Table 1, there was no correlation between stage and PKM2 expression ($P = 0.811$). PKM2 expres-

Table 2 Prognosis analysis of recurrence-free survival and overall survival of total patients (*n* = 366)

Characteristics	RFS		OS	
	HR (95% CI)	<i>P</i> value	HR (95% CI)	<i>P</i> value
Age (yr)				
≤ 60				
> 60	1.12 (0.80-1.57)	0.488	1.16 (0.83-1.64)	0.373
Gender				
M				
F	1.18 (0.85-1.65)	0.315	1.09 (0.78-1.53)	0.600
PKM2				
Negative				
Positive	0.93 (0.66-1.32)	0.713	0.92 (0.65-1.30)	0.637
T stage				
T1/2/3				
T4	6.12 (4.25-8.81)	< 0.001	5.04 (3.51-7.22)	< 0.001
N stage				
N0/1/2				
N3	6.02 (4.29-8.46)	< 0.001	5.64 (4.01-7.95)	< 0.001
Stage				
I / II				
III	8.42 (5.48-12.94)	< 0.001	6.70 (4.41-10.16)	< 0.001

RFS: Recurrence-free survival; OS: Overall survival; HR: Hazard ratio; PKM2: The M2 isoform of pyruvate kinase; T: Tumor; N: Node; 95% CI: 95% confidence interval.

Table 3 Univariate and multivariate analysis of overall survival in signet ring cell carcinoma (*n* = 79)

Characteristics	Groups	HR (95% CI)	<i>P</i> value
Univariate analysis			
Age (yr)	> 60 vs ≤ 60	1.11 (0.52-2.37)	0.785
Gender	F vs M	1.08 (0.56-2.07)	0.817
PKM2	Positive vs negative	2.13 (1.02-4.44)	0.042
T stage	T4 vs T1/2/3	6.25 (3.03-12.85)	< 0.001
N stage	N3 vs N0/1/2	5.70 (2.90-11.22)	< 0.001
Stage	III vs I / II	6.84 (2.83-16.53)	< 0.001
Multivariate analysis			
PKM2	Positive vs negative	2.12 (1.02-4.42)	0.044
Stage	III vs I / II	6.84 (2.83-16.55)	< 0.001

HR: Hazard ratio; PKM2: The M2 isoform of pyruvate kinase; 95% CI: 95% confidence interval; F: Female; M: Male; T: Tumor; N: Node.

sion was strongly correlated with gastric cancer differentiation (*P* < 0.001). Differentiated type cancers expressed more PKM2 protein than did the undifferentiated ones. Well differentiated adenocarcinoma showed 63.6% PKM2-positive cells; in contrast, signet-ring cell cancers showed only 17.7% PKM2-positive cells.

Association between PKM2 expression and patient prognosis

We evaluated whether PKM2 expression could be a prognostic factor for gastric cancer. Two out of 368 patients died of non-cancer and were excluded from analysis. Table 2 shows that recurrence-free survival (RFS) and overall survival (OS) are significantly different between patients with different clinical stage (*P* < 0.001), T classification (*P* < 0.001), and N classification (*P* < 0.001). There was no significant difference in prognosis according to PKM2 expression.

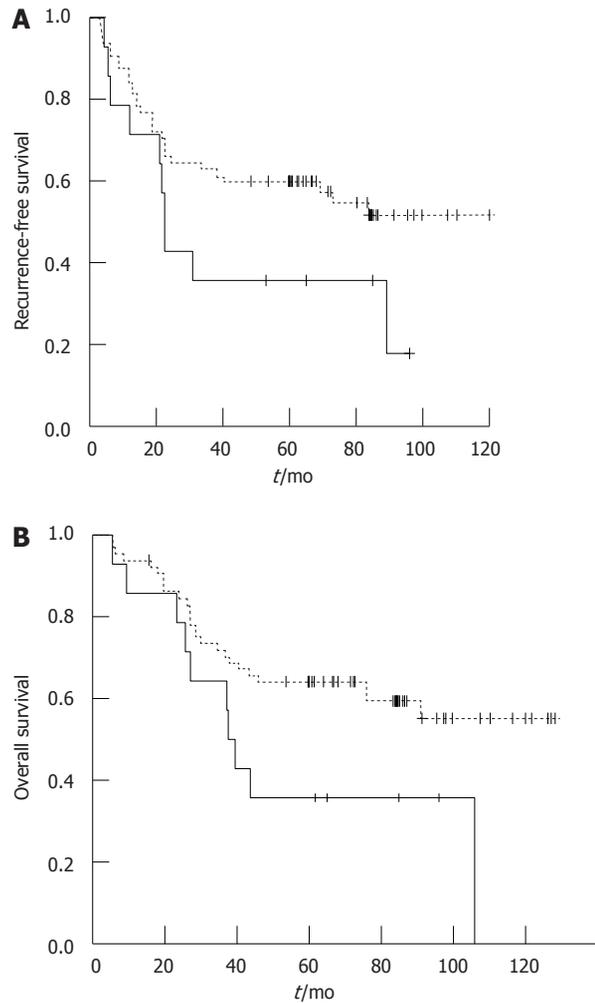


Figure 3 Recurrence-free survival (A) and overall survival (B) curves according to the M2 isoform of pyruvate kinase expression in signet ring cell gastric cancer after curative resection (*n* = 79). Positive M2 isoform of pyruvate kinase (PKM2) expression, real line; Negative PKM2 expression, dotted line.

We performed subgroup analysis at each tumor stage. In stages II or III patients, PKM2 expression showed no significant correlation with RFS or OS. However, in stage I early gastric cancer patients (*n* = 99), PKM2 expression was significantly correlated with poor RFS (*P* = 0.006) and OS (*P* = 0.015). Based on the observation that PKM2 expression rate was remarkably different according to cancer histology (Table 1), the prognostic value of PKM2 expression in patient subgroups was evaluated according to the histology. We found that in signet-ring cell carcinomas PKM2 expression correlated with poor prognosis (*P* = 0.042 for OS, Table 3 and Figure 3). Moreover, univariate and multivariate analyses showed that PKM2 expression, as well as clinical stage, were independent prognostic factors for survival (Table 3).

DISCUSSION

In this study, we report the characterization of PKM2 expression in human gastric cancers, and present its correlation with clinicopathological parameters and patients' prognosis. First, our study revealed that PKM2 is overex-

pressed in gastric cancers both at the mRNA and protein levels compared to normal gastric tissues. Well and moderately differentiated adenocarcinoma showed significantly higher expression of PKM2 (60% PKM2-positive cells); in contrast, signet-ring cell cancers showed only 17.7% PKM2-positive cells (Table 1). Because PKM2 is mainly localized in the cytoplasm of primary cancer cells and signet-ring cells contain a large amount of mucin and scanty cytoplasm, we hypothesize that this might explain the lower levels of PKM2 expression in these cells. This finding might be explained by the different glucose utilization rates of the various gastric cancer subtypes. Fluorine-18 fluoro deoxy-D-glucose positron emission tomography detected glucose uptake of tumor cells, and differentiated gastric cancers showed higher fluoro deoxy-D-glucose uptake rates than did undifferentiated ones^[19].

Second, PKM2 protein expression was found to negatively correlate with survival in signet-ring cell gastric cancer patients, as higher expression of PKM2 is associated with patients' shorter survival time ($P = 0.042$) after curative resection (Figure 3). Signet-ring cell carcinomas have a distinct biology and generally have worse prognosis than do other types of gastric cancer^[20]. A recent study reported that higher glucose uptake was indicative of a more aggressive disease especially in advanced signet-ring cell cancers^[21], although no biological mechanism was proposed to explain it. This finding is in agreement with our results. Thus, our study suggests that higher PKM2 expression, which indicates a higher rate of glycolysis in the tumor, might represent a novel prognostic marker for the clinical outcome of these types of gastric cancers.

PKM2 expression was related with poor prognosis only in stage I gastric cancer patients who did not receive chemotherapy. Only 4 of 99 patients showed relapse after curative gastrectomy, and in all cases, cancer cells were positive for PKM2 expression compared to the 36% patients overall who expressed PKM2. Furthermore, 3 patients had early relapses, within 1 year from the surgery, and all expressed high levels of PKM2 in the resected tissues. As cancer relapse in stage I patients are rare and four recurrent cases in our result are small number, it seems too early to conclude that PKM2 expression correlated with poor prognosis of stage I gastric cancer. However, it is clinical value to expand investigation in large cases. For stages II and III patients, there were no significant differences in survival. In a previous study, PKM2 was shown to positively correlate with the response to cisplatin in human gastric cancer cell lines^[18]. Cisplatin is the main chemotherapeutic agent for gastric cancers as either adjuvant or palliative aim. As cisplatin was administered as adjuvant therapy to 62.8% (147/234) of stages II or III gastric cancer patients after curative gastrectomy, the negative prognostic effect of PKM2 might be cancelled by cisplatin-based chemotherapy.

The possibility of using PKM2 as a target for the development of anti-cancer therapies has been evaluated in the preclinical setting^[22,23]. PKM2 knockdown by short hairpin RNA reduced the ability of human cancer cell lines to form tumors in nude mouse xenografts^[10,24]. If

anti-cancer strategies based on targeting PKM2 treatment are feasible, stage I or signet-ring cell cancer patients with PKM2 expression would be suitable candidates for such treatments.

The molecular function of PKM2 and its role in cancer are not completely understood yet. It was recently shown that PKM2 allows cancer cells to mount an anti-oxidant response and thereby support cell survival under acute oxidative stress^[25] and also induces epidermal growth factor receptor (EGFR)-dependent β -catenin transactivation, which leads to cell proliferation and tumorigenesis^[26]. These data are in agreement with our microarray study in which we also identified EGFR and β -catenin signaling, and hypoxic stress are linked to gastric cancer. Altogether, these studies suggest that the function of PKM2 in gastric cancer is very complex and needs to be further elucidated. In addition, the mechanisms of the regulation of PKM2 expression specifically in gastric tumors should be studied.

In conclusion, this study showed that PKM2 was overexpressed in gastric cancers. Moreover, PKM2 expression is an independent prognostic factor for signet ring cell carcinomas. The biological role of PKM2 in the development of these tumors needs to be further elucidated.

COMMENTS

Background

Gastric cancer is the major cause of cancer-related deaths worldwide. It is important to identify molecular markers to predict patients' outcomes and personalize treatments according to the individual biology. Clinical and prognostic implications of M2 isoform of pyruvate kinase (PKM2) as a marker for gastric cancer were unclear. The authors evaluated whether PKM2 expression is correlated with cancer progression and prognosis in human gastric cancer patients.

Research frontiers

PKM2 was identified as a driver of aerobic glycolysis, and has been shown to be overexpressed in tumor cells. PKM2 was selectively expressed in cancer cells and suggested valuable tumor marker for diagnosis or monitoring of various cancers. The biological function of PKM2 in cancer has been elucidated and PKM2 might be a candidate for anti-cancer target.

Innovations and breakthroughs

This study revealed that PKM2 was overexpressed in gastric cancers both at the mRNA and protein levels compared to normal gastric tissues and was found to negatively correlate with survival in signet-ring cell gastric cancer patients. PKM2 expression might be an adverse prognostic factor for signet-ring cell carcinomas. Its function and potential as a prognostic marker should be further verified in gastric cancer.

Applications

It is plausible to use PKM2 as an adverse prognostic marker of signet-ring cell cancer patients. If anti-cancer strategies based on targeting PKM2 treatment are feasible, signet-ring cell cancer patients with PKM2 expression would be suitable candidates for such treatments.

Terminology

Aerobic glycolysis: Many tumor cells have elevated rates of glucose uptake but reduced rates of oxidative phosphorylation. This persistence of high lactate production by tumors in the presence of oxygen was known as aerobic glycolysis. This metabolic switch may be required to support cell growth. High aerobic glycolysis by malignant tumors is utilized clinically to diagnose and monitor treatment responses of cancers and also to treat cancer using antagonist.

Peer review

This study investigated PKM2 expression in 368 gastric cancers and evaluated its potential as a prognostic biomarker based on relapse and survival data of patients. The results indicate that PKM2 positive expression could be used as an adverse prognostic marker in signet-ring cell gastric cancer.

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Effects of vagus nerve preservation and vagotomy on peptide YY and body weight after subtotal gastrectomy

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Abstract

AIM: To investigate the relationship between the function of vagus nerve and peptide YY₃₋₃₆ and ghrelin levels after subtotal gastrectomy.

METHODS: We enrolled a total of 16 patients who underwent subtotal gastrectomy due to gastric cancer. All surgeries were performed by a single skilled surgeon. We measured peptide YY₃₋₃₆, ghrelin, leptin, insulin, growth hormone levels, and body weight immediately before and one month after surgery.

RESULTS: Vagus nerve preservation group showed less body weight loss and less increase of peptide YY₃₋₃₆ compared with vagotomy group (-5.56 ± 2.24 kg vs -7.85 ± 1.57 kg, $P = 0.037$ and 0.06 ± 0.08 ng/mL vs 0.19 ± 0.12 ng/mL, $P = 0.021$, respectively). Moreover, patients with body weight loss of less than 10% exhib-

ited reduced elevation of peptide YY₃₋₃₆ level, typically less than 20% [6 (66.7%) vs 0 (0.0%), $P = 0.011$, odd ratio = 3.333, 95% confidence interval (1.293, 8.591)].

CONCLUSION: Vagus nerve preservation contributes to the maintenance of body weight after gastrectomy, and this phenomenon may be related to the suppressed activity of peptide YY₃₋₃₆.

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Key words: Peptide YY; Ghrelin; Vagotomy; Gastrectomy; Body weight

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INTRODUCTION

Body weight loss is a common and serious outcome in patients with gastric cancer who are treated by gastrectomy^[1]. Weight loss is correlated with declines in postoperative quality of life and is the most reliable indicator of malnutrition, which impairs immune function, infection susceptibility, and survival^[2,3]. Postoperative body weight loss can be explained by mechanisms such as reduced food intake, appetite loss caused by the reduced reservoir or delayed gastric emptying^[4,5], diarrhea^[6] and malabsorp-

tion. Malabsorption, in turn, is linked to reduced secretion of gastric acid^[7] and pancreatic insufficiency^[8,9]. In addition, it was recently suggested that alterations of endocrine status, such as reduced gastrin^[8] or ghrelin^[10], and increased cholecystokinin levels^[5,8] might be involved in weight loss after gastrectomy. However, the mechanism of body weight loss after gastrectomy has not been fully clarified.

The 28-amino acid peptide ghrelin is the endogenous ligand for the growth hormone secretagogue receptor 1a, which stimulates the release of growth hormone from the pituitary gland^[11]. The majority of ghrelin is produced by X/A-like endocrine cells of the gastric oxyntic mucosa, and smaller amounts are secreted by other organs, such as the intestine, pancreas, kidney and hypothalamus^[12,13]. Ghrelin has a number of physiologic effects that result in positive energy balance, such as promoting the appetite signal in the hypothalamus as an antagonist to leptin^[14], stimulating gastrointestinal activities such as peristalsis, gastric acid secretion, and pancreatic excretion through the vagal nerves^[15], and regulation of fat metabolism. Peptide YY₃₋₃₆, a 36 amino acid gut-derived hormone, reduces food intake over the short term in animals^[16,17] and in humans^[18] by stimulating hypothalamic neuropeptide Y receptors. Preprandial decreases and postprandial increases in plasma peptide YY₃₋₃₆ concentrations suggest that peptide YY₃₋₃₆ is one of satiety signals. Peptide YY₃₋₃₆ is suggested to be involved in intermediate term inhibition of food intake, in contrast to the classical short term regulators such as cholecystokinin^[16]. Dose-dependent reductions in food intake following peripheral peptide YY₃₋₃₆ administration are observed in both fasting and freely feeding rodents^[16,17,19,20]. In healthy human volunteers, intravenous infusion of peptide YY₃₋₃₆ caused a sustained decrease in appetite and food intake for more than 24 h^[18]. Moreover, gastric bypass results in a more robust peptide YY₃₋₃₆ response to caloric intake, which, in conjunction with decreased ghrelin levels, may contribute to the sustained efficacy of this procedure^[21]. One animal study suggested that peptide YY release is inhibited through a vagal cholinergic mechanism due to significant elevations of basal and food-induced release of peptide YY after truncal vagotomy^[22].

Recently, one study reported that reductions in visceral fat were significantly lower in patients in whom the vagus nerve was preserved than in patients who had undergone vagotomy, and concluded that the vagus nerve locally regulates amounts of intra-abdominal fat tissue^[23]. However, they did not mention the hormonal changes regarding the effect of vagotomy and vagus nerve preservation on body weight loss. Therefore, we aimed to reveal the correlation between the effect of vagus nerve preservation and vagotomy on peptide YY₃₋₃₆ or ghrelin levels after subtotal gastrectomy in relation to body weight loss.

MATERIALS AND METHODS

Patients

Sixteen patients who underwent subtotal gastrectomy at Gospel Hospital, Kosin University College of Medicine,

Busan, South Korea between January 2008 and January 2010 were enrolled in the study. The inclusion criteria were as follows: (1) adenocarcinoma of the stomach confirmed by histopathologic examination; (2) preoperative clinical staging of less than stage IIIA (International Union Against Cancer tumor, node, metastasis stage classification); (3) curative surgical treatment (R0) (i.e., subtotal gastrectomy with D1 or D2 lymph node dissection); and (4) age between 20 and 80 years. The exclusion criteria were the presence of any of the following: (1) cardiopulmonary, liver, or renal dysfunction; (2) active dual malignancy; (3) pregnancy; (4) past history of gastrointestinal surgery; and (5) postoperative complications after subtotal gastrectomy that could affect oral food intake, such as anastomotic leakage, pancreatitis, and mechanical ileus. Sixteen patients were randomized by sealed-envelope selection and divided into two study groups. The random allocation sequence was concealed until interventions were assigned. Nine patients were treated by subtotal gastrectomy with vagus nerve preservation (vagus nerve preservation group), and seven patients underwent both subtotal gastrectomy and vagotomy (vagotomy group). The study was approved by the Kosin University Ethics Committee, and all patients provided written informed consent before study entry in accordance with the Declaration of Helsinki.

Operative procedures

In seven cases, subtotal gastrectomy and bilateral truncal vagotomy were performed with D1 or D2 lymph node dissection followed by Billroth-I or Roux-en-Y reconstruction. The hepatic and celiac branches of the vagus nerve were preserved in nine patients who underwent Billroth-I or Roux-en-Y reconstruction after subtotal gastrectomy with D1 or D2 lymph node dissection. The greater omentum was largely preserved in all cases. All operations were performed by a surgeon with a history of over 1000 gastric cancer operations over the course of 20 years. An ultrasonic knife (Ultracision[®], Ethicon Endo Surgery, Cincinnati, OH, United States) was used to prevent nerve damage in the vagus nerve preservation group. Electrical impulses were produced by a high-frequency ultrasound generator, transferred to a hand piece and converted into mechanical movement at a frequency of 55.5 kHz. This instrument was chosen because of the relatively low temperatures generated, ranging from 50 °C to 100 °C, which limit damage to adjacent tissue compared to conventional diathermy. Diathermy produces temperatures up to 400 °C, resulting in char formation and deleterious thermal effects to a distance of up to 1 cm from the blade, as well as the extensive formation of necrotic tissue.

Body weight and blood sampling for hormone measurement in the fasted state

Peptide YY₃₋₃₆, ghrelin, leptin, insulin, and growth hormone levels as well as body weights were measured 1 d before surgery (day 1) and 1 mo after surgery (day 30). Venous fasting blood samples were taken early in the

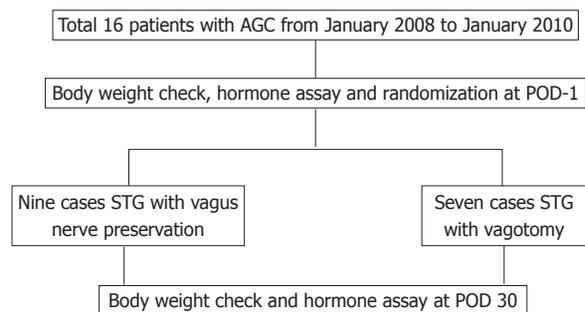


Figure 1 Flowchart for investigating effects of vagus nerve preservation and vagotomy on body weight and hormones such as peptide YY, ghrelin, leptin, insulin and growth hormone after total gastrectomy due to gastric cancer. AGC: Advanced gastric cancer; POD: Postoperative day; STG: Subtotal gastrectomy.

morning on day 1 and day 30 for the measurement of plasma concentrations of the following hormones: peptide YY₃₋₃₆ (enzyme-linked immunosorbent assay, Phoenix Pharmaceuticals Inc., Belmont, CA, United States), total ghrelin (radioimmunoassay, Linco Research Inc., St. Louis, MO, United States), leptin (radioimmunoassay, Linco Research Inc.), insulin (two-site sandwich immunoassay, Siemens Medical Solutions Diagnostics, Los Angeles, CA, United States), and human growth hormone (radioimmunoassay, Packard Instruments Inc., Chicago, IL, United States). Peptide YY₃₋₃₆, insulin, and growth hormone levels were measured with the V-MAX 220 VAC enzyme-linked immunosorbent assay reader (Molecular Devices, Sunnyvale, CA, United States), ADVIA Centaur XP (Siemens, Tarry-town, NY, United States), and COBRA II Gamma counter (PacKard, Waltham, MA, United States), respectively. Ghrelin and leptin were analyzed with the COBRA II Gamma Counter (PacKard, United States). Total protein, albumin, total cholesterol, and triglyceride levels were tested with the ADVIA 2400 (Siemens, Tarry-town, NY, United States).

Statistical analysis

Statistical analysis was performed using the SPSS software (version 16.0, SPSS, Chicago, IL, United States). Differences between the vagus nerve preservation group and the vagotomy group, including sex, age, stages of gastric cancer, body weight, body mass index (BMI), and laboratory values were assessed by Fisher's exact tests and Mann-Whitney *U* test. Wilcoxon signed rank test was used to calculate the changes between pre and post-operative values in body weight, BMI, peptide YY₃₋₃₆, ghrelin, leptin, insulin, growth hormone, and other laboratory profiles of all patients. The differences in body weight, BMI, hormones, and other laboratory values between two groups one month after operation were calculated by Mann-Whitney *U* test. Fisher's exact tests were used to validate the correlation among vagus nerve preservation, body weight loss of less than 10%, and peptide YY₃₋₃₆ level increases of less than 20%. Statistical significance was set at a *P* value of < 0.05.

Table 1 Baseline characteristics of patients in two groups

Operative procedure	Vagus nerve preservation (<i>n</i> = 9)	Vagotomy (<i>n</i> = 7)	<i>P</i>
Age (yr)	59.01 ± 13.12	52.74 ± 6.40	NS
Sex (male/female)	6/3	4/3	NS
Body weight (kg)	62.01 ± 11.22	63.13 ± 6.01	NS
BMI (kg/m ²)	23.21 ± 2.40	24.41 ± 3.53	NS
TNM stage			NS
II	5 (55.5)	3 (42.8)	
IIIa	4 (45.5)	4 (57.2)	
Pathologic type			NS
Differentiated	6 (66.6)	4 (57.2)	
Undifferentiated	3 (33.4)	3 (42.8)	
Reconstruction			NS
Billroth-1	5 (55.5)	5 (71.4)	
Roux-en-Y	4 (45.5)	2 (28.6)	
Laboratory values			
Peptide YY ₃₋₃₆ (ng/mL)	0.38 ± 0.10	0.42 ± 0.07	NS
Ghrelin (pg/mL)	693.71 ± 211.25	703.90 ± 238.89	NS
Leptin (ng/mL)	3.88 ± 4.59	5.50 ± 7.99	NS
Insulin (mIU/L)	6.36 ± 3.95	6.47 ± 4.66	NS
Growth hormone (ng/mL)	1.51 ± 2.34	1.4 ± 0.18	NS
HOMA index	1.70 ± 1.24	1.68 ± 1.38	NS
Albumin (g/dL)	3.71 ± 0.43	3.63 ± 0.52	NS
Total protein (g/dL)	6.93 ± 0.50	6.92 ± 1.04	NS
Total cholesterol (mg/dL)	204.91 ± 25.23	209.63 ± 21.11	NS
Triglyceride (mg/dL)	171.72 ± 49.21	140.41 ± 45.24	NS

Data were expressed as mean ± SD or *n* (%). BMI: Body mass index; TNM: Tumor, node, metastasis; HOMA: Homeostatic model assessment; NS: Not significant.

RESULTS

Patient characteristics

The study flow diagram is summarized in Figure 1. There was no significant difference in age, sex, body weight, BMI, clinical stage of gastric cancer, pathologic types, and laboratory profiles including hormone values between the two groups. Table 1 summarizes the clinical and laboratory background of the 16 patients who completed the study.

Changes of body weight and hormones

All patients demonstrated body weight loss (preoperative body weight: 62.51 ± 9.02 kg *vs* postoperative body weight: 56.02 ± 8.32 kg, *P* < 0.001) with decreased BMI (23.71 ± 2.94 kg/m² *vs* 21.33 ± 2.62 kg/m², *P* < 0.001). All patients have increased peptide YY₃₋₃₆ (0.41 ± 0.09 ng/mL *vs* 0.52 ± 0.15 ng/mL, *P* = 0.020), and decreased ghrelin (787.34 ± 421.33 pg/mL *vs* 506.21 ± 201.10 pg/mL, *P* = 0.007) postoperatively. Insulin levels were significantly increased in most patients (4.59 ± 6.12 mIU/L *vs* 1.86 ± 1.49 mIU/L, *P* = 0.001). There was no correlation between the basal values of peptide YY₃₋₃₆ and the extent of body weight loss. There were no significant changes in leptin and growth hormone levels after surgery. No significant differences were found in albumin, protein, triglyceride, and total cholesterol levels either. Vagus nerve preservation group showed less decrease in

Table 2 Changes of body weight, body mass index and laboratory values between pre-operation and one month after operation and parameters at 1 mo after operation in two groups

Operative procedure	Changes from pre-operation to one month after operation			One month after operation		
	Vagus nerve preservation (n = 9)	Vagotomy (n = 7)	P	Vagus nerve preservation (n = 9)	Vagotomy (n = 7)	P
Body weight (kg)	-5.56 ± 2.24	-7.85 ± 1.57	0.037 ¹	56.44 ± 10.40	55.29 ± 4.99	NS
BMI (kg/m ²)	-1.91 ± 1.04	-2.91 ± 0.39	0.031 ²	21.21 ± 2.08	21.44 ± 3.39	NS
Peptide YY ₃₋₃₆ (ng/mL)	0.06 ± 0.08	0.19 ± 0.12	0.021 ³	0.44 ± 0.07	0.62 ± 0.17	0.020 ⁴
Ghrelin (pg/mL)	-229.23 ± 196.69	-174.47 ± 174.58	NS	464.47 ± 235.49	529.43 ± 134.86	NS
Leptin (ng/mL)	-2.08 ± 3.38	-3.56 ± 7.41	NS	1.79 ± 1.31	1.94 ± 1.79	NS
Insulin (mIU/L)	9.5 ± 12.37	11.01 ± 8.26	NS	15.86 ± 10.89	17.48 ± 7.48	NS
HOMA index	0.33 ± 0.15	0.35 ± 0.43	NS	2.10 ± 1.79	2.25 ± 1.99	NS
Growth hormone (ng/mL)	0.07 ± 3.32	0.19 ± 0.12	NS	1.58 ± 2.20	1.95 ± 2.44	NS
Albumin (g/dL)	-0.05 ± 0.32	-0.14 ± 0.66	NS	3.64 ± 0.31	3.50 ± 0.43	NS
Total protein (g/dL)	-0.17 ± 0.71	-0.18 ± 0.83	NS	6.77 ± 0.41	6.78 ± 0.88	NS
Total cholesterol (mg/dL)	-4.00 ± 15.21	-6.57 ± 10.42	NS	200.88 ± 22.31	203.00 ± 27.82	NS
Triglyceride (mg/dL)	-14.33 ± 20.40	-11.71 ± 18.93	NS	157.44 ± 38.29	128.71 ± 36.66	NS

All data were expressed as mean ± SD. ¹95% confidence interval (CI) = -4.445 to -0.157; ²95% CI = -1.902 to -0.103; ³95% CI = -0.224 to -0.022; ⁴95% CI = -0.320 to -0.032. BMI: Body mass index; HOMA: Homeostatic model assessment; NS: Not significant.

body weight (-5.56 ± 2.24 kg *vs* -7.85 ± 1.57 kg, $P = 0.037$) and BMI (-1.91 ± 1.04 kg/m² *vs* -2.91 ± 0.39 kg/m², $P = 0.031$) than vagotomy group. Moreover, less elevation of peptide YY₃₋₃₆ was observed in vagus nerve preservation group (0.06 ± 0.08 ng/mL *vs* 0.19 ± 0.12 ng/mL, $P = 0.021$) than vagotomy group. Total protein, albumin, total cholesterol, and triglyceride levels did not show significant changes between two groups after surgery. These results were presented in Table 2. Vagus nerve preservation group showed significantly lower post-operative peptide YY₃₋₃₆ value than vagus nerve preserved group (0.44 ± 0.07 ng/mL *vs* 0.62 ± 0.17 ng/mL, $P = 0.020$). However, there were no differences in other post-operative values between two groups. These post-operative findings were described in Table 2.

Relationships among vagus nerve preservation, body weight change, and peptide YY₃₋₃₆ change

Patients were separated into groups based on the changes of body weight and peptide YY₃₋₃₆. Body weight loss of less than 10% was more frequently observed in vagus nerve preservation group [6 (66.7%) *vs* 0 (0.0%), $P = 0.011$, odd ratio (OR) = 3.333, 95% confidence interval (95% CI) (1.293, 8.591); Figure 2A]. Increases of peptide YY₃₋₃₆ less than 20% was more frequently noticed in the vagus nerve preservation group after surgery [5 (55.6%) *vs* 0 (0.0%), $P = 0.034$, OR = 2.750, 95% CI (1.258, 6.010); Figure 2B]. Patients with body weight loss of less than 10% exhibited significant correlation with reduced elevation of peptide YY₃₋₃₆ level, typically less than 20% [5 (83.3%) *vs* 0 (0.0%), $P = 0.001$, OR = 11.000, 95% CI (1.697, 71.282); Figure 2C].

DISCUSSION

Weight loss is a common complication after gastrectomy. However, the effects of different surgical procedures associated with gastrectomy on postoperative body weight

loss and hormonal changes are not well understood. In the present study, we examined changes in body weight, peptide YY₃₋₃₆, ghrelin, leptin, growth hormone, and insulin levels in vagus nerve preservation and vagotomy groups.

Although all 16 patients who underwent subtotal gastrectomy exhibited body weight loss, the vagus nerve preservation group demonstrated significantly less decreases in body weight and BMI than the vagotomy group. This study reproduces the findings of Melissas *et al*^[7] who postulated that this phenomenon might be explained by the energy saving function of the vagus nerve as a major parasympathetic nerve innervating visceral organs. The sympathetic nervous system predominates during energy-spending catabolic states, whereas in energy-saving anabolic states the parasympathetic nervous system prevails^[24,25]. Anatomical and physiological studies have demonstrated the innervation of adipose tissue by sympathetic nerves, which in turn accelerate lipolysis of adipocytes^[26,27]. The vagotomy group showed significantly greater visceral fat mass reduction than the vagus nerve preservation group^[7]. In our study, vagus nerve preservation presented 3.333 fold more chance than vagotomy group for body weight loss less than 10% (95% CI from 1.293 to 8.591).

All patients in this study showed increased peptide YY₃₋₃₆ levels after subtotal gastrectomy. A previous study demonstrated that stomach gastrin inhibited peptide YY secretion in rats^[28]. All study patients lost the antrum after subtotal gastrectomy, therefore reducing the effect of stomach gastrin on peptide YY. This is a possible explanation for the elevation of peptide YY levels in these patients. An alternative explanation for this phenomenon is that there is a negative correlation between fasting peptide YY₃₋₃₆ and markers of adiposity^[18,29-31]. In addition, fasting peptide YY levels are significantly higher in anorexia nervosa sufferers than normal weight controls. In rodent studies, mice exposed to a high-fat diet develop

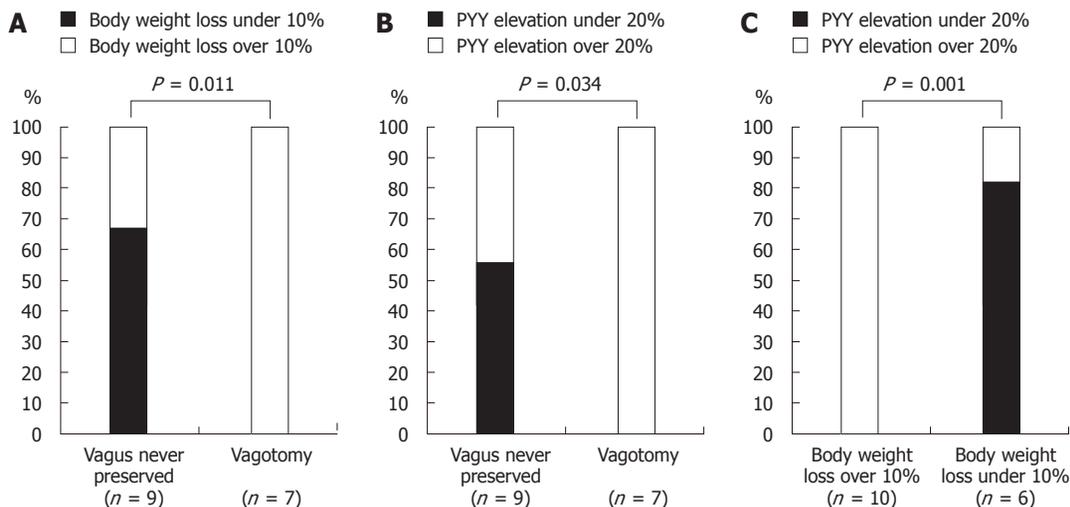


Figure 2 Correlations among vagus nerve preservation, body weight loss and peptide YY₃₋₃₆ increase. A: Vagus nerve preservation was correlated with body weight loss less than 10% [66.7% vs 0.0%, $P = 0.011$, odd ratio (OR) = 3.333, 95% confidence interval (95% CI) (1.293, 8.591)]; B: The vagus nerve preservation group showed significant correlation with peptide YY₃₋₃₆ level increases of less than 20% [55.6% vs 0.0%, $P = 0.034$, OR = 2.750, 95% CI (1.258, 6.010)]; C: Patients with body weight loss of less than 10% exhibited tight correlation with reduced elevation of peptide YY₃₋₃₆ level, typically less than 20% [83.3% vs 0.0%, $P = 0.001$, OR = 11.000, 95% CI (1.697, 71.282)]. PYY: Peptide YY.

obesity and a concomitant reduction in circulating peptide YY^[32,33].

Vagus nerve preservation group demonstrated significantly less increase in peptide YY₃₋₃₆ than vagotomy group in the present study. Moreover, the vagus nerve preservation group demonstrated a tight correlation with increases in peptide YY₃₋₃₆ levels of less than 20%. According to a previous animal study, truncal vagotomy resulted in significant elevations of basal and food-induced release of peptide YY^[22]. This suggests that peptide YY release is inhibited tonically, probably through a vagal cholinergic mechanism. Adrenergic pathways did not participate in food-stimulated peptide YY release. However, electrical stimulation of the splanchnic nerves increased basal levels of peptide YY, suggesting that the sympathetic nervous system affects the release of peptide YY^[22]. This agrees with our finding that the vagus nerve preservation group showed reduced elevation of peptide YY₃₋₃₆ levels in comparison to the vagotomy group, although increased peptide YY₃₋₃₆ levels were observed in both groups. An alternative explanation for more elevation of peptide YY₃₋₃₆ in the vagotomy group could be a compensatory increase in peptide YY₃₋₃₆ secretion in response to reduced peptide YY₃₋₃₆ signaling to the hindbrain *via* the vagus. Thus peptide YY₃₋₃₆ may exert its effects on body weight by acting centrally, *via* vagal stimulation, or both. Many lines of evidence suggest that peptide YY₃₋₃₆ exerts its effects on feeding *via* the hypothalamus; intra-arcuate injection of peptide YY₃₋₃₆ reduces feeding, whereas Y2-antagonist injection has the opposite effect^[34]. Thus, vagotomy, transection of hindbrain-hypothalamic pathways, can cause compensatory increase of peripheral peptide YY₃₋₃₆.

When correlations between body weight loss and increase of peptide YY₃₋₃₆ levels were performed, patients who demonstrated body weight loss of less than 10% ex-

hibited lower increases in peptide YY₃₋₃₆ levels, less than 20%. Because vagus nerve preservation is associated with lower increases in peptide YY₃₋₃₆ levels and reductions in body weight loss, it is not clear whether the lower increase in peptide YY₃₋₃₆ levels actually caused less body weight loss. However, we cannot exclude the possibility that peptide YY₃₋₃₆ levels influence body weight, as peptide YY₃₋₃₆ has been shown to suppress appetite and promote weight loss^[16,17,19,20,35]. In healthy human volunteers, intravenous infusion of peptide YY₃₋₃₆ causes sustained decreases in appetite and food intake for more than 24 h^[18]. Energy intake by obese subjects during a buffet lunch was reduced by 30% after intravenous infusion of peptide YY₃₋₃₆^[18]. Chronic administration of peptide YY₃₋₃₆ inhibits food intake and reduced body weight gain in mice, rabbits, and rhesus macaques^[16,35,36]. In addition, daily food intake, body weight, and body fat are increased in peptide YY knockout mice in comparison to wild-type mice^[32]. Although the number of patients enrolled in the present study was limited, these findings suggest that elevated basal peptide YY₃₋₃₆ levels may contribute to body weight loss after subtotal gastrectomy.

All patients in this present study demonstrated increased plasma insulin after operation. A previous study showed the partial gastrectomy and intestinal resection induced impaired oral glucose tolerance despite normal insulin concentrations^[37]. Increased basal level insulin might reflect the impaired insulin tolerance in the present study although it was not proved by oral glucose tolerance test.

There are five limitations in our study. First, patient appetite was not assessed. Assessing subjects' appetite on a visual analogue scale before and after surgery would have allowed us to evaluate the relationship between changes in peptide YY₃₋₃₆ levels and changes in appetite. Second, unfortunately, this present study did not include

data from meal-stimulated secretions of peptide YY or other hormones such as glucagon like peptide-1, which could have shed more light on the true interaction effects between vagus nerve preservation/vagotomy and gastrointestinal hormonal functions and body weight. Third, we did not evaluate body composition. Evaluating changes in body composition may have helped to elucidate the correlations between vagus nerve preservation, changes in peptide YY₃₋₃₆ levels, and changes in specific body composition, especially visceral fat levels. Fourth, only total ghrelin was measured, since active octanoylated ghrelin is unstable. Although both total and active ghrelin appear to be regulated in a similar and parallel manner, future studies will need to focus on measurement of the biologically active form. Finally, small number of patients was enrolled in the present study.

In summary, body weight loss, increased peptide YY₃₋₃₆ levels, and decreased ghrelin levels were observed in all patients after subtotal gastrectomy. Vagus nerve preservation group showed less decrease in body weight and BMI than vagotomy group. Less increase of peptide YY₃₋₃₆ was observed in vagus nerve preservation group. Moreover, patients with body weight loss of less than 10% exhibited reduced elevation of peptide YY₃₋₃₆ level, typically less than 20%. Based on these results and those of previous studies, we concluded that vagus nerve preservation resulted in reduced body weight loss after subtotal gastrectomy, in direct relation with peptide YY₃₋₃₆ activities and suggest that vagus nerve should be preserved for preventing excessive body weight loss after subtotal gastrectomy due to gastric cancer.

COMMENTS

Background

Body weight loss is a common and serious outcome in patients with gastric cancer who are treated by gastrectomy. Weight loss is correlated with declines in postoperative quality of life and is the most reliable indicator of malnutrition, which impairs immune function, infection susceptibility, and survival. Patients who underwent vagus nerve-preserving procedures lose less body weight than patients treated with vagotomy after gastrectomy.

Research frontiers

Ghrelin has a number of physiologic effects that result in positive energy balance, such as promoting the appetite signal in the hypothalamus as an antagonist to leptin. Peptide YY₃₋₃₆ is suggested to be involved in intermediate term inhibition of food intake, in contrast to the classical short term regulators such as cholecystokinin. Recently, one study reported that reductions in visceral fat were significantly lower in patients in whom the vagus nerve was preserved than in patients who had undergone vagotomy, and concluded that the vagus nerve locally regulates amounts of intra-abdominal fat tissue.

Innovations and breakthroughs

This study is the first to evaluate relationship between the differences in weight loss between patients treated with vagus nerve-preserving procedures and vagotomy and the changes of peptide YY₃₋₃₆ and ghrelin levels after subtotal gastrectomy. Vagus nerve preservation group showed less decrease in body weight and BMI than vagotomy group. Less increase of peptide YY₃₋₃₆ was observed in vagus nerve preservation group. Moreover, patients with body weight loss of less than 10% exhibited reduced elevation of peptide YY₃₋₃₆ level, typically less than 20%. Based on these results, the authors concluded that vagus nerve preservation resulted in reduced body weight loss after subtotal gastrectomy, in direct relation with peptide YY₃₋₃₆ activities

Applications

Present study showed that vagus nerve preservation resulted in less decrease

in body weight and BMI than vagotomy group. Furthermore, this study suggested plausible peptide YY₃₋₃₆ activities in this phenomenon. Considering these findings, the authors cautiously suggest to preserve vagus nerve during subtotal gastrectomy for less body weight loss.

Peer review

This is a good experiment study in which authors analyze the cause of the differences in weight loss between patients treated with vagus nerve-preserving procedures and vagotomy in the view of the changes of peptide YY₃₋₃₆ and ghrelin levels after subtotal gastrectomy. The findings that vagus nerve preservation resulted in reduced body weight loss after subtotal gastrectomy, in direct relation with peptide YY₃₋₃₆ activities suggesting the possible role of peptide YY₃₋₃₆ in this phenomenon.

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Human papilloma virus 16 E6 oncoprotein associated with p53 inactivation in colorectal cancer

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papilloma virus (HPV) infection and colorectal cancer.

METHODS: Sixty-nine patients with pathologically confirmed primary colorectal cancer including 6 stage I, 24 stage II, 21 stage III, and 18 stage IV patients were enrolled in this study to investigate whether HPV 16 could be involved in colorectal tumorigenesis. Nested-polymerase chain reaction (nested-PCR) was used to detect HPV16 DNA in colorectal tumor tissues and further confirmed by *in situ* hybridization (ISH). In addition, immunohistochemistry analysis was performed to examine the E6 oncoprotein in colorectal tumors. To verify whether E6 could inactivate the p53 transcriptional function, the levels of p21 and Mdm2 mRNA expression were evaluated by real-time reverse transcription (RT)-PCR.

RESULTS: Of the 69 colorectal tumors, HPV16 DNA was detected in 11 (16%) by nested-PCR, and HPV16 DNA was present in 8 of the 11 (73%) tumors which was confirmed by ISH. The presence of HPV16 DNA in colorectal tumors was not associated with patients' clinical parameters including age, gender, smoking status, tumor site; however, HPV16 infection was more common in stage I patients than in late-stages patients (II, III and IV). We next asked whether HPV16 infection could be linked with colorectal cancer development. Immunohistochemical data indicated that 8 of the 11 HPV16 DNA-positive tumors had E6 oncoprotein expression. Moreover, we also observed that the adjacent normal tissues including endothelial cells, lymphocytes, fibroblasts, and gland cells in E6-positive tumors had E6 oncoprotein expression. In addition, 3 of the 4 (75%) E6-positive tumors carrying p53 wild-type had negative immunostaining, but one tumor had less p53 immunostaining. We further examined whether E6-positive and/or p53 mutated tumors reduce p53 transcriptional activity. Real-time RT-PCR analysis indicated that p21 and mdm2 mRNA expression levels in E6/p53-wildtype tumors were significantly lower than in their adjacent

Abstract

AIM: To investigate the association between human

normal tissues; as expected, E6-positive/p53-mutated tumors had lower p21 and mdm2 mRNA expression levels compared with their adjacent normal tissues. These results clearly indicate that the E6 oncoprotein expressed in p53 wildtype tumors may reduce p21 and mdm2 expression *via* p53 inactivation.

CONCLUSION: These results suggest that HPV16 infection may be involved in a subset of colorectal cancer, and we suggest that the transmission of HPV to the colon and rectum might occur through peripheral blood lymphocytes.

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Key words: Human papilloma virus; Colorectal cancer; p53; p21; Blood lymphocytes

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INTRODUCTION

A high risk of human papilloma virus (HPV) 16/18 infection has been documented to be involved in the development of cervical and anal genital cancers^[1]. Among non-genital cancers, the association of HPV16/18 infection and oropharyngeal cancer was recently evidenced^[2-4]. However, other non-genital cancers, including lung, breast, and colorectal cancers have not yet been identified^[5-12]. This is due to a lack of clarity as to how HPV transmits to internal organs even though blood circulation has been suggested as a possible route of infection^[13,14].

In the past two decades, a large body of research has demonstrated an association between HPV infection and colorectal cancer^[5,6,15-17]. Studies have shown HPV16 to be the major HPV subtype in colorectal tumors^[16,17]. However, inconsistent conclusions have been drawn on this issue because only HPV DNA detection is used to associate HPV and colorectal cancer; there is no evidence to demonstrate that HPV is involved in colorectal cancer development. It is well known that HPV DNA integration into the host chromosome plays a crucial role in HPV-associated tumorigenesis^[18]. When HPV DNA was spliced at E2, E6 and E7 oncoproteins were expressed which inactivated the p53 and Rb pathways^[18]. Therefore, in the present study, 69 tumors resected from colorectal cancer patients were enrolled to determine the presence

of HPV16 DNA by nested polymerase chain reaction (PCR) and *in situ* hybridization (ISH), and expressions of E6 and p53 proteins were evaluated by immunohistochemistry (IHC) in colorectal tumor paraffin serial sections. We explored whether HPV16 DNA could exist and express E6 oncoprotein to inactivate the p53 pathway in colorectal tumors. We next asked whether HPV16 DNA and E6 oncoprotein could be expressed in adjacent normal tissue cells, such as endothelial cells and lymphocytes, to understand whether colorectal tumors infected with HPV16 could be spread through blood circulation.

MATERIALS AND METHODS

Study subjects

We collected tumor specimens from 69 patients with colorectal cancer. All of these patients, including 34 females and 35 males who were admitted to the Department of Surgery at Chung Shan Medical University Hospital, Taichung, Taiwan between 2000 and 2005, were asked to submit a written informed consent approved by the Institutional Review Board. A series of examinations for pathological stages were conducted for each case by board-certified pathologists based on the criteria in the 7th edition of the American Joint Committee on Cancer. We collected information pertaining to personal characteristics from hospital reports. Smokers were defined as those who were active smokers or previous smokers and nonsmokers were those who had never smoked.

Immunohistochemistry

Formalin-fixed and paraffin-embedded specimens were sectioned at a thickness of 3 μ m. All sections were then deparaffinized in xylene, rehydrated through serial dilutions of alcohol, and washed in phosphate-buffered saline (pH 7.2), the buffer that was used for all subsequent washes. For HPV16 E6 and p53 detection, sections were heated in a microwave oven twice for 5 min in citrate buffer (pH 6.0), and then incubated with a monoclonal anti-human p53 antibody (DAKO, DO7, Denmark; at a dilution of 1:250) for 60 min at 25 °C or with monoclonal anti-HPV16 (Santa Cruz, CA, United States) for 90 min at 25 °C. The conventional streptavidin peroxidase method (DAKO, LSAB Kit K675, Copenhagen, Denmark) was performed to develop signals and the cells were counter-stained with hematoxylin. Negative controls were obtained by leaving out the primary antibody. The intensities of signals were evaluated independently by three observers. The results were evaluated independently by three observers and scored for the percentage of positive nuclei: score 0, no positive staining; score +, from 1% to 10%; score ++, from 1% to 50%; score +++, more than 50% positive cells. Positive control slides for p53 protein detection were purchased from DAKO (Denmark) and the cervical cancer tumor tissues with HPV16 were used as a positive control for HPV16 E6. The antibody dilution buffer replaced the antibodies to serve as a negative control.

Direct sequencing

Mutations in exons 5-8 of the *p53* gene were determined by direct sequencing of PCR products amplified from the DNA of tumor cells isolated by microdissection of the colorectal tumor tissues. DNA lysis buffer was used to lyse cells and then the solution was subjected to proteinase K digestion and phenol-chloroform extraction. Finally, the DNA was precipitated by ethanol. Target sequences were amplified in a 50 μ L reaction mixture containing 20 pmol of each primer, 2.5 units of Taq polymerase (TAKARA Shuzo, Shiga, Japan), 0.5 mmol/L dNTPs, 5 μ L PCR reaction buffer, and 1 μ L genomic DNA as the template. Genomic DNA sequences extracted from the frozen sections were not adequate for an amplification of long fragment DNA sequences, and therefore, PCR products ranging from 200 to 400 bp were amplified for *p53* mutation analysis. Primers for β -actin, which act as an internal control, were included in each amplification reaction. The primers used in the reactions were E5S (5'TGCCCTGACTTTCAACTCTG3') and E5AS (5'GCTGCTCACCATCGCTATC3') for exon 5, E6S (5'CTGATTCCCTCACTGATTGCT3') and E6AS (5'AGTTGCAAACCAGACCTCAGG3') for exon 6, E7S (5'CCTGTGTTATCTCCTAGGTTG3') and E7AS (5'GCACAGCAGGCCAGTGTGCA3') for exon 7, and E8S (5'GACCTGATTTCCCTTACTGCC3') and E8AS (5'TCTCCTCCACCGCTTCTTGT3') for exon 8. An initial cycle was performed for 5 min at 94 °C; followed by 35 cycles of 40 s at 94 °C, 40 s at 54 °C, and 1 min at 72 °C. The PCR products were sequenced using an Applied Biosystems 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, United States), and the same primers were used for both the PCR and for the DNA sequencing. All *p53* mutations were confirmed by direct sequencing of both DNA strands.

Nested polymerase chain reaction

Genomic DNA was prepared from a tissue section and isolated by conventional phenol-chloroform extraction, ethanol precipitation, and was finally dissolved in 20 μ L of sterile distilled water. HPV viral DNA was first amplified with consensus primers MY09 and MY11^[19] followed by a second round of amplification with type-specific primers flanking the L1 region to identify the subtype. Ten microliters of the final PCR product were loaded onto a 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet-visible illumination. Appropriate negative and positive controls were included in each PCR reaction. A part of the β -actin gene in all samples was amplified to exclude false-negative results while DNA preparations from the SiHa cell (containing HPV16) were used as positive controls.

ISH

ISH for the detection of HPV type 16 DNA was performed using digoxigenin-labeled (DIG-labeled) oligonucleotide probes and a commercially available hybridization kit (Boehringer Mannheim, Indianapolis, IN). Briefly,

the hybridizing probes were prepared by PCR amplification using HPV 16 type-specific primers with DIG-deoxyuridine triphosphate as a substrate according to the manufacturer's instructions^[19]. The deparaffinized and rehydrated 5 μ m sections were digested with proteinase K, rinsed with PBS, and dehydrated. The hybridization was performed in a humidified chamber at 48 °C for 16 h followed by a wash with sodium chloride-sodium citrate. Thereafter, the detection reagent (anti-DIG antibody conjugated with peroxidase) was applied to the sections and then the sections were incubated in diaminobenzidine solution to allow the signals to develop. After the signal development, the sections were counterstained with hematoxylin, rinsed briefly in absolute ethanol, mounted, and observed for signals under a microscope.

Preparation of RNA and real-time quantitative RT-PCR

Total RNA was extracted from the colorectal tumors after homogenization in 1 mL TRIzol reagent, followed by chloroform re-extraction and isopropanol precipitation. Three micrograms of total RNA from the colorectal tumor tissues were reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, CA, United States) and oligo d(T)₁₅ primer. Real-time RT-PCR was performed in a final volume of 25 μ L containing 1 μ L of each cDNA template, 10 pmoles of each primer, and 12.5 μ L of a SYBR-Green master mix. The primers were designed using ABI Prism 7000 SDS Software. Quantification was carried out using the comparative threshold cycle (CT) method and water was used as the negative control. An arbitrary threshold was chosen on the basis of the variability of the baseline. CT values were calculated by determining the point at which the fluorescence exceeded the threshold limit. CT was reported as the cycle number at this point. The average of the target gene was normalized to *18S rRNA* as the endogenous housekeeping gene.

Statistical analysis

Statistical analysis was performed using the SPSS statistical software program (Version 11.0 SPSS Inc., Chicago, IL, United States). The χ^2 test, Fisher's exact test (two tailed), and Mann-Whitney test were applied for statistical analysis.

RESULTS

HPV16 DNA does indeed exist in a subset of colorectal tumors

Sixty-nine colorectal tumors were collected to evaluate HPV16 DNA. As shown in Figure 1, the presence of HPV16 DNA in colorectal tumors was determined by nested PCR using MY09/MY11 and specific primers, and 11 of 69 tumors (16%) possessed positive HPV16 DNA signals. The relationships between HPV16 DNA and clinicopathological features were statistically analyzed (Table 1). Our data showed that HPV16 DNA was present more frequently in stage I tumors than in late-stage tumors (66.7% for stage I vs 12.5% for stage II, 9.5% for stage III,

Table 1 Relationships between human papilloma virus 16 infection and clinical parameters of colorectal cancer patients *n* (%)

Parameters	HPV16		<i>P</i> value
	Negative	Positive	
Gender			0.782
Male (<i>n</i> = 35)	29 (82.9)	6 (17.1)	
Female (<i>n</i> = 34)	29 (85.3)	5 (14.7)	
Age (yr)			0.322
< 68 (<i>n</i> = 31)	28 (90.3)	3 (9.7)	
> 68 (<i>n</i> = 38)	30 (78.9)	8 (21.1)	
Smoking status			0.718
No (<i>n</i> = 49)	42 (85.7)	7 (14.3)	
Yes (<i>n</i> = 20)	16 (80.0)	4 (20.0)	
Stage			0.017
I (<i>n</i> = 6)	2(33.3)	4 (66.7)	
II (<i>n</i> = 24)	21 (87.5)	3 (12.5)	
III (<i>n</i> = 21)	19 (90.5)	2 (9.5)	
IV (<i>n</i> = 18)	16 (88.9)	2 (11.1)	
Tumor site			0.554
Ascending colon (<i>n</i> = 22)	19 (86.4)	3 (13.6)	
Transverse colon (<i>n</i> = 5)	5 (100.0)	0 (0)	
Descending colon (<i>n</i> = 5)	3 (60.0)	2 (40.0)	
Sigmoid colon (<i>n</i> = 10)	9 (90.0)	1 (10.0)	
Rectum (<i>n</i> = 27)	22 (81.5)	5 (18.5)	

HPV: Human papilloma virus.

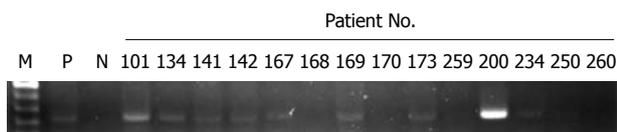


Figure 1 Representatives of positive and negative human papilloma virus 16 DNA detected by nested polymerase chain reaction in tumors of colorectal cancer patients. M: 100-bp ladder DNA marker; P: Positive control; DNA of SiHa cell line served as positive control for human papilloma virus 16, respectively; N: Negative control, the DNA template was replaced with distilled water.

and 11.1% for stage IV, *P* = 0.017). However, the presence of HPV16 DNA in colorectal tumors was not associated with other clinicopathological features including gender, age, smoking status, and tumor site (Table 1). To further confirm the presence of HPV16 DNA, ISH was conducted to determine the presence of HPV16 DNA in 11 colorectal tumor paraffin sections. ISH data indicated that 8 of the 11 tumors (72.7%) had positive HPV16 DNA signals (Table 2 and Figure 2). Collectively, these results clearly indicate that HPV16 DNA exists in a subset of colorectal tumors, at least in this study population.

E6 oncoprotein is expressed in HPV16 DNA-positive colorectal tumors and related to p53 inactivation

To explore whether HPV16 infection could be linked with colorectal cancer development, HPV16 E6 oncoprotein was evaluated by IHC in HPV16 DNA-positive colorectal tumors. Our data showed that E6 oncoprotein expression was detected in all HPV16 DNA-positive tumors (Table 2). We next examined whether E6 oncoprotein expressed in four p53 wild-type colorectal tumors (P101, P134, P141 and P169) could degrade p53 protein to produce tumors

Table 2 p53 mutation, p53 protein, E6 oncoprotein and clinical information in human papilloma virus 16 DNA-positive colorectal patients

Patient No.	Gender	Age (yr)	Stage	Site	p53		HPV16	
					Mutation	IHC	ISH	E6
101	F	65	I	d-colon	No	-	+	+
134	F	54	II A	Rectum	No	+	+	+
141	M	72	I	Rectum	No	-	+	+
142	F	56	II A	d-colon	Yes	++	+	+
167	M	70	III B	d-colon	Yes	+++	+	+
169	M	78	IV	Rectum	No	-	+	+
173	F	83	II A	s-colon	Yes	+	+	+
200	M	74	IV	a-colon	Yes	+	+	+
226	F	78	I	Rectum	No	-	-	-
234	F	76	I	a-colon	No	+	-	-
259	F	79	III B	Rectum	Yes	++	-	-

HPV: Human papilloma virus; IHC: Immunohistochemistry; ISH: *In situ* hybridization. -: No positive staining; +: 1%-10%; ++: 1%-50%; +++: > 50% positive cells.

Table 3 p21 and Mdm2 mRNA expression levels in tumor tissues and adjacent normal tissues of E6-positive colorectal patients

Variable	E6-positive (<i>n</i> = 8)			
	p53 wild-type (<i>n</i> = 4)	<i>P</i>	p53-mutation (<i>n</i> = 4)	<i>P</i>
P21 mRNA		0.025		0.061
Adjacent normal tissues	348.46 ± 143.89		241.12 ± 84.13	
Tumor tissues	49.62 ± 16.58		122.96 ± 45.28	
Mdm2 mRNA		0.032		0.043
Adjacent normal tissues	563.74 ± 252.28		541.50 ± 100.56	
Tumor tissues	111.60 ± 75.99		317.74 ± 137.59	

with p53 negative immunostaining (Figure 2). IHC analysis clearly showed p53 negative immunostaining in three out of four colorectal tumors (P101, P141, and P169), but one tumor (P134) had p53 positive immunostaining (< 10%). The other four E6-positive tumors with p53 mutations (P142, P167, P173 and P200) still had p53 positive immunostaining (Figure 2). To explore whether p53 inactivation could have occurred in p53 wild-type E6-positive tumors, expression levels of the p53-downstream targets Mdm2 and p21 mRNA were decreased compared with adjacent normal tissues. Real-time RT-PCR analysis showed that Mdm2 and p21 mRNA expression levels in these E6-positive tumors with p53 wild-type were significantly lower than in their adjacent normal tissues (*P* = 0.025 for p21 mRNA; *P* = 0.032 for Mdm2 mRNA; Table 3). As expected, both gene mRNA expression levels in E6-positive tumors with p53 mutations were lower than in the adjacent normal tissues (*P* = 0.043 for Mdm2 mRNA); however, p21 mRNA levels were marginally different between tumors and adjacent normal tissues (*P* = 0.061). These results clearly indicate that E6 oncoprotein is expressed in HPV16 DNA-positive colorectal tumors and may be linked with p53 inactivation in these HPV16 DNA positive tumors, which had the p53 wild-type gene.

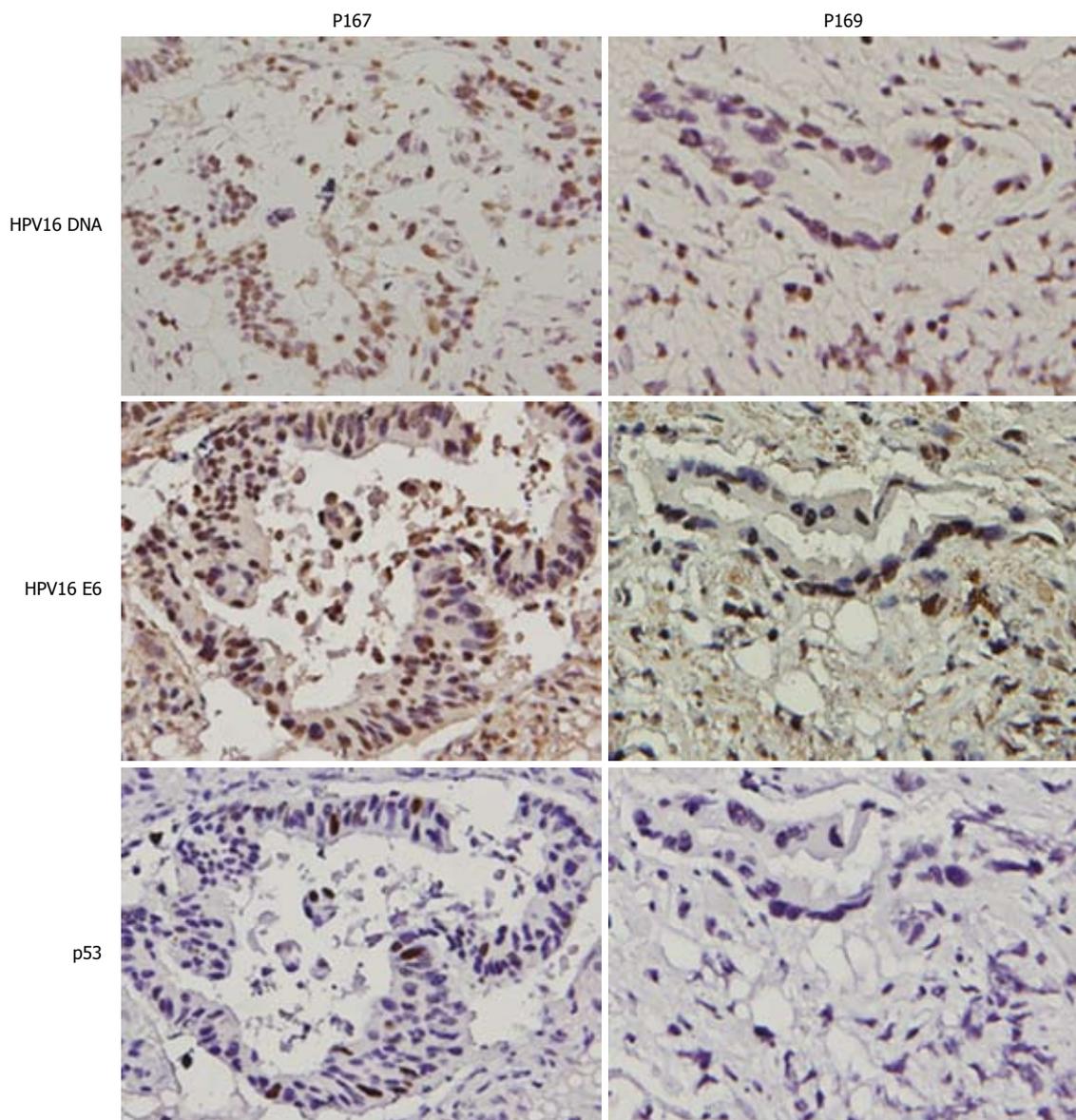


Figure 2 The representative reciprocal relationships between human papilloma virus 16 E6 and p53 immunostainings in human papilloma virus 16-infected colorectal (400 ×). HPV: Human papilloma virus.

E6 oncoprotein is expressed in endothelial cells and lymphocytes of colorectal tumors

To understand whether HPV-infected colorectal tumors could be mediated through blood circulation, E6 oncoprotein expression in normal parts of the colorectal tumors were examined by IHC (Figure 3). Our data showed that E6 oncoprotein is indeed expressed in endothelial cells of blood vessels and in lymphocytes infiltrating colorectal tumors. In addition, E6 oncoprotein was expressed in normal glands and dysplastic glands in colorectal tumors (Figure 3). These results seem to support the possibility that HPV16 infection in colorectal tumors may occur partially through blood circulation.

DISCUSSION

The presence of HPV is commonly detected by nested PCR in human tumors including colorectal tumors. In

previous studies, only one report has shown the concomitant detection of HPV16 DNA in three colorectal tumors by nested-PCR and *in situ* PCR^[15]. In the present study, nested PCR detected 8 of 11 HPV16-positive colorectal tumors and ISH confirmed this finding as well. Detection of HPV DNA by ISH had markedly lower sensitivity than *in situ* PCR, suggesting that the HPV16 DNA copy number in the tumors studied herein could be higher than previously reported^[19]. In the current study, the association between HPV infection and tumor stage was observed; however, only 6 stage I patients were enrolled in this study. Since the sample size of stage I patients is so small, it might be biased to conclude that HPV infection might play a role in the development of colorectal cancer. In addition, among 4 of the 6 stage I tumors with HPV16 DNA, HPV infection was confirmed by ISH in only 2 tumors. Therefore, the association between HPV infection and tumor stage should be verified by a larger

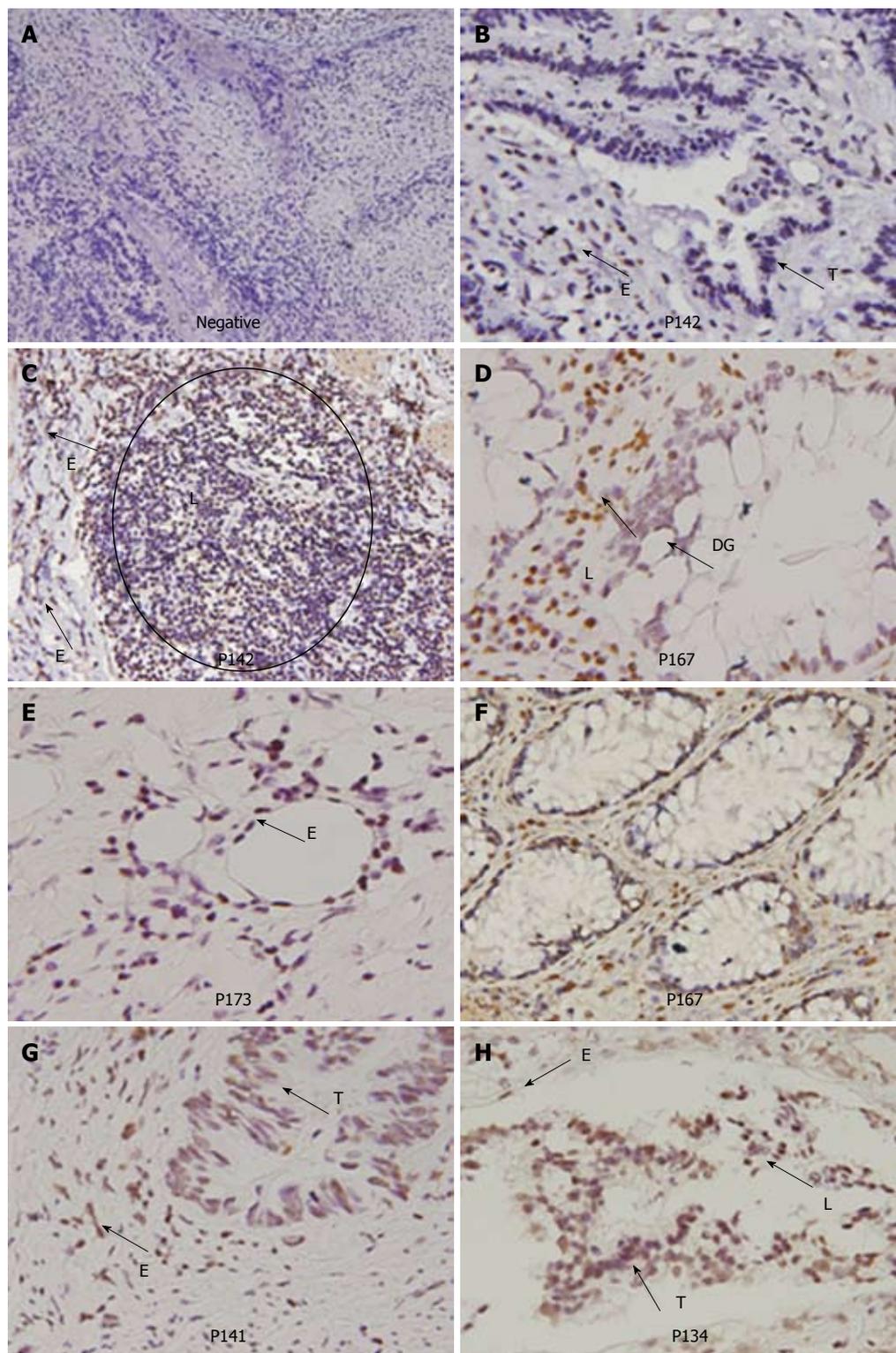


Figure 3 Immunohistochemical analysis of human papilloma virus 16 E6 protein in colorectal tumors and adjacent normal tissues. A: A negative result of immunostaining in tumor cells (100 ×); B: Human papilloma virus 16 (HPV16) E6 protein expressed in endothelial cells (400 ×); C: HPV16 E6 protein expressed in endothelial cells and lymphocytes (100 ×); D: HPV16 E6 protein expressed in lymphocytes and dysplastic gland (400 ×); E: HPV16 E6 protein expressed in endothelial cells and tumor cells (400 ×); F: HPV16 E6 protein expressed in normal gland (400 ×); G: HPV16 E6 protein in endothelial cells and Fibroblast (400 ×); H: HPV16 E6 protein in endothelial cells, lymphocytes and tumor cells (400 ×). E: Endothelial cells; T: Tumor cells; L: Lymphocytes; DG: Dysplastic gland.

study population.

To explore whether the existence of HPV16 DNA could be involved in colorectal cancer development, the presence of E6 and p53 proteins in tumor tissues and

p21 and Mdm2 mRNA expression in tumors and adjacent normal tissues were evaluated in a subset of colorectal tumors (11 of 69, 16%). E6 oncoprotein expression was detected in 8 of the 11 HPV16 DNA-positive tumors.

Among these eight E6-positive tumors, p21 and Mdm2 mRNA expression levels were markedly decreased in four E6-positive tumors carrying the wild-type *p53* gene; however, no significant decrease in both gene mRNA expression levels was noted in the other four E6-positive tumors carrying the mutated *p53* gene. Therefore, these results clearly show that E6 oncoprotein may be involved in this small subset of colorectal cancer development *via* the inactivated p53 pathway (Table 3).

Sexual activity has been considered to be a major route of transmission for HPV resulting in genital cancers, such as cervical, anal, and oropharyngeal cancers^[1,2]. However, there is evidence of HPV infections in infants and female university students who are virgins, revealing that HPV transmission *via* other routes than sexual intercourse may exist^[3,13,14,20-25]. In addition, peripheral blood lymphocytes (PBLs) from healthy donors have been shown to be infected with HPV^[13]. Therefore, it has been suggested that HPV infection in internal organ tissues might occur through blood circulation. Our previous lung cancer studies have indicated that HPV16/18 DNA and E6 oncoprotein not only exist in lung tumors but are also expressed in adjacent normal tissues including lymphocytes, endothelial cells, macrophages, and bronchial epithelial cells^[26]. We therefore speculated that PBLs may first be infected by HPV to act as a mediator of HPV infection to lung tissues *via* blood circulation^[14,26]. In the present study, IHC analysis clearly indicates that E6 oncoprotein was expressed in tumor-infiltrating lymphocytes of HPV16 DNA-positive colorectal tumors. In addition, E6 oncoprotein expression was also detected in the endothelial cells of the HPV16 DNA-positive colorectal tumors. These results seem to support our previous hypothesis that HPV infection in lung tumor tissues may be mediated through blood circulation and not as a direct contact transmission in cervical and oropharyngeal tumor tissues^[14,19,26].

To our knowledge, this is the first report to indicate that HPV16 E6 oncoprotein may downregulate p21 and Mdm2 transcription *via* inactivation of p53 in the involvement of colorectal cancer development. Similar observations of E6 oncoprotein expression in adjacent normal lung and colorectal tissue cells seem to support the possibility that HPV infection in colorectal tumors might be mediated through blood circulation. Notably, even though our present study provides support for the association between HPV infection and colorectal cancer, the involvement of HPV infection in colorectal cancer development is limited to a small subset of the population.

COMMENTS

Background

Human papilloma virus (HPV) DNA integration into the host chromosome plays a crucial role in HPV-associated tumorigenesis. When HPV DNA was spliced at E2, E6 and E7 oncoproteins were expressed to inactivate p53 and Rb pathways. A high risk of HPV16/18 infection has been documented to be involved in the development of cervical and anal genital cancers. Among non-genital cancers, the association of oropharyngeal cancer with HPV16/18 infection was recently evidenced. However, other non-genital cancers, including lung, breast, and colorectal cancers have not yet been identified.

Research frontiers

The authors provide the evidence to indicate that HPV16 may be involved in a small subset of colorectal cancer development. Therefore, HPV vaccination not only prevents cervical cancer but also reduces HPV-associated colorectal cancer development.

Innovations and breakthroughs

The association of HPV infection with colorectal cancer has been extensively investigated; however, no strong evidence supports the involvement of HPV in colorectal cancer development. Herein, nested-polymerase chain reaction and *in situ* hybridization were used to detect the presence of HPV16 DNA in colorectal tumors. Immunohistochemical data further showed that E6 oncoprotein is expressed in HPV16 DNA-positive tumors and E6 expression was negatively correlated with p53 expression. These results suggest that HPV16 might contribute to a small subset of colorectal cancer development.

Applications

HPV infection is not only involved in cervical and oropharyngeal cancers but is also linked with internal organ cancers, such as lung and colorectal cancers. Therefore, blood transmission of HPV infection in internal organs might be noted in public.

Peer review

The authors present an interesting work on the expression of HPV16 E6 oncoprotein in colorectal cancer. The manuscript is generally well written. The topic is very interesting and to some extent provocative. The manuscript is well structured and the cited literature is comprehensive and up-to-date.

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Excisional hemorrhoidal surgery and its effect on anal continence

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Abstract

AIM: To investigate the role of anal cushions in hemorrhoidectomy and its effect on anal continence of the patients.

METHODS: Seventy-six consecutive patients (33 men and 43 women) with a mean age of 44 years were included. They underwent Milligan-Morgan hemorrhoidectomy because of symptomatic third- and fourth-degree hemorrhoids and failure in conservative treatment for years. Wexner score was recorded and liquid continence test was performed for each patient before and two months after operation using the techniques described in our previous work. The speed-constant rectal lavage apparatus was prepared in our laboratory. The device could output a pulsed and speed-constant saline stream with a high pressure, which is capable of overcoming any rectal resistance change. The patients were divided into three groups, group A (< 900 mL), group B (900-1200 mL) and group C (> 1200 mL) according to the results of the preoperative liquid continence test.

RESULTS: All the patients completed the study. The average number of hemorrhoidal masses excised was 2.4. Most patients presented with hemorrhoidal symptoms for more than one year, including a mean duration of incontinence of 5.2 years. The most common symptoms before surgery were anal bleeding ($n = 55$), prolapsed lesion ($n = 34$), anal pain ($n = 12$) and constipation ($n = 17$). There were grade III hemorrhoids in 39 (51.3%) patients, and grade IV in 37 (48.7%) patients according to Goligher classification. Five patients had experienced hemorrhoid surgery at least once. Compared with postoperative results, the retained volume in the preoperative liquid continence test was higher in 40 patients, lower in 27 patients, and similar in the other 9 patients. The overall preoperative retained volume in the liquid continence test was 1130.61 ± 78.35 mL, and postoperative volume was slightly decreased (991.27 ± 42.77 mL), but there was no significant difference ($P = 0.057$). Difference was significant in the test value before and after hemorrhoidectomy in group A (858.24 ± 32.01 mL vs 574.18 ± 60.28 mL, $P = 0.011$), but no obvious difference was noted in group B or group C. There was no significant difference in Wexner score before and after operation (1.68 ± 0.13 vs 2.10 ± 0.17 , $P = 0.064$). By further stratified analysis, there was significant difference before and 2 months after operation in group A (2.71 ± 0.30 vs 3.58 ± 0.40 , $P = 0.003$). In contrast, there were no significant differences in group B or group C (1.89 ± 0.15 vs 2.11 ± 0.19 , $P = 0.179$; 0.98 ± 0.11 vs 1.34 ± 0.19 , $P = 0.123$).

CONCLUSION: There is no difference in the continence status of patients before and after Milligan-Morgan hemorrhoidectomy. However, patients with preoperative compromised continence may have further deterioration of their continence, hence Milligan-Morgan hemorrhoidectomy should be avoided in such patients.

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Key words: Anal cushion; Anal incontinence; Liquids continence test; Wexner score; Hemorrhoidectomy

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INTRODUCTION

Hemorrhoids are very common and it occurs in men and women of all ages. It is estimated that 50% of the people older than 50 years have hemorrhoids symptoms at least for a period of time^[1]. The most common symptoms include rectal bleeding, pain, anal irritation and anal mass prolapse and a disrupted quality of life. There has been much speculation over the years as to the nature of “hemorrhoids”. It is now generally accepted that “hemorrhoids” are a disorder of the anal cushions^[2]. Thomson demonstrated that in patients suffering from hemorrhoids, the specialized “cushions” of submucosal tissue lining the anal canal slide downwards, together with the anal mucosa, due to the fragmentation of Parks’ ligament^[3-5]. This means that hemorrhoids result from distal enlargement of the anal cushions. The anal cushions are connective tissue complexes that contain smooth cells and vascular channels; they are thought to provide an effective tight seal to close the anal in concert with the internal anal sphincter^[6].

For patients with grades III and IV hemorrhoids, surgical excision remains the most common choice of treatment. Two main approaches have been used, one removes the cushions (e.g., Milligan-Morgan hemorrhoidectomy) and the other retains the cushion (e.g., stapled hemorrhoidopexy or procedure for prolapse and hemorrhoids). The expensive stapled hemorrhoidopexy has become a widely accepted alternative to excisional hemorrhoidectomy for treating the third- and fourth-degree hemorrhoids in China over the recent decade, it even has a trend to replace the traditional hemorrhoidectomy^[7]. According to Thomson’s theory, impairment of the anal cushions may lead to anal incontinence. Some previous studies^[8-13] indicate that hemorrhoidectomy might be complicated with fecal incontinence. Therefore, many non-excisional options have become available to overcome the disadvantages of traditional surgery, which have given rise to dispute around the world. However, to our knowledge, there has been no direct evidence supporting the function of the anal cushion. Our study aims to define the role of the anal cushions in hemorrhoidectomized patients by performing a liquid continence test simulating analorectal continence of liquid stool and using the Wexner score (the Cleveland clinic continence scoring system)^[14,15].

MATERIALS AND METHODS

Patients

Consecutive patients with hemorrhoids were included in the study. Inclusion criteria for the cohort were: the existence of symptomatic third- and fourth-degree hemorrhoids, and failure in conservative treatment for years and intention for Milligan-Morgan hemorrhoidectomy. Patients younger than 18 or older than 80 years, who had experienced concomitant anal diseases (fissure, abscess, fistula, inflammatory bowel disease, rectal cancer) were excluded. Eligible patients were asked for signed informed consent. The study was approved by the local ethics committee.

Eighty patients who referred to our hospital between April 2005 and September 2010 were recruited. Four patients, who lost to follow-up and did not complete the second Wexner scoring and liquid continence test, were excluded. Eventually, 76 patients were eligible and completed the study. The demographic and clinical data, and the results of liquid continence test were obtained. The anal continence was assessed using the Wexner scoring system.

Liquid continence test was performed and Wexner score was recorded for each patient before and two months after operation. The patients were divided into three groups: group A (< 900 mL), group B (900-1200 mL) and group C (> 1200 mL) according to the retained volume in the liquid continence test done before operation.

Each patient underwent the standard Milligan-Morgan hemorrhoidectomy using conventional instruments for dissection and a monopolar coagulator for hemostasis by experienced surgeons.

Liquid continence test

Liquid continence test was performed preoperatively in all the patients. At 2 mo follow-up after operation, wounds were healed in all the patients. The same tests were repeated two months after surgery (60 ± 3 d).

This test was performed using the techniques described in our previous work^[16]. The speed-constant rectal lavage apparatus was prepared in our laboratory^[16]. The device could output a pulsed and speed-constant saline stream with a high pressure, capable of overcoming any rectal resistance change.

The first liquid continence test was performed at the preoperative days 1 and 2. Patients were advised to empty their rectums before the examination. The patient sat on the device. After a F16 balloon urethral catheter was introduced into the rectum about 8-cm deep, the balloon was inflated with 5 mL air. The warm saline (37 °C) was infused at a rate of 60 mL/min. The patients were instructed to hold the liquid as long as possible. If the device was alarmed when a leakage amount reached 10 mL or the infusing liquid reached the maximum (1500 mL), the test stopped. The total volume retained was recorded. After the end-point was recorded, the balloon was deflated and the catheter was extracted.

Table 1 Demographic characteristics of hemorrhoid patients

Variables	Values
Age (yr) ¹	44 (22-72)
Gender (male/female)	33/43
Chief complaints, <i>n</i> (%)	
Prolapse	32 (42.1)
Anal bleeding	55 (72.3)
Anal pain	12 (15.8)
Constipation	17 (25.4)
Hemorrhoids stage, <i>n</i> (%)	
Third-degree	39 (51.3)
Fourth-degree	37 (48.7)
Resected piles (<i>n</i>) ²	2.4 ± 0.3
Disease duration (yr) ²	3.6 ± 1.1

¹Data are median (range); ²Data are means ± SD.

Wexner score

Anal incontinence was assessed using the Wexner grading system^[14]. Wexner score contains three items about the type and frequency of incontinence (scored from zero to four) and items on pad usage and lifestyle alteration (both scored from zero to four). Data were collected by physicians through a patient interview.

Statistical analysis

Statistical analysis was performed with SPSS 16.0 software. The data were expressed as mean ± SD or median (range). Continuous data were compared using paired Student's *t* test. Difference was considered to be significant when the *P* value was < 0.05.

RESULTS

The demographics and clinical details of the 76 patients are shown in Table 1. The mean age of patients was 44 years (range: 22-72 years) and the male to female ratio was 1:1.3. The number of hemorrhoidal masses excised varied from 2-4 (mean: 2.4 ± 0.3). The mean duration of incontinence was 5.2 years. Most patients had hemorrhoidal symptoms for more than one year, and some patients even for more than 20 years. The most common symptoms observed in the patients before surgery was anal bleeding (55 cases), prolapsed lesion (34 cases), anal pain (12 cases) and constipation (17 cases) (Table 1). Thirty-nine (51.3%) patients had grade III hemorrhoids, and 37 patients (48.7%) had grade IV hemorrhoids according to the Goligher classification. Five patients experienced hemorrhoid surgery at least once.

Compared with the postoperative results, the retained volume in the preoperative liquid continence test was higher in 40 patients, lower in 27 patients, and similar in the rest 9 patients. The overall preoperative volume in the liquid continence test was 1130.61 ± 78.35 mL, and postoperative values were slightly decreased (991.27 ± 42.77 mL), but there was no significant difference (*P* = 0.057).

According to the results of preoperative test, patients were divided into three groups: 17 patients in group A (<

900 mL), 26 in group B (900-1200 mL) and 32 in group C (> 1200 mL) (Table 2). Interestingly, significant difference was found in the test results before and after hemorrhoidectomy in group A (858.24 ± 32.01 mL *vs* 574.18 ± 60.28 mL, *P* = 0.011), but no obvious difference was noted in group B or group C (Table 2).

There was no significant difference in the Wexner score before and after operation (1.68 ± 0.13 *vs* 2.10 ± 0.17, *P* = 0.064; Table 2).

By further stratified analysis, there was significant difference in the Wexner score before and two months after operation in group A (2.71 ± 0.30 *vs* 3.58 ± 0.40, *P* = 0.003). In contrast, there were no significant differences in group B or group C (1.89 ± 0.15 *vs* 2.11 ± 0.19, *P* = 0.179; 0.98 ± 0.11 *vs* 1.34 ± 0.19, *P* = 0.123; Table 2).

DISCUSSION

To evaluate accurately the anal continence is still a clinical challenge. Parks^[17] pointed out that it is difficult to evaluate postoperative anal continence due to the flaws related to subjective and objective factors. We, therefore, used liquid continence test and Wexner scoring system in combination to better assess the anal continence status. The liquid continence test could simulate liquid stool; compared with anorectal manometry, it is more applicable, which can yield objective assessment with quantitative data^[16,18,19]. The Wexner Continence Grading Scale has been widely used for evaluating anal continence^[20-22]. It is convenient in practice and easily acceptable by the patients. Consequently, our assessments based on liquid continence test and questionnaire scoring system, are likely to be more reliable.

In our study, the mean Wexner Continence Grading Scale did not vary significantly after surgery in the overall patients. By further subgroup analysis, after hemorrhoidectomy, the Wexner score significantly increased in patients with preoperative continence defect or subclinical incontinence (group A), while no significant difference was observed in the group with normal anal continence (groups B and C). Similar results were obtained by means of the liquid continence test. While the liquid continence test and Wexner scoring system yielded the similar results, the former is more direct and objective. Baxter *et al*^[23] insisted that fecal incontinence is manifested as a symptom, so any evaluation of incontinence must be built on the perception of the patient. This is one of the reasons why we prefer the liquid continence test in the evaluation.

What is the role of anal cushion in hemorrhoidectomy? In spite of the high incidence of hemorrhoidal diseases worldwide, some aspects of its pathophysiology still remain unknown. According to Thomson's attractive theory, the anal cushions serve as a conformable plug to ensure complete closure of the anal canal and contribute to the anal continence mechanism^[5]. Hemorrhoidectomy is associated with the removal of the anal cushions, and may occasionally lead to anal incontinence^[7,11]. Jóhannsson *et al*^[12] concluded from his questionnaire that 29% of

Table 2 Results of liquid continence test before and after hemorrhoidectomy and Wexner score assessments (mean)

	Liquid continence test (pre-operation) (mL)	Liquid continence test (post-operation) (mL)	P value	Wexner score (pre-operation)	Wexner score (post-operation)	P value
Total patients	1130.61 ± 78.35	991.27 ± 42.77	0.057	1.68 ± 0.13	2.10 ± 0.17	0.064
Group A (n = 17)	858.24 ± 32.01	574.18 ± 60.28	0.011	2.71 ± 0.30	3.58 ± 0.40	0.003
Group B (n = 26)	977.96 ± 15.96	927.31 ± 53.23	0.061	1.89 ± 0.15	2.11 ± 0.19	0.179
Group C (n = 33)	1391.21 ± 16.95	1257.79 ± 51.53	0.124	0.98 ± 0.11	1.34 ± 0.19	0.123

the patients reported the incontinence after hemorrhoidectomy. Thekkinkattil *et al.*^[24] demonstrated that the cushion: canal ratio was reduced in patients with idiopathic fecal incontinence. However, the theory is controversial by the fact that submucosal hemorrhoidectomy does not yield better functional outcome than excisional hemorrhoidectomy^[25]. Our study revealed that hemorrhoidectomy (excised anal cushions) did not impair the function of anal continence. Our findings agree with a previous report^[26] that no incontinence occurred after conventional hemorrhoidectomy. However, there is still much uncertainty regarding the role of anal cushion in fecal incontinence. It is important to note, although no obvious change was observed in anal continence in the patients after excising the anal cushion, further subgroup analysis showed that the patients with a lower value of liquid continence test (< 900 mL) after surgery did impair the fecal continence. This may be partly attributed to the fact that this group of patients had been complicated with continence defect or subclinical incontinence.

Our data support that the traditional hemorrhoidectomy, which necessitates excision of anal cushions, is a safe procedure for patients with normal fecal continence. From the perspectives of cost-effectiveness, the traditional hemorrhoidectomy should be recommended, especially in the developing countries. Nevertheless, surgeons should keep in mind that this kind of surgery may increase the risk of complicated anal incontinence in the patients with anal function defect or subclinical incontinence. Best of all, this study enhances the awareness of surgeons that preoperative evaluation of hemorrhoid patients is important regarding the choice of surgical procedure.

Our study had several limitations, such as a small sample size, short follow-up, and saline continence test could not assess the solid stool, which might result in improper findings. Further studies are being conducted to work out an objective test for solid and flatus stool, and compare the anal cushion preserving and non-preserving procedures as well.

In conclusion, removing anal cushions does not obviously impair the fecal continence in patients with a proper indication for the operation, and therefore it is a safe procedure. It is not necessary to pay excessive attention to anal cushion in hemorrhoid patients. Thorough investigations should be carried out on anal continence so as to prevent the occurrence of postoperative complications.

COMMENTS

Background

Traditional hemorrhoidectomy is tended to be replaced by stapled hemorrhoidopexy with anal cushion retained, because the anal cushion theory considers that the removal of the anal cushions will damage the anal rectal control function. In this study, water retention test and Wexner scoring were used to observe the changes of the anus and rectum control function in the patients treated with traditional hemorrhoidectomy.

Research frontiers

The authors evaluated the anal continence in the patients with hemorrhoids before and after operation using liquid continence test and Wexner scoring system in combination so as to define the role of anal cushions in hemorrhoidectomy.

Innovations and breakthroughs

To evaluate accurately the anal continence is still a clinical challenge, and it is especially difficult to evaluate the postoperative anal continence due to flaws related to subjective and objective factors. The authors, therefore, used liquid continence test and Wexner scoring system in combination in an attempt to better assess the anal continence. The liquid continence test could simulate liquid stool, thus yielding objective assessment with quantitative data. The Wexner scoring system is convenient in practice and easily acceptable by the patients. Consequently, the assessments obtained by this study based on liquid continence test and questionnaire scoring system, are likely to be more reliable. The authors also found that removing anal cushions does not obviously impair the fecal continence in patients with a proper indication for the traditional hemorrhoidectomy, and therefore it is a safe procedure.

Applications

According to this study, that the traditional hemorrhoidectomy, which necessitates excision of anal cushions, is a safe procedure for patients with normal fecal continence. From the perspectives of cost-effectiveness, the traditional hemorrhoidectomy should be recommended, especially in the developing countries. Nevertheless, surgeons should keep in mind that this kind of surgery may increase the risk of complicated anal incontinence in the patients with anal function defect or subclinical incontinence.

Peer review

This study provides new evidences on the safety of Milligan-Morgan hemorrhoidectomy which is less expensive for patients. Some elements may substantiate the study, such as fecal continence test for not only liquid and but also for solid and flatus stools, and comparison between anal cushion preserving and non-preserving procedures.

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Rapidly deforming gastric carcinosarcoma with osteoblastic component: An autopsy case report

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Abstract

Carcinosarcomas are rare, malignant, biphasic tumors simultaneously comprising carcinoma and sarcoma in a single tumor. We present an extremely rare case of gastric carcinosarcoma with an osteoblastic component that drastically changed its shape within 2 mo. A 59-year-old male patient presented to the emergency outpatient unit with a complaint of black stool. Gastrointestinal endoscopy showed an ulcerated mass in the cardia of the lesser curvature of the stomach. Biopsy specimens revealed only adenocarcinoma. Two months later, the ulcerated lesion drastically changed its shape into an exophytic tumor. Total gastrectomy was performed. In the resected specimen, the gastric tumor contained both adenocarcinoma and sarcoma components with lace-like osteoid. The patient died 7 mo after the operation, and an autopsy was performed. In the autopsy, widespread metastases were present in the liver, lung, lymph nodes and peritoneum. In this report, we describe a case of gastric carcinosarcoma and presume its tumorigenesis based on the autopsy findings.

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INTRODUCTION

Carcinosarcomas are rare, malignant, biphasic tumors simultaneously comprising carcinoma and sarcoma in a single tumor. Although carcinosarcomas of the esophagus are most frequently observed in the upper gastrointestinal tract^[1], they are very rarely found in the stomach. We performed a keyword search of the literature for gastric carcinosarcoma. Published articles, limited to English abstracts indexed primarily in the PubMed database, through to 2012, were reviewed. Until 2012, at least 46 cases of gastric carcinosarcoma have been reported^[2-20]; most of them in Japanese patients. **In this article, we present a rare case of gastric carcinosarcoma with an osteoblastic component that drastically changed its shape.**

CASE REPORT

Case history

A 59-year-old Japanese male patient presented to the emergency outpatient unit with a complaint of black stool. He was an office worker. He had no apparent past

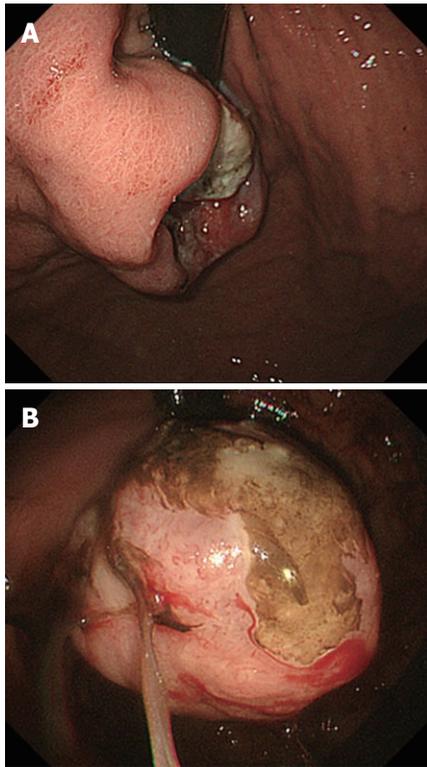


Figure 1 Retroflexed endoscopic view of the lesion in the gastric cardia. A: Ulcerated lesion; B: Two months later, the lesion changed its shape into an exophytic mass.

medical history and took no medications. There was no family history of malignant neoplasm. On physical examination, the abdomen was flat and not tender. Laboratory data were normal with the exception of a white blood cell count of $13\,100/\mu\text{L}$ and an elevated serum carcinoembryonic antigen (CEA) level of 12.5 ng/mL (normal, $< 5.0\text{ ng/mL}$).

Gastrointestinal endoscopy showed an ulcerated mass in the cardia of the lesser curvature of the stomach (Figure 1A). The esophagogastric junction was not involved with the tumor. Biopsy from the lesion showed tubular adenocarcinoma. Two months later, upper gastrointestinal endoscopy was performed again and showed that the ulcerated lesion had drastically changed its shape into an exophytic mass (Figure 1B). Although the patient had multiple liver metastases, palliative total gastrectomy was performed to control gastric bleeding and ameliorate anemia.

After the palliative operation, systemic chemotherapy with S-1 and cisplatin was performed. As a result of tumor progression, the patient received second-line chemotherapy of irinotecan (CPT-11) and mitomycin C. Although three cycles of second-line chemotherapy were performed, liver metastases continued to develop. His general condition gradually worsened, and he died 7 mo after the operation. An autopsy was performed.

Pathological findings

On macroscopic examination of a $27\text{ cm} \times 21\text{ cm}$ total gastrectomy specimen, an $11.5\text{ cm} \times 10.5\text{ cm} \times 5.0\text{ cm}$

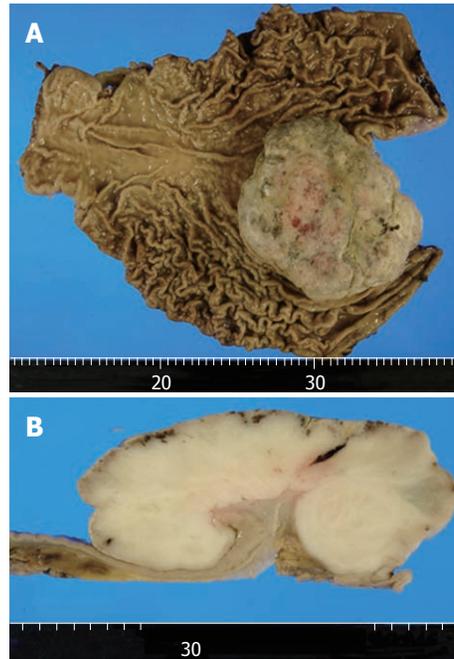


Figure 2 Macroscopic view of the resected specimen. A: An exophytic tumor located in the gastric cardia; B: Cross section of the tumor.

exophytic tumor was found in the cardia (Figure 2A). Erosion and necrosis were observed on the tumor surface. The cut surfaces of the tumor were tan-gray and fleshy (Figure 2B).

On light microscopy, the tumor contained both epithelial and spindle cell components. Spindle cell clusters and fascicles with the appearance of stromal sarcomas were observed in large regions. A small amount of lace-like osteoid was found in the sarcoma component (Figure 3A). Irregular fused glands with cuboidal and columnar cells appearing to overlap these regions were observed (Figure 3B). The spindle cells generally had coarse chromatin, elongated oval nuclei, small nucleoli, pale eosinophilic cytoplasm, and frequent mitotic activity. In adjacent regions of the tumor, dysplastic glands and goblet cell metaplasia were seen within the mucosa. No *Helicobacter pylori* (*H. pylori*) was observed. The tumor infiltrated the subserosal layer. Lymph node metastases with an adenocarcinoma component were detected in seven of 22 resected lymph nodes. Venous and lymphatic involvements with adenocarcinoma components were also observed. Immunohistochemically, cytokeratin AE1/AE3 positivity was observed in the tubular adenocarcinoma component (Figure 4A). There was no staining with cytokeratin AE1/AE3 in spindle cells. In spindle cells, positive staining with vimentin and negative staining with desmin, smooth muscle actin, c-kit, S-100, CD34, and HHF-35 was observed (Figure 4B). Widespread positive nuclear staining with p53 and more limitedly with Ki-67 was observed in both glandular and spindle tumor cells.

In the autopsy, widespread metastases were present in the liver, lungs, lymph nodes, and peritoneum. Although liver metastases showed both adenocarcinoma and spindle cell components, only the adenocarcinoma compo-

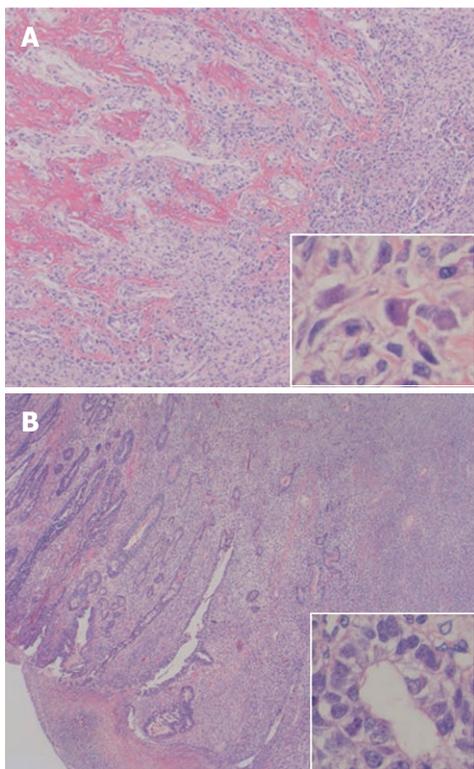


Figure 3 Representative microphotographs. A: Hematoxylin and eosin stain of the tumor (4 ×), a large part of the tumor comprised spindle cells producing lace-like osteoid; B: Tubular adenocarcinoma coexisting in the tumor. High-power view of tumor cells (40 ×) is shown in the insets.

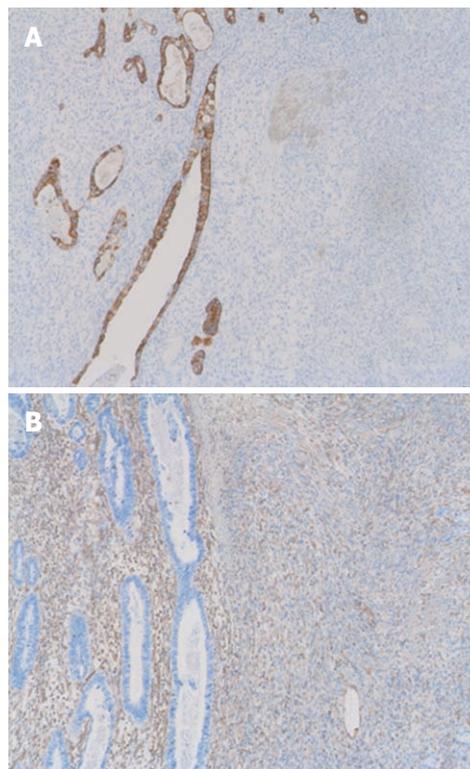


Figure 4 Immunohistochemical staining of the tumor. A: Cytokeratin AE1/AE3 staining showing positive expression in the adenocarcinoma component but not in the sarcomatous component (4 ×); B: Vimentin demonstrating opposite staining pattern (4 ×).

ment was present in lung metastases. Carcinosarcoma and pulmonary thromboembolism were the major causes of death in this case.

DISCUSSION

Gastric carcinosarcomas are rare tumors. They have also been referred to in the literature as sarcomatoid carcinomas. The first report of sarcomatoid carcinoma of the stomach was by Queckenstadt in 1904. Additional cases have been subsequently reported^[2-19]. In 2007, Ikeda *et al*^[20] reported one case and reviewed 43 cases from the literature. Until 2012, at least 46 cases of gastric carcinosarcoma have been reported; most of them in Japanese patients. We reported gastric carcinosarcoma containing an osteoblastic component, which is extremely rare. Based on our knowledge, there has been only one report of gastric carcinosarcoma with osteoblastic differentiation^[14]. No previous report has described gastric carcinosarcoma as a tumor that rapidly deformed within 2 mo.

The histogenesis of biphasic tumors remains unclear. Some researchers have argued that a primary carcinoma stimulates excessive stromal proliferation, resulting in a carcinosarcoma^[21]. Other researchers, however, are of the opinion that the spindle cell component reflects anaplasia within the carcinoma^[4,22-24].

There are findings supporting the idea that gastric biphasic tumors are epithelial in origin^[3,5,11]. In a number of studies, cells with an intermediate appearance between

that of epithelial and sarcomatous components have been seen, and these cells have been reported to stain positively with epithelial differentiation markers such as CEA. However, there have been cases in which spindle cells were differentiated as entirely smooth muscle or cartilage. In the literature, gastric carcinosarcomas differentiated as rhabdomyoblastic, neuroblastic, or osteoblastic have been reported^[14,25-27]. A combination of carcinosarcoma and carcinoid tumor with neuroendocrine malignancy was reported in two cases^[13,25]. Therefore, it may be that in their beginning, these tumors developed with diverse differentiation of a multipotent stem cell.

The histological findings observed in our case may support the theory that gastric biphasic tumors are epithelial in origin. All biopsy specimens obtained at the initial endoscopy showed only adenocarcinoma. However, in the surgically resected specimen, the spindle cell component occupied > 90% of the tumor, and a small number of adenocarcinoma cells were observed in the peripheral zone of the tumor. In the autopsy, the liver metastases that had been detected in the ante-mortem examination were composed of both spindle cell and adenocarcinoma components. In contrast, small metastases of the lung first diagnosed at autopsy showed only adenocarcinoma. Although these differences in tumor components at each metastatic site might have resulted from differences in affinities of each tumor component for lymphatics or veins, we believe that the development of the adenocarcinoma component might have preceded

that of the sarcoma component in our case. Rapid proliferation of the sarcoma component may have accounted for the drastic deformation of the tumor in our case. Although the tumorigenesis of this lesion is interesting from the perspective of epithelial-mesenchymal transition^[28,29], a more sophisticated approach will be required to elucidate this problem.

From a clinical standpoint, gastric carcinosarcomas share clinicopathological features with gastric adenocarcinomas. The age range of the 46 cases previously reported was 29 to 83 years with a mean of 56 years^[8,20,29]. There were 33 male and 13 female patients. Tumors that showed polypoid growth and ulcerated lesions were present in 22 and 19 cases, respectively. In our patient, the tumor exhibiting the ulcerative lesion rapidly changed its shape into an exophytic mass and was located in the cardia.

Gastric carcinosarcomas have a low incidence of metastasis, but mortality is high^[5,11,13,14,25,26,30]. Approximately 50% of previously reported patients with gastric carcinosarcoma died of the disease within 6 mo^[8,20,27]. In those cases that metastasize, the metastases may show pure carcinoma, pure sarcoma, or a mixture of both^[16]. In our case, both components were present.

Histologically, carcinosarcomas include epithelial and sarcomatous regions, and the two tumor components may be separate or overlapping^[3,5,13,14,25,26]. The tumor is often accompanied by atrophy, dysplasia, and intestinal metaplasia^[5,11]. Several types of differentiation of the sarcoma component have been reported, including smooth muscle, cartilage, striated muscle, and neuroendocrine cells. However, production of osteoid by the sarcoma component is extremely rare. We observed staining with cytokeratin AE1/AE3 only in the epithelial component in the case presented. Vimentin positivity and production of lace-like osteoid suggested that spindle cells had differentiated as osteosarcoma. In our patient, widespread p53 and Ki-67 positivity confirmed the high proliferation and malignant potential of this tumor. *H. pylori* may not be strongly associated with carcinosarcoma^[51,32].

In summary, we reported a case of gastric carcinosarcoma with an osteoblastic component that rapidly deformed within 2 mo. Carcinosarcoma of the stomach is a rare tumor and has a poor prognosis. It is impossible to establish standard therapy for this rare disease with a large-scale clinical trial, therefore, detailed descriptions and collections of individual cases should be continued.

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Cerebral lipiodol embolism after transarterial chemoembolization for hepatic carcinoma: A case report

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Abstract

We report a case of cerebral lipiodol embolism (CLE) after transarterial chemoembolization (TACE) for unresectable hepatic carcinoma (HCC). A 54-year-old man with unresectable HCC underwent TACE *via* the right hepatic artery and right inferior phrenic artery using a mixture of 40 mg pirarubicin and 30 mL lipiodol. His level of consciousness deteriorated after TACE, and non-contrast computed tomography revealed a CLE. The cerebral conditions improved after supportive therapy. The complication might have been due to hepatic arterio-pulmonary vein shunt caused by direct invasion of the tumor. Even though CLE is an uncommon complication of TACE, we should be aware of these rare complications in patients with high risk factors.

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Key words: Hepatic carcinoma; Cerebral lipiodol embolism; Chemoembolization

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INTRODUCTION

Hepatic carcinoma (HCC) is one of the leading causes of cancer-related death. It has a tendency to invade the tissue around the tumor, such as diaphragm, portal and hepatic veins, which may result in formation of hepatic arteriopulmonary vein or hepatic arteriovenous shunts. Transarterial chemoembolization (TACE) is one of the most common treatment modalities as a palliative and preoperative method for patients with advanced HCC. Although various complications of TACE have been reported, cerebral lipiodol embolism (CLE) after TACE is rare. In this paper, we report a case of CLE after TACE for advanced HCC.

CASE REPORT

A 54-year-old man was admitted with right upper quadrant pain. He was a hepatitis B virus carrier for 30 years. The α -fetoprotein level was 1200 ng/mL. Enhance computed tomography (CT) revealed a 13-cm mass of the right liver lobe. These clinical signs indicated that the patient had unresectable HCC and Child-Pugh class A. As revealed by angiography, the huge hypervascular tumor located in the right liver was supplied by the right hepatic artery (RHA) and right inferior phrenic artery (RIPA) without arteriovenous shunt. TACE was performed *via* the RHA and RIPA using a mixture of 40 mg pirarubicin and 30 mL lipiodol. Toward the end of the procedure, the lipiodol was deposited in the tumor densely. The embolism process was monitored by fluoroscopy all the way and no abnormal flow of the lipiodol was found. Twenty

minutes after TACE, the patient complained of a serious headache and followed by confusion. Non-contrast enhanced CT scanning showed no hyper-intense lesions in the bilateral lungs, but multiple disseminated hyper-intense lesions in the brain, consistent with the deposition of lipiodol. His neurologic symptoms disappeared completely when discharged 9 d later.

DISCUSSION

TACE can result in various severe complications, including acute hepatic failure, intrahepatic biloma, pseudoaneurysm formation, and ectopic infarction, which occur in less than 1% of the patients. Although CLE is a rare complication of TACE, sporadic cases of CLE after TACE have been reported^[1,2]. We encountered a case of CLE after TACE, which was probably associated with hepatic or right inferior phrenic arteriopulmonary vein shunt. The patient had no specific respiratory symptoms such as cough, dyspnoea, but had neurological symptoms including headache and followed by confusion. CT scanning showed some positive findings, indicating deposition of lipiodol, and the diagnosis of CLE was confirmed clinically.

The underlying mechanisms of CLE after TACE are still obscure. Hepatic arterio-pulmonary vein shunt, which is associated with pulmonary vein invasion of HCC, may be the reasonable explanation for CLE. Vascular abnormalities, referred to as hepatic vein invasion, pulmonary arteriovenous shunt, can be found in patients with advanced HCC^[3]. An intracardiac right-to-left shunt *via* a patent foramen ovale or intrapulmonary arteriovenous shunt can lead to CLE. Patients with advanced HCC are likely to have a pulmonary arteriovenous shunt^[4], and a right-to-left shunt from the RIPA to the pulmonary vasculature is also a possible route^[5]. It has been shown that fat globules < 7 μm in diameter can pass directly through the pulmonary arteriolar network (i.e., transpulmonary shunt) and result in cerebral injury^[6]. Therefore, presence of intracardiac shunt may not be a requisite for CLE as has been demonstrated in mongrel dogs^[7]. But this kind of patients usually had specific respiratory symptoms such as cough, dyspnoea and so on. Wu *et al*^[1] thought that pulmonary and CLE might be correlated closely with the bypass between tumor feeding artery and pulmonary vessels due to the tumor invading the thoracic cavity. Matsumoto *et al*^[2] concluded that communication between tumor feeding artery and pulmonary vein might have occurred *via* adhesive pleural or tumor invasion into the diaphragm. Therefore, a small dose of lipiodol could enter into the systemic circulation quickly and caused CLE. Thus, we hypothesize that lipiodol passed through the hepatic or right inferior phrenic arteriopulmonary vein shunt or hepatic arteriovenous shunt, and then traveled to the cerebral artery through intrapulmonary arte-

riovenous shunt.

Although CLE is a rare complication of TACE in patients with HCC, we should keep alert when we observe complications of TACE. When angiogram shows any hepatic arteriovenous or hepatic arteriopulmonary vein shunts, we should decrease the dose of lipiodol during the procedure and pay attention to the respiratory and neurological symptoms after the procedure, which may be caused by ectopic embolism.

In addition to after-embolization syndrome, with its symptoms manifested as fever, pain, nausea and vomiting, there are also some severe complications of TACE, including acute hepatic failure, intrahepatic biloma, pseudoaneurysm formation, ectopic infarction, *etc.* Clinicians should keep in mind that a small number of patients after TACE will suffer from some severe and rare complications. Our patient developed a severe headache and followed by confusion 20 min after the procedure. If some symptoms such as cough, chest pain, chest distress, headache, nausea, and vomiting occur in patients after TACE, physical examinations should be done so as to exclude pulmonary and cerebral complications.

In conclusion, even though CLE is an uncommon complication of TACE, we should be aware of the rare complications in patients with high-risk factors such as a large-size tumor, hepatic vein or diaphragm invasion of tumor and congenital cardiovascular disease, and reduce the dose of lipiodol or stop the procedure accordingly. To reduce the risk of lipiodol embolism, a small lipiodol dose and detection of intracardiac shunt before TACE should be considered in the HCC patients with high risk factors.

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 Asian Pacific *Helicobacter pylori*
 Meeting 2012
 Kuala Lumpur, Malaysia

January 19-21, 2012
 American Society of Clinical
 Oncology 2012 Gastrointestinal
 Cancers Symposium
 San Francisco, CA 3000,
 United States

January 19-21, 2012
 2012 Gastrointestinal Cancers
 Symposium
 San Francisco, CA 94103,
 United States

January 20-21, 2012
 American Gastroenterological
 Association Clinical Congress of
 Gastroenterology and Hepatology
 Miami Beach, FL 33141,
 United States

February 3, 2012
 The Future of Obesity Treatment
 London, United Kingdom

February 16-17, 2012
 4th United Kingdom Swallowing
 Research Group Conference
 London, United Kingdom

February 23, 2012
 Management of Barretts
 Oesophagus: Everything you need
 to know
 Cambridge, United Kingdom

February 24-27, 2012
 Canadian Digestive Diseases Week
 2012
 Montreal, Canada

March 1-3, 2012
 International Conference on
 Nutrition and Growth 2012
 Paris, France

March 7-10, 2012
 Society of American Gastrointestinal
 and Endoscopic Surgeons Annual
 Meeting
 San Diego, CA 92121, United States

March 12-14, 2012
 World Congress on
 Gastroenterology and Urology
 Omaha, NE 68197, United States

March 17-20, 2012
 Mayo Clinic Gastroenterology and
 Hepatology
 Orlando, FL 32808, United States

March 26-27, 2012
 26th Annual New Treatments in
 Chronic Liver Disease
 San Diego, CA 92121, United States

March 30-April 2, 2012
 Mayo Clinic Gastroenterology and
 Hepatology
 San Antonio, TX 78249,
 United States

March 31-April 1, 2012
 27th Annual New Treatments in
 Chronic Liver Disease
 San Diego, CA 92121, United States

April 8-10, 2012
 9th International Symposium on
 Functional GI Disorders
 Milwaukee, WI 53202, United States

April 13-15, 2012
 Asian Oncology Summit 2012
 Singapore, Singapore

April 15-17, 2012
 European Multidisciplinary
 Colorectal Cancer Congress 2012
 Prague, Czech

April 18-20, 2012
 The International Liver Congress
 2012
 Barcelona, Spain

April 19-21, 2012
 Internal Medicine 2012
 New Orleans, LA 70166,
 United States

April 20-22, 2012
 Diffuse Small Bowel and Liver
 Diseases
 Melbourne, Australia

April 22-24, 2012
 EUROSON 2012 EFSUMB Annual

Meeting
 Madrid, Spain

April 28, 2012
 Issues in Pediatric Oncology
 Kiev, Ukraine

May 3-5, 2012
 9th Congress of The Jordanian
 Society of Gastroenterology
 Amman, Jordan

May 7-10, 2012
 Digestive Diseases Week
 Chicago, IL 60601, United States

May 17-21, 2012
 2012 ASCRS Annual Meeting-
 American Society of Colon and
 Rectal Surgeons
 Hollywood, FL 1300, United States

May 18-19, 2012
 Pancreas Club Meeting
 San Diego, CA 92101, United States

May 18-23, 2012
 SGNA: Society of Gastroenterology
 Nurses and Associates Annual
 Course
 Phoenix, AZ 85001, United States

May 19-22, 2012
 2012-Digestive Disease Week
 San Diego, CA 92121, United States

June 2-6, 2012
 American Society of Colon and
 Rectal Surgeons Annual Meeting
 San Antonio, TX 78249,
 United States

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 Challenges
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July 25-26, 2012
 PancreasFest 2012
 Pittsburgh, PA 15260, United States

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 OESO 11th World Conference
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Neurogastroenterology and Motility
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 Bologna, Italy

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 Surgery
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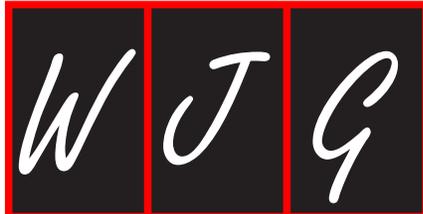
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 Modern Technologies in
 Diagnosis and Treatment of
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 The Liver Meeting
 San Francisco, CA 94101,
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 of Liver Diseases
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December 1-4, 2012
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In press

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

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

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No volume or issue

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

Books

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

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- 15 Morse SS. Factors in the emergence of infectious dis-

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- 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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Write as mean \pm SD or mean \pm SE.

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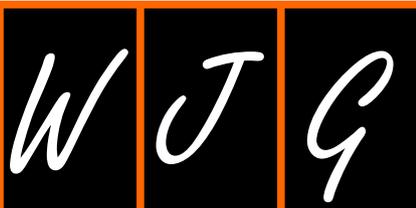
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Inflammation- and stress-related signaling pathways in hepatocarcinogenesis

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Abstract

It has been established that cancer can be promoted and exacerbated by inflammation. Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, and its long-term prognosis remains poor. Although HCC is a complex and heterogeneous tumor with several genomic mutations, it usually develops in the context of chronic liver damage and inflammation, suggesting that understanding the mechanism(s) of inflammation-mediated hepatocarcinogenesis is essential for the treatment and prevention of HCC. Chronic liver damage induces a persistent cycle of necroinflammation and hepatocyte regeneration, resulting in genetic mutations in hepatocytes and expansion of initiated cells, eventually leading to HCC development. Recently, several inflammation- and stress-related signaling pathways have been identified as key players in these processes, which include the nuclear factor- κ B, signal transducer and activator of transcription, and stress-activated mitogen-activated protein kinase pathways. Although these pathways may suggest potential therapeutic targets, they have a wide range of functions and complex crosstalk occurs among them.

This review focuses on recent advances in our understanding of the roles of these signaling pathways in hepatocarcinogenesis.

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Key words: Hepatocellular carcinoma; Inflammation; Nuclear factor- κ B; Mitogen-activated protein kinase; Signal transducer and activator of transcription; c-Jun NH₂-terminal kinase; p38; Transforming growth factor-activated kinase 1; Apoptosis signal-regulating kinase 1

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INTRODUCTION

Various types of cancer arise in the setting of chronic inflammation, suggesting a strong link between inflammation and carcinogenesis^[1,2]. Although Virchow first suggested this relationship in the 19th century, clear evidence for it has been obtained only during the last decade^[3]. The development of hepatocellular carcinoma (HCC) is one of the most extensively investigated inflammation-based carcinogenic processes because more than 90% of HCCs develop in the context of chronic liver damage and inflammation.

HCC is diagnosed in more than half a million people each year and is the third most common cause of cancer mortality worldwide^[4]. The short-term prognosis of patients with HCC has improved recently due to advances in early diagnosis and treatment, but long-term prognosis remains unsatisfactory, as indicated by the low overall survival of 22%-35% at 10 years after curative treatment^[5,6]. Thus, understanding the molecular carcinogenic mechanisms and the unique pathogenic biology of HCC has become an important issue worldwide.

HCC is a complex and heterogeneous tumor with several genomic mutations, but even the most frequent genetic mutations, such as those in p53 and β -catenin, are seen in 30%-50% of HCC cases at most^[7]. On the other hand, as mentioned above, more than 90% of HCC develops based on chronic inflammation, indicating that understanding the mechanism(s) of inflammation-mediated hepatocarcinogenesis is necessary for the treatment and prevention of HCC.

The main cause of HCC is viral hepatitis caused by the hepatitis B virus (HBV) or hepatitis C virus (HCV); other major etiologies include hemochromatosis, alcoholic hepatitis, and non-alcoholic steatohepatitis (NASH)^[7]. Most of these diseases are known to cause chronic inflammation in the liver, which plays a critical role in hepatocarcinogenesis. For example, in chronic viral hepatitis, the host immune responses to HBV or HCV are often insufficiently strong to completely clear the infection, resulting in chronic stimulation of an antigen-specific immune response^[8]. Virus-infected hepatocytes are killed by host immune cells as well as the intrinsic cytopathic effects of the hepatitis viruses, triggering the production of various cytokines and growth factors and subsequently inducing compensatory hepatocyte regeneration. This persistent cycle of necro-inflammation and hepatocyte regeneration is thought to increase the risk of genetic mutation in hepatocytes, and, furthermore, to promote survival and expansion of initiated cells^[9-11]. Additionally, reactive oxygen species (ROS) and nitrogen oxygen species, generated by both initiated cells and inflammatory cells, could accelerate hepatocarcinogenesis through several mechanisms, such as the induction of oxidative DNA damage, DNA methylation, and hepatocyte injury^[11].

Multiple signaling pathways are involved in these processes. Among them, recent *in vivo* studies have shown that several inflammation- and stress-related signaling pathways are key players in hepatocarcinogenesis, including the nuclear factor- κ B (NF- κ B), signal transducer and activator of transcription (STAT), and stress-activated mitogen-activated protein kinase (MAPK) pathways. Mutations in genes involved in these signaling pathways are currently thought to be rare. Nevertheless, constitutive activation of these pathways is frequently seen in the tumor and surrounding liver tissues, and may be due to the inflammatory microenvironment. Interestingly, these signaling pathways do not act independently, but are linked through extensive crosstalk. This review highlights

advances in the understanding of these interesting but complex signaling pathways in hepatocarcinogenesis.

INFLAMMATION-RELATED SIGNALING IN HEPATOCARCINOGENESIS

Role of the I κ B kinase/NF- κ B pathway in hepatocytes

The NF- κ B family of transcription factors consists of five members: p65/RelA, c-Rel, RelB, p50/NF- κ B1, and p52/NF- κ B2. Two of the five members dimerize and are held in the cytoplasm by the inhibitor of NF- κ B (I κ B) proteins^[12]. In response to many kinds of proinflammatory stimuli, the I κ B kinase (IKK) complex, which consists of two catalytic subunits, IKK α and IKK β , and a regulatory component, IKK γ /I κ B kinase (NEMO), phosphorylates I κ B and subsequently induces degradation of it. Once activated, NF- κ B dimers translocate into the nucleus and stimulate the transcription of various genes, such as those encoding cytokines and anti-apoptotic factors^[13]. Mice lacking RelA, IKK β , or NEMO reveal embryonic lethality with extensive liver apoptosis and degeneration^[14-16]. This liver apoptosis is induced by tumor necrosis factor (TNF)- α , and intercrossing with TNF receptor 1 knockout mice prevents liver damage and the lethal phenotype^[14,17,18]. Furthermore, hepatocyte-specific IKK β or NEMO knockout mice are not embryonic-lethal, but are more sensitive to TNF- α -mediated liver injury^[19,20]. Thus, NF- κ B plays a key role in liver homeostasis by preventing hepatocyte death.

The role of IKK β in hepatocarcinogenesis has been examined using the diethylnitrosamine (DEN)-induced mouse HCC model^[21]. DEN is the most commonly used genotoxic chemical carcinogen to investigate the mechanism of hepatocarcinogenesis, because it is easy to induce HCC and DEN-induced HCC shows histology and gene expression similar to human HCC, especially with a poor prognosis^[22,23]. A single dose of DEN given to 2-wk-old male mice is sufficient to induce HCC. However, when DEN is administered to mice older than 4 wk of age, it cannot induce HCC and requires assistance from tumor promoters, such as phenobarbital, because hepatocyte proliferation is rare in adult mice^[24]. Thus, some stimulation that induces hepatocyte proliferation is indispensable as a tumor promoter in this model.

Strikingly, DEN-induced HCC was markedly increased in hepatocyte-specific IKK β knockout mice^[21]. Hepatocyte-specific knockout of IKK β induced a greater extent of hepatocyte death with ROS accumulation after DEN administration, because NF- κ B activation is required for the up-regulation of antioxidative genes, such as ferritin heavy chain and manganese-dependent superoxide dismutase. Excess ROS accumulation promotes cell death through various mechanisms, including prolonged c-Jun NH₂ terminal kinase (JNK) activation^[25]. Cell death is accompanied by an inflammatory reaction, and the elevated hepatocyte death rate enhances compensatory proliferation. Thus, the hepatocyte-specific deletion of

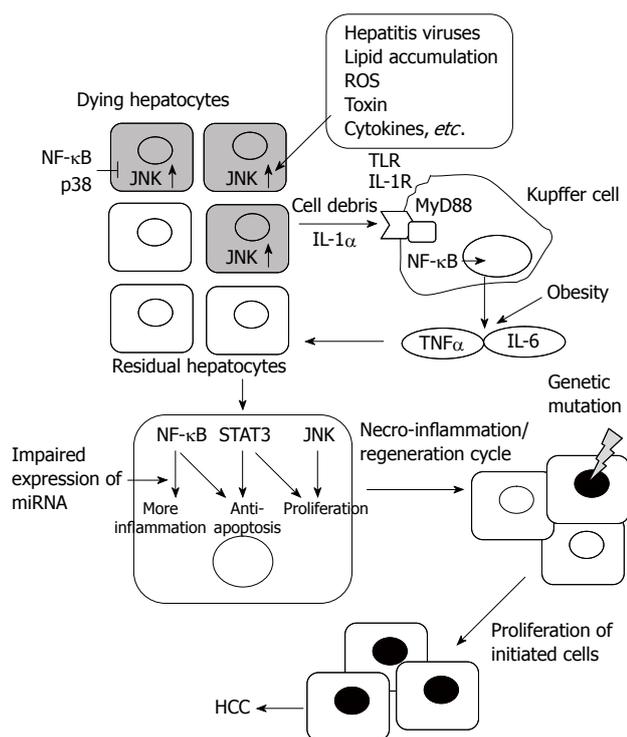


Figure 1 Role of inflammation- and stress-related signaling pathways in hepatocarcinogenesis. Chronic liver damage induces a persistent cycle of necro-inflammation and hepatocyte regeneration, resulting in genetic mutations in hepatocytes and expansion of initiated cells, eventually leading to hepatocellular carcinoma (HCC) development. As shown in the figure, nuclear factor- κ B, signal transducer and activator of transcription 3, and stress-activated mitogen-activated protein kinase pathways play critical roles in these processes. Furthermore, other factors, such as obesity and impaired expression of microRNA, can modify these inflammatory processes and accelerate HCC development. ROS: Reactive oxygen species; TLR: Toll-like receptor; NF- κ B: Nuclear factor- κ B; TNF: Tumor necrosis factor; IL: Interleukin; JNK: c-Jun NH₂ terminal kinase; STAT: Signal transducer and activator of transcription.

IKK β augments DEN-induced hepatocyte death and cytokine-driven compensatory proliferation, which acts as a tumor promoter and eventually leads to increased HCC development (Figure 1). Similar findings were obtained in mice lacking IKK γ /NEMO, the hepatocyte-specific deletion of which results in spontaneous liver damage, hepatosteatosis, fibrosis, and HCC development^[20].

Although the experiments described above demonstrate a tumor-suppressive role of NF- κ B in the hepatocyte, hepatocyte NF- κ B has been also identified to have tumor-promoting roles in other HCC models that depend on chronic inflammation rather than liver damage and death-driven compensatory proliferation. Experiments crossing transgenic mice expressing a non-degradable I κ B α mutant in hepatocytes with *MDR2* knockout mice, which show low-grade chronic inflammation in the portal area and subsequent cancer development, revealed that the inhibition of NF- κ B activation resulted in reduced HCC development^[26]. In this model, NF- κ B activation in the hepatocyte promoted a low degree of TNF- α production and paracrine TNF- α signaling maintained NF- κ B activation in the malignant cells, leading to

the expression of anti-apoptotic genes and the survival of malignant cells. Notably, NF- κ B activation is more important in the progression of hepatocarcinogenesis than in the initiation step in this model. A similar tumor-promoting role of NF- κ B in the hepatocyte has been reported in hepatocyte-specific lymphotoxin $\alpha\beta$ transgenic mice^[27]. Lymphotoxin $\alpha\beta$ transgenic hepatocytes produce chemokines, such as CCL2, CCL7, CXCL1, and CXCL10, in an IKK β -dependent manner, recruiting inflammatory cells into the liver and inducing spontaneous chronic inflammation and the subsequent development of HCC. These findings suggest that NF- κ B activation in the hepatocyte is involved in the production of cytokines and chemokines that maintain the inflammatory environment as a tumor promoter. Thus, the NF- κ B pathway in the hepatocyte plays dual roles in hepatocarcinogenesis, according to the disease model and the carcinogenesis stage.

Role of the IKK/NF- κ B pathway in myeloid cells

The role of the NF- κ B pathway in myeloid cells has also been investigated using a DEN-induced HCC model^[21]. In contrast to hepatocyte-specific IKK β knockout mice, deletion of IKK β in both hepatocytes and myeloid cells, including Kupffer cells, strongly inhibited DEN-induced HCC development. This phenotype was derived from the markedly reduced production of cytokines, such as TNF- α , interleukin-6 (IL-6), and hepatocyte growth factor, which are secreted by non-parenchymal cells in response to dying hepatocytes and induce compensatory proliferation of residual hepatocytes. Thus, IKK β /NF- κ B in myeloid cells is required for the production of liver growth factors and subsequent hepatocarcinogenesis. Furthermore, IKK β in myeloid cells, especially in Kupffer cells, has also been implicated in the development of metastatic liver tumors through IL-6 production^[28]. Thus, the IKK β /NF- κ B pathway orchestrates inflammatory crosstalk between hepatocytes and myeloid cells in liver cancer development (Figure 1).

Role of inflammatory cytokines in hepatocarcinogenesis

As mentioned above, NF- κ B activation-mediated production of inflammatory cytokines plays an important role in the inflammation-carcinogenesis axis of the liver. Various inflammatory cytokines, including TNF- α , IL-1 α , IL-1 β , IL-6, and IL-8, have been implicated in chronic liver inflammation, among which IL-6 is thought to be one of the most important^[8,29,30]. In chronic hepatitis, IL-6 is considered to be produced mainly by activated Kupffer cells and to intensify local inflammatory responses, and then induce compensatory hepatocyte proliferation, facilitating malignant transformation of hepatocytes^[30]. Hepatocytes express high amounts of the IL-6 receptor and a signal-transducing element (gp130) that, upon IL-6 binding, activates two signaling pathways, Janus activated kinase (JAK)-STAT and MAPK, which are important in the regulation of cell survival and proliferation^[31]. In fact, serum IL-6 levels are elevated in patients with chronic liv-

er diseases, including alcoholic hepatitis, HBV and HCV infections, and NASH^[32-34]. Additionally, a higher serum IL-6 level correlates with future HCC development in patients with chronic hepatitis B or C^[35,36]. These findings suggest that IL-6 plays a role linking chronic inflammation and hepatocarcinogenesis in humans.

In a mouse HCC model, IL-6 knockout mice showed a marked reduction in DEN-induced HCC development, indicating that IL-6 signaling is directly involved in hepatocarcinogenesis^[37]. This study also demonstrated the key role played by the toll-like receptor (TLR) adapter protein MyD88. Necrotic hepatocyte-induced IL-6 production was reduced significantly in MyD88-deficient Kupffer cells. Furthermore, deletion of MyD88 suppresses DEN-induced carcinogenesis, indicating that IL-6 production through the TLR/MyD88/NF- κ B pathway in Kupffer cells is essential for HCC development. Another study showed that the DEN-induced acute inflammatory response is triggered by IL-1 α release from necrotic hepatocytes, and IL-1 α subsequently induces IL-6 production by Kupffer cells^[38]. Indeed, IL-1 receptor knockout mice showed significantly reduced DEN-induced IL-6 production and subsequent HCC development^[38]. Of note, a clinical study revealed that higher serum IL-6 levels correlated with higher aspartate aminotransferase levels in chronic hepatitis C, suggesting that IL-6 may be produced in response to HCV-induced hepatocyte injury^[36].

HCC develops much more frequently in males than in females in almost all populations, with a male-to-female ratio of 2:1-4:1^[39]. Interestingly, although this sex disparity is also found in this DEN-induced HCC model, ablation of IL-6 abolished the sex differences in hepatocarcinogenesis^[37]. However, ovariectomized female mice revealed enhanced IL-6 production and aggravated HCC development. Furthermore, estrogen administration to Kupffer cells inhibits necrotic hepatocyte-induced IL-6 production. These results suggest that estrogen-mediated down-regulation of IL-6 may partly explain the sex disparity in HCC development. However, more recently, Li *et al*^[40] reported that transcription factors Foxa1 and Foxa2 in the hepatocyte played important roles in the sex disparity in hepatocarcinogenesis through an interaction with the estrogen and androgen receptors, independent of IL-6 signaling. Thus, the sex disparity in hepatocarcinogenesis may have several causes in addition to estrogen-mediated down-regulation of IL-6.

Several epidemiologic studies have shown that obesity and metabolic syndrome increase the risk of HCC^[41-43]. Although the mechanism by which obesity and metabolic syndrome promote hepatocarcinogenesis is not fully understood, it seems likely to be mediated, in part, by a state of chronic inflammation. A recent report by Park *et al*^[44] demonstrated that dietary- or genetically induced obesity promoted DEN-induced HCC along with low-grade inflammation, and ablation of IL-6 or the TNF receptor 1 abrogated their tumor-promoting effects, suggesting that IL-6 and TNF- α are required for the promotion of obesity-associated HCC. IL-6 and TNF- α produced by

adipose tissue or Kupffer cells activate hepatocyte STAT3 and NF- κ B, respectively, promoting cell proliferation and survival of initiated hepatocytes. In fact, recent clinical studies have suggested that visceral fat accumulation, insulin resistance, and dysregulation of adipokines, which induce the activation of the inflammatory response, play important roles in hepatocarcinogenesis^[45-47]. As the incidence rate of such obesity-associated hepatocarcinogenesis is likely to increase in the near future, inflammatory cytokines have also attracted considerable attention as a mediator of the association between obesity and hepatocarcinogenesis^[48].

Role of JAK-STAT signaling

JAK/STAT signaling pathways are important components of many cytokine receptor systems. Cytokines function by specifically recognizing their receptors, which, as a result of binding to their ligand, undergo conformational changes, resulting in the displacement of JAKs, and subsequently JAKs phosphorylate and activate STATs. The STAT protein family consists of seven members encoded by distinct genes. Among them, STAT3 is the most important IL-6 signaling pathway molecule and is recognized as a key player linking inflammation and cancer^[49,50]. In response to IL-6 signaling through gp130/JAK, STAT3 forms homodimers that translocate to the nucleus. STAT3 is constitutively active in many tumor cells, but not in normal cells, and regulates the expression of genes involved in tumor progression, such as cell survival, proliferation, invasion, and angiogenesis^[51].

Clinical and experimental evidence suggest the involvement of the STAT3 signaling pathway in hepatocarcinogenesis. Activated nuclear STAT3 is found in 60% of HCC and is more pronounced in aggressive tumors^[52]. In contrast, suppressors of this pathway, such as suppressor of cytokine signaling 3 (SOCS3), are down-regulated in HCC^[53]. In a mouse model, hepatocyte-specific STAT3 ablation prevented DEN-induced HCC development^[52], whereas hepatocyte-specific SOCS3 knockout mice were susceptible to HCC development through the enhanced activation of JAK/STAT and MAPK signaling^[53]. Hepatocyte-specific IL-6 and IL-6R transgenic mice spontaneously develop hepatocellular hyperplasia and adenomas, which are considered precancerous lesions in humans, accompanying STAT3 activation^[54]. Furthermore, in a human study, gain-of-function mutations in gp130 have been identified in 60% of benign hepatocellular adenomas with an inflammatory phenotype, and when combined with β -catenin-activating mutation, lead to HCC development^[55]. Thus, the IL-6-gp130-JAK-STAT3 signaling axis is an important contributor to HCC development, making it an attractive target for the treatment and/or prevention of hepatocarcinogenesis.

Interaction between STAT3 and NF- κ B has been reported at several levels in tumors. Some studies showed that STAT3 and NF- κ B co-regulate numerous oncogenic and inflammatory genes^[50]. Additionally, STAT3 directly interacts with RelA, trapping it in the nucleus, thereby

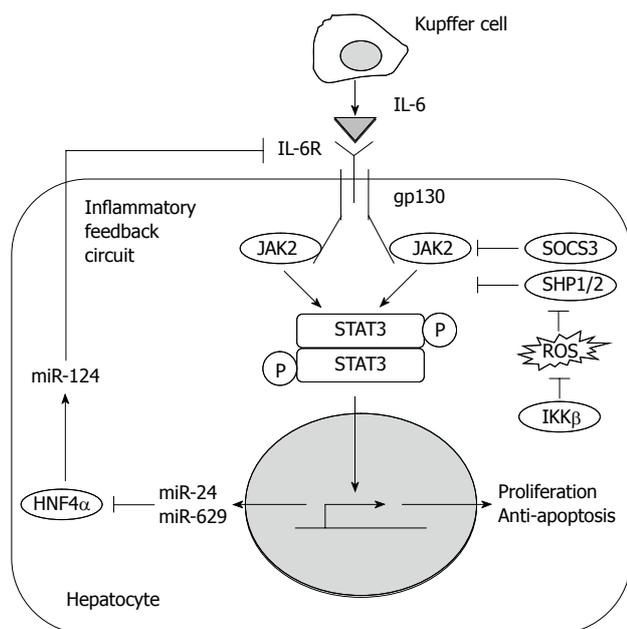


Figure 2 Implications and regulatory system of interleukin-6/gp130/janus activated kinase/signal transducer and activator of transcription 3 signaling in hepatocarcinogenesis. Interleukin (IL)-6 secreted by Kupffer cells activates signal transducer and activator of transcription (STAT) 3, which promotes the proliferation and survival of initiated hepatocytes. STAT3 activation is suppressed by I κ B kinase β through the prevention of reactive oxygen species. However, once STAT3 is activated, STAT3 activation becomes sustained through a microRNA feedback inflammatory loop. JAK: Janus activated kinase; SOCS3: Suppressor of cytokine signaling 3; IKK: I κ B kinase; HNF: Hepatocyte nuclear factor; ROS: Reactive oxygen species.

contributing to constitutive NF- κ B activation^[56]. On the other hand, a recent *in vivo* study revealed that IKK β /NF- κ B in the hepatocyte negatively regulated STAT3 activation in a DEN-induced HCC model^[52]. Inactivation of IKK β caused ROS accumulation and ROS were found to oxidize protein tyrosine phosphatases, including SHP1 and SHP2, which dephosphorylated JAK2 and STAT3. Oxidation of SHP1 and SHP2 results in the loss of their catalytic activity and the constitutive activation of STAT3. In fact, an inverse correlation between NF- κ B and STAT3 has been found in human HCC samples. This crosstalk may be a reason for the aggravation of DEN-induced HCC in hepatocyte-specific IKK β knockout mice. Furthermore, Bard-Chapeau *et al.*^[57] showed that hepatocyte-specific SHP2 knockout mice revealed spontaneous liver inflammation and tumorigenesis, accompanied by STAT3 activation. These mice also showed enhanced DEN-induced HCC, which was decreased significantly by intercrossing with hepatocyte-specific STAT3 knockout mice. These results suggest that the inhibition of STAT3 activation by SHP2 plays an important tumor-suppressing role in hepatocarcinogenesis (Figure 2).

Implications of microRNA in inflammatory signaling

MicroRNAs (miRNAs) are endogenous 20-23-nucleotide RNAs that play important gene-regulatory roles by pairing with the messenger RNAs of protein-coding genes to

direct their post-translational repression^[58]. Recently, miRNAs have been reported to be implicated in hepatocarcinogenesis through modulating inflammatory signaling pathways. Ji *et al.*^[59] found that miR-26 was significantly reduced in human HCC tissues, compared with surrounding non-tumor tissues, and a gene network analysis revealed that miR-26 expression was inversely correlated with the activation of NF- κ B and IL-6 signaling pathways. Although a causal relationship between miR-26 and hepatocarcinogenesis could not be evaluated in this study, Kota *et al.*^[60] showed that low miR-26 expression played a causal role in hepatocarcinogenesis using a myc-induced mouse HCC model, and induction of miR-26 by gene therapy suppressed HCC development.

More recently, hepatocyte nuclear factor 4 α (HNF4 α), a transcription factor that is essential for the development of hepatocytes, was reported to play a key role in hepatocarcinogenesis, linking miRNAs and inflammatory signaling pathways^[61]. Transient suppression of HNF4 α induces decreased miR-124 expression, leading to increased IL-6R expression and subsequent STAT3 activation. STAT3 activation not only plays the tumor-promoting roles described above, but also up-regulates HNF4 α -targeting miRNAs, miR-24 and miR-629, resulting in continued suppression of HNF4 α . Thus, transient suppression of HNF4 α initiates an IL-6/STAT3-mediated hepatocarcinogenesis process through a miRNA feedback loop circuit (Figure 2). The authors also showed that systemic administration of miR-124 prevented hepatocarcinogenesis by inhibiting the feedback loop and inducing tumor-specific apoptosis in mice. Thus, a therapeutic strategy targeting miRNA may be useful for the prevention and treatment of HCC.

STRESS-RELATED SIGNALING IN HEPATOCARCINOGENESIS

Stress-activated MAPK

MAPK cascades are signaling systems that transmit intracellular signals initiated by extracellular stimuli to the nucleus^[62,63]. The MAPK family consists of three major MAPK cascades, converging on extracellular signal-regulated kinases (ERKs), JNKs, and p38 MAPKs. Each MAPK signaling system comprises at least three components: MAPK, MAPK kinase (MAPKK), and MAPKK kinase (MAP3K). MAP3K phosphorylates, and thereby activates, MAPKK, and activated MAPKK, in turn, phosphorylates and activates MAPK. Among the three MAPKs, ERKs are activated predominantly by growth factors, whereas JNKs and p38 MAPKs, also called stress-activated MAPKs, are activated by stresses. We discuss the function and regulation of the stress-activated MAPKs in hepatocarcinogenesis in detail below.

Role of JNK signaling

JNK has three isoforms (JNK1, JNK2, JNK3) encoded by three loci. JNK1 and JNK2 are expressed ubiquitously, including in the liver, whereas JNK3 is expressed primar-

ily in the brain^[64]. JNK is phosphorylated and activated by two MAPKs, MKK4 and MKK7, and subsequently phosphorylates transcription factors, such as c-Jun and JunD, which compose the AP-1 complex^[65]. Additionally, JNK phosphorylates other proteins, such as Bcl-2 family members, and exerts various kinds of functions, depending on the cell type and stimuli^[66]. Furthermore, although JNK1 and JNK2 isoforms play redundant roles in many physiological processes, they also have distinct biological activities in some situations^[67,68].

The major functions of JNK in the liver are thought to be the induction of hepatocyte proliferation and cell death. JNK is involved in cell cycle progression, mostly through the activation of c-Jun. In this function, JNK1 is considered to be more important than JNK2, because proliferation of hepatocytes after partial hepatectomy is significantly impaired in JNK1 knockout mice, but not in JNK2 knockout mice^[69]. Additionally, hepatocyte death due to TNF- α , lipotoxicity, ER stress, ischemia-reperfusion, and drug toxicity, such as that from acetaminophen, are also considered to be JNK-dependent^[19,70-73]. JNK1 and JNK2 are, to some extent, redundant in this function. Although the downstream targets of JNK are not fully understood, most studies have demonstrated that JNK is required for the activation of the mitochondrial apoptotic pathway, through the activation of pro-apoptotic Bcl-2 family members^[74,75].

The role of JNK in hepatocarcinogenesis has been investigated using the DEN-induced HCC model^[76]. As mentioned above, hepatocyte-specific knockout of IKK β markedly promotes DEN-induced HCC, through enhanced hepatocyte death and compensatory proliferation^[21]. These phenomena can be partly explained by enhanced JNK activation in the setting of IKK β depletion. Because prolonged JNK activation is closely related to cell death, systems for the regulation of JNK activity are needed for tissue homeostasis. In this regard, NF- κ B plays an important role. Although several mechanisms have been proposed in the NF- κ B-mediated inhibition of JNK activation, ROS is one of the most important mediators^[25,77-79]. ROS accumulation, caused by the reduced expression of NF- κ B-dependent antioxidative enzymes, extends JNK activation by inactivating MAPK phosphatases that are essential for the dephosphorylation of activated JNK^[25]. In fact, the administration of antioxidants to hepatocyte-specific IKK β knockout mice decreased sustained JNK activation and hepatocyte death after DEN injection, and furthermore, intercrossing hepatocyte-specific IKK β knockout mice with JNK1 knockout mice significantly reduced DEN-induced hepatocyte death and compensatory proliferation, eventually suppressing HCC development^[21,76]. Additionally, JNK1 knockout mice showed a significant reduction of DEN-induced HCC, compared with wild-type controls. Thus, JNK1 is involved in hepatocarcinogenesis through hepatocyte death and proliferation, which are key components of necro-inflammatory cycles (Figure 1). Furthermore, in addition to the initial phase, JNK1 plays a tumor-

promoting role by enhancing cancer cell proliferation and neovascularization through the increased expression of cyclin D1 and vascular endothelial growth factor, respectively^[76]. Another study showed that JNK1 promoted HCC cell proliferation *in vivo* through the up-regulation of c-myc expression and the down-regulation of p21 expression^[69]. This study, however, also showed that JNK2 was not involved in hepatocarcinogenesis. In fact, JNK1, but not JNK2, is activated in approximately half of human HCC tissues, compared with adjacent non-tumor tissues^[69,80]. These results suggest that JNKs, especially JNK1, play an important role in the development of HCC. Notably, the pharmacological inhibition of JNK suppressed DEN-induced HCC and the growth of xenografted human HCC cells, suggesting that JNK may be a promising therapeutic target for HCC^[69].

On the other hand, a recent study using conditional JNK knockout mice showed that the ablation of both JNK isoforms, JNK1 and JNK2, in hepatocytes increased DEN-induced HCC, whereas the ablation of JNK1 and JNK2 in both hepatocytes and myeloid cells reduced hepatic inflammation and the development of HCC, indicating that JNK plays dual roles in hepatocarcinogenesis, depending on cell type and carcinogenesis stage^[81].

JNK plays a pivotal role in the development of metabolic syndrome-related disorders, including NASH^[41]. Inflammatory cytokines and ROS accumulation in the liver caused by obesity and fatty liver disease induce JNK activation, leading to insulin resistance by increasing inhibitory insulin receptor substrate 1 ser307 phosphorylation^[82]. As a clinical study showed that insulin resistance was a major contributor to obesity-mediated hepatocarcinogenesis, JNK may be a candidate therapeutic target in such situations^[83]. Furthermore, ROS-mediated JNK activation in the liver is linked not only to liver disease, but also to systemic disorders, such as atherosclerotic cerebrovascular diseases; thus, further elucidation of this process is important^[84,85].

Role of p38 signaling

The p38 MAPK family consists of four members: p38 α , p38 β , p38 γ , and p38 δ ^[86]. Among them, p38 α is abundant in most cell types, and its function has been investigated in most published studies of p38 MAPKs. p38 is activated through phosphorylation, primarily by MKK3 and MKK6, but phosphorylation by MKK4 and autophosphorylation are also involved in some stimuli^[87]. p38 can activate not only transcription factors, such as ATF2, p53, and Mitf, but also protein kinases, such as MAPKAP kinase 2 (MK2) and MK5^[88]. Although p38 was initially discovered as a regulator of inflammatory cytokine production, recent studies have revealed that it has tumor-suppressing properties. p38 inhibits tumorigenesis by the down-regulation of cyclins, up-regulation of cyclin-dependent kinase inhibitors, and modulation of the tumor suppressor p53, resulting in cell cycle arrest, oncogene-induced senescence, apoptosis induction, and contact inhibition^[86].

Although the roles of p38 in the liver have yet to be clarified, compared with JNK, major roles reported to date are the inhibition of hepatocyte death and proliferation. These effects of p38 are partially mediated by negative regulation of the JNK/c-Jun pathway. For example, hepatocyte-specific p38 α knockout mice showed much stronger lipopolysaccharide (LPS)-induced JNK activation in the liver, and intercrossing with hepatocyte specific IKK β knockout mice induced severe liver injury after LPS administration, suggesting that p38 α and IKK β act synergistically to protect the liver from TNF- α -induced hepatocyte death^[89].

The role of p38 in hepatocarcinogenesis has also been investigated using the DEN-induced HCC model^[38]. Similar to hepatocyte-specific IKK β knockout mice, hepatocyte-specific p38 α knockout mice showed enhancement of DEN-induced ROS accumulation, JNK activation, liver damage, and compensatory hepatocyte proliferation, eventually resulting in enhanced carcinogenesis. Another study showed the tumor-suppressing role of p38 through interaction with the JNK/c-Jun pathway by focusing on the antiproliferative effects in the advanced stage^[90]. However, in contrast to IKK β knockout mice, enhanced activation of the JNK pathway in p38 α knockout mice was accompanied by MAPKK activation, suggesting that the targets of p38 α may be upstream of JNK, such as MAPKKs and MAP3Ks^[38]. Consistent with these animal experiments, in human samples, the activity of the MKK6/p38 pathway is decreased in HCC tissues, compared with adjacent non-tumor tissues, and is significantly lower in larger HCC tissues^[91]. These findings suggest that the p38 pathway may play an anti-proliferative role in human HCC.

Regulatory system of stress-activated MAPK signaling by MAP3Ks

The evidence presented above suggests that JNK acts generally as a tumor promoter and p38 acts generally as a tumor suppressor in hepatocarcinogenesis, but some studies have shown opposite roles in HCC and other cancers. For example, JNK plays tumor-suppressing roles in mouse skin cancer and mammalian cancer models^[92,93]. Additionally, JNK has been reported to act as a tumor suppressor by inducing cancer cell apoptosis in HCC^[94]. As mentioned above, JNK was also reported to play dual roles in hepatocarcinogenesis, depending on cell type and carcinogenesis stage^[81]. p38 may also have oncogenic effects, facilitating cell invasion, inflammation, and angiogenesis^[95,96]. Furthermore, crosstalk among JNK, p38, and molecules involved in other signaling pathways, such as NF- κ B, further complicates their roles^[97]. Thus, understanding of the regulatory system of stress-activated MAPK signaling is necessary for the potential use of these molecules as therapeutic targets. Importantly, only two molecules, JNK and p38, are downstream in this pathway, whereas more than 10 molecules have been identified for upstream MAP3Ks^[98]. Each MAP3K is activated by several different kinds of stimuli, and integrated

into a unique pattern of MAPK activation and substrate phosphorylation, leading to a specific cellular response to the stimulus. Thus, the activities of JNK and p38 are tightly regulated by MAP3Ks. Several recent studies have uncovered roles of MAP3Ks in the regulation of stress-activated MAPK signaling in hepatocarcinogenesis.

Role of apoptosis signal-regulating kinase 1

Apoptosis signal-regulating kinase 1 (ASK1), one of the most important MAP3Ks, selectively activates JNK and p38 signaling in response to a variety of stimuli, including ROS and cytokines^[99]. In particular, ASK1 plays a key role in oxidative stress-induced cell death. In the absence of oxidative stress, thioredoxin (Trx), a reduction/oxidation regulatory protein, inhibits ASK1 kinase activity *via* direct binding to the N-terminal region of ASK1. However, once oxidative stress occurs in the cell, Trx is converted to its oxidized form and dissociates from ASK1, resulting in ASK1 kinase activation^[100]. ASK1 is considered to induce cell death through stress-activated MAPK-mediated activation of the mitochondrial cell death pathway^[101]. In fact, ASK1 is involved in acetaminophen-induced hepatocyte death, a typical ROS-mediated liver injury, through mechanisms involving Trx-ASK1 dissociation^[102]. Furthermore, ASK1 is involved in hepatocyte death mediated by death receptors, such as TNF-R and Fas^[75].

ASK1 knockout mice showed significantly increased DEN-induced HCC, suggesting that ASK1 plays tumor-suppressing roles in hepatocarcinogenesis in this model^[75]. Activation of JNK and the pro-apoptotic Bcl-2 family member Bim, which are required for death receptor-mediated apoptosis, are attenuated in ASK1 knockout HCC, resulting in decreased cancer cell apoptosis. On the other hand, ASK1 plays a minor role in the tumor-promoting effects of JNK, such as the DEN-induced acute phase reaction, cancer cell proliferation, and neovascularization. Thus, ASK1 is considered to play major roles in the tumor-suppressing part of JNK activity in hepatocarcinogenesis. Furthermore, DNA damage-induced p38 activation and subsequent p21 up-regulation is impaired in ASK1 knockout mice. Thus, ASK1 controls the tumor-suppressing function in stress-activated MAPK signaling through the induction of apoptosis and the DNA damage response.

Another study indicated that ASK1 and Bim are also required for sorafenib-induced apoptosis in HCC cells^[103]. Sorafenib is a small-molecule multikinase inhibitor that is currently the sole therapeutic drug effective for the treatment of HCC^[104]. Most recently, somatic mutations in the ASK1 gene, which reduce the kinase activity of ASK1, have been identified in melanoma^[105]. Thus, it may be important to clarify whether similar mutations are found in HCC, from the point of view of not only the carcinogenesis mechanism, but also possible therapeutic effects of anticancer drugs.

Role of transforming growth factor β -activated kinase 1

Another major MAP3K, activated kinase 1 (TAK1), is

activated through TNF receptor, TLR, IL-1 receptor, and transforming growth factor β receptor signaling, and then activates the JNK and NF- κ B pathways, which play opposing roles in cell death^[106]. Interestingly, hepatocyte-specific TAK1 knockout mice show spontaneous hepatocyte death, and this phenotype is partially rescued by crossing with TNF receptor 1 knockout mice, suggesting that TAK1 knockout hepatocytes are highly sensitive to endogenous TNF- α -induced apoptosis^[107]. This spontaneous cell death subsequently causes compensatory hepatocyte proliferation, inflammation, fibrosis, and the eventual development of HCC in aged mice^[107,108]. These phenomena resemble the phenotype observed in hepatocyte-specific NF- κ B knockout mice. Furthermore, JNK activation is rather enhanced in TAK1 knockout mice, indicating that TAK1 in the hepatocytes acts as a tumor suppressor, mainly by regulating the activation of the NF- κ B pathway. However, enhanced JNK activation in TAK1 knockout mice occurs partially through the activation of another MAP3K, TAO2, suggesting that TAK1 may interact with other MAP3Ks^[108]. Interestingly, crossing hepatocyte-specific TAK1 knockout mice with NEMO knockout mice attenuated JNK activation and prevented hepatocyte death and the development of HCC, suggesting that NEMO has a tumor-promoting function in the setting of TAK1 deletion^[108]. Additionally, this function of NEMO is considered to be independent of NF- κ B. Furthermore, a recent study showed that TAK1 inhibits ASK1-mediated apoptosis through a direct interaction between the C-terminal domain of TAK1 and the N-terminal or C-terminal domain of ASK1 in HEK 293 cells^[109]. Thus, in the setting of TAK1 deletion, ASK1 may play a tumor-promoting role by accelerating hepatocyte apoptosis and subsequent inflammation. Because crosstalk among MAP3Ks is less well understood, further studies are needed to clarify the whole picture of stress-activated MAPK signaling pathways.

CONCLUSION

One of the most important reasons for the poor prognosis of HCC is its frequent recurrence. Once HCC has developed, the recurrence rate does not decline with time, suggesting that most cases of late-phase recurrence are due to metachronous multicentric carcinogenesis caused by persistent chronic inflammation^[110]. Thus, determining the molecular mechanism(s) of inflammation-mediated hepatocarcinogenesis is important in preventing not only the occurrence, but also the recurrence, of HCC. As discussed in this review, recent studies have indicated that NF- κ B, STAT3, and stress-activated MAPK signaling pathways play key roles in inflammation-mediated hepatocarcinogenesis. These findings may prompt their introduction into the clinical setting as therapeutic targets. However, these pathways have a wide range of functions and exhibit complex crosstalk, and furthermore, may play opposing roles, depending on the cell type and carcinogenesis stage. Thus, alternative strategies, such as

targeting particular isoforms, including JNK1; upstream regulators, including MAP3K; and other modulators, including miRNA; may be more beneficial than targeting the entire pathway. In this regard, further studies clarifying the entire picture of the signaling network are needed to translate these signaling pathways into clinical practice.

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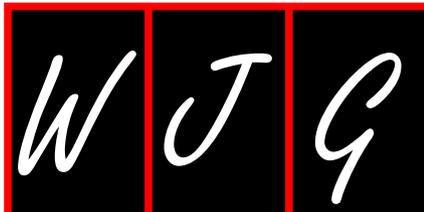
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Indomethacin for post-endoscopic retrograde cholangiopancreatography pancreatitis prophylaxis: Is it the magic bullet?

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Abstract

Acute pancreatitis is a common complication of endoscopic retrograde cholangiopancreatography (ERCP). Pancreatic duct stent insertion after ERCP has been widely accepted as the standard of care for the prevention of this complication in high-risk patients. Unfortunately, the placement of pancreatic stents requires higher level of endoscopic expertise and is not always feasible due to anatomic considerations. Therefore, effective non-invasive pharmacologic prophylaxis remains appealing, particularly if it is inexpensive, easily administered, has a low risk side effect profile and is widely available. There have been multiple studies evaluating potential pharmacologic candidates for post-ERCP pancreatitis (PEP) prophylaxis, most of them yielding disappointing results. A recently published large, multi-center, randomized controlled trial reported that in high risk patients a single dose of rectal indomethacin administered immediately after the ERCP significantly decreased the incidence of PEP compare to placebo.

INVITED COMMENTARY ON HOT ARTICLES

Acute pancreatitis is the most frequent complication of endoscopic retrograde cholangiopancreatography (ERCP). The incidence of post-ERCP pancreatitis (PEP) varies between 1%-10%, with incidence exceeding 25% being reported in certain high-risk patient populations^[1,2]. The wide range for this incidence is mostly due to the heterogeneous interplay of patient characteristics, procedure-related, and operator-related factors^[3,4].

Numerous agents and interventions have been studied so far in the prevention of PEP. These can be divided into sphincter relaxants, protease inhibitors, types of contrast, anti-inflammatory/anti-oxidant agents, anti-secretory agents, electrosurgical techniques, and placement of various types of pancreatic stents^[5]. The results have generally been disappointing and at present the only fea-

sible option to decrease the rate of PEP is the insertion of pancreatic stent in high risk patients. Unfortunately, the placement of pancreatic stents requires higher level of endoscopic expertise and is not always feasible due to anatomic considerations. Indeed, a recent survey reported that more than 20% of physicians performing ERCP never place pancreatic stents^[6]. Therefore, effective pharmacologic prophylaxis remains appealing, particularly if it is inexpensive, easily administered, has a low risk side effect profile and is widely available. Intravenous gabexate, a protease inhibitor, and somatostatin, an anti-secretory agent, have been shown to prevent PEP^[7]. However, both of these therapies are not readily available and require continuous intravenous infusion. As such, the search for effective, cheap and feasible pharmacologic prophylaxis for PEP has been continued.

Phospholipase A₂ is presumed to play a pivotal role in the inflammatory cascade associated with acute pancreatitis^[8]. This has been the basis for several prospective placebo-controlled randomized controlled trials (RCTs) evaluating non-steroidal anti-inflammatory drugs (NSAIDs), potent inhibitors of phospholipase A₂ activity, in the prevention of PEP. In 2003, Murray *et al*^[9] showed that rectal diclofenac given immediately after ERCP reduced the incidence of PEP in a high-risk patient population. These findings were corroborated by Khoshbaten *et al*^[10], who also demonstrated that immediate administration of rectal diclofenac after ERCP reduced the incidence of acute pancreatitis in patients undergoing pancreatogram. Studies evaluating rectal indomethacin have also suggested a protective effect against PEP in patients undergoing pancreatography or ERCP for biliary obstruction^[11,12]. In spite of these promising findings, the relatively small sample size of each of these studies and the heterogeneous study groups, have yielded overall inconclusive results.

A meta-analysis published in 2008 attempted to further validate the role of prophylactic rectal NSAIDs on PEP^[13]. Assuming a two-tailed $\alpha = 0.05$, a power of 0.9 and PEP incidences of 12.06% and 4.38% in the placebo and NSAID groups respectively, the authors concluded that a total of 586 patients would be required to demonstrate the intended decrease in the incidence of PEP. By compiling the results of four previous RCTs, an adequate pooled sample size was achieved to detect a statistically significant 64% (95% CI: 0.22-0.60) reduction in acute pancreatitis in patients who received NSAIDs immediately after ERCP when compared to placebo. The results of this meta-analysis further emphasized the apparent benefit of rectal administered NSAIDs for PEP prophylaxis and the need of large prospective multi-center trials to confirm these findings.

On the background of these promising results, Elmunzer *et al*^[14], report the results of a multi-center, randomized, placebo-controlled, double-blind clinical trial conducted to determine the effect of a single dose of rectal indomethacin administered immediately after ERCP in patients at elevated risk for PEP. Inclusion cri-

teria selected patients with an elevated baseline risk of PEP as defined by one or more of the following major criteria: clinical suspicion of sphincter of Oddi dysfunction (SOD), a history of PEP, pancreatic sphincterotomy, precut sphincterotomy, more than eight cannulation attempts, pneumatic dilatation of an intact biliary sphincter, or ampullectomy. Patients were also eligible if they met two or more of the following minor criteria: an age of less than 50 years and female sex, history of recurrent pancreatitis (≥ 2 episodes), three or more injections of contrast agent into the pancreatic duct with at least one injection to the tail of the pancreas, excessive injection of contrast agent into the pancreatic duct resulting in opacification of pancreatic acini, or the acquisition of a cytologic specimen from the pancreatic duct with the use of a brush. The study design consisted of patients randomly assigned to receive either two 50 mg indomethacin suppositories or two identical-appearing placebo suppositories immediately after ERCP. The randomization was concealed by using centralized location and stratified by study center. The primary and secondary outcomes of the study were the development of PEP^[15] and moderate or severe PEP, respectively. Patients with post-ERCP abdominal pain were hospitalized, followed clinically, and had their serum amylase and lipase measured at least once 24 h after the procedure. Patients discharged after uneventful ERCP were contacted within 5 d and again at 30 d to capture delayed occurrence of primary outcome and to assess for any delayed adverse events.

The study enrolled a total of 602 subjects from February 2009 through July 2011. An interim analysis recommended the study to be terminated early on the basis of the benefit of indomethacin compared with placebo. A total of 295 patients received indomethacin, and 307 patients received placebo. Baseline characteristics were similar in the two study groups. The majority of patients (82%) had a clinical suspicion of sphincter of Oddi dysfunction. The overall incidence of PEP was 13.1% (79 of 602 patients). The incidence of PEP was 9.2% (27 of 295 patients) in the indomethacin group compared to 16.9% (52 of 307) in the placebo group ($P = 0.005$), corresponding to an absolute risk reduction of 7.7 percentage points, relative risk reduction of 46%, with a number needed to treat to prevent one additional episode of PEP of 13. The secondary outcome of moderate or severe PEP occurred in 40 patients, 13 (4.4%) in the indomethacin group compared to 27 (8.8%) in the placebo group ($P = 0.03$). Among patients hospitalized for PEP, the median length of hospital stay was 0.5 d shorter in the indomethacin group (3.5 d) than in the placebo group (4 d) ($P < 0.001$). A persistent protective effect of indomethacin against PEP was noted in the post-hoc analysis of patients stratified based on their pre-treatment risk of PEP^[16], regardless of whether patients had undergone pancreatic stenting, clinical suspicion of SOD, and in all subtypes of SOD. The authors concluded that among patients at high risk for post-ERCP pancreatitis, rectal indomethacin significantly reduced the incidence of the

condition.

Acute pancreatitis remains the most common major complication of ERCP. NSAIDs represent an attractive pharmacological agent for PEP prophylaxis because they are inexpensive, can be easily administered and have a relatively low risk profile. Previous efforts to endorse NSAIDs for PEP prophylaxis have been limited by small single-center studies with conflicting results.

This study by Elmunzer *et al*^[14] is the first large multi-center, randomized, controlled trial that demonstrates the protective effects of a single dose rectal indomethacin against PEP in high-risk patients. The validity of the conclusions is supported by a number of the study methodological strengths including double blinded randomized design, adequate allocation concealment, strict clinically meaningful definition of PEP, thorough follow up with very low lost-to-follow-up rate and intention-to-treat analysis. The authors should also be commended for following the patients thirty days post-procedure to evaluate for any delayed pancreatitis or adverse events. A reduction in the incidence of PEP with rectal NSAIDs in the study group consisting primarily of patients with clinical suspicion of SOD (82%) confirms the benefit of this prophylactic agent in this challenging patient population. This finding is congruent with previous trials suggesting a maximal benefit from prophylactic NSAIDs in high-risk patients. Moreover, this study showed that the relative treatment effect of indomethacin remained across the spectrum of patient's risk of PEP. These results, in conjunction with a trend toward benefit with respect to rates of PEP in patients without clinical suspicion of SOD treated with indomethacin, suggest the need of additional studies to confirm a potential protective effect even in low-risk patients. The very high prevalence of patients with suspected SOD in this trial should be considered when interpreting the external validity of the results and applying them to other high risk PEP patients.

Prophylactic temporary pancreatic duct stenting has been widely accepted for PEP prophylaxis^[17]. One of the main limitations of previous prospective trials regarding the effects of NSAIDs for PEP prophylaxis is their failure to report the use of prophylactic pancreatic stents in their study population. A particular strength of this study is that the majority of patients (> 80%) underwent pancreatic stent placement in addition to the study intervention (indomethacin or placebo). Indomethacin reduced the risk of PEP to a similar degree irrespective to whether the patient received a pancreatic stent or not. These findings highlight the additive protective effect of NSAIDs for PEP prophylaxis in high-risk patients receiving temporary pancreatic duct stenting. Furthermore, it suggests that NSAIDs may be an alternate non-invasive prophylactic measure for PEP in those patients in whom pancreatic stenting may not be feasible or not recommended; however, this requires further investigation.

Elmunzer *et al*^[14] also reported that prophylactic indomethacin was associated with decreased severity of PEP, which is congruent with previous findings by

Sotoudehmanesh and colleagues. In the subgroup of patients that were hospitalized post-ERCP, the indomethacin group also had a shorter hospital stay when compared to placebo. These results suggest that the benefits of NSAIDs are not limited to reducing the incidence of PEP; but potentially also includes disease severity modulation presumably by regulating the inflammatory response and clinical manifestation of PEP. If further validated, these findings have both clinical as well as economic implications, given the substantial morbidity with increasing severity of PEP and associated health care expenditures.

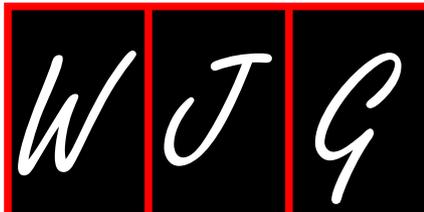
In summary, this multi-center double blinded randomized controlled trial further supports the use of prophylactic rectal NSAIDs in the prevention of PEP and addresses several limitations of previous studies that have been met with general skepticism. This study demonstrates that rectal indomethacin can reduce the incidence and severity of PEP in high-risk population consisting mostly of patients with suspected SOD and could potentially have a benefit even in low-risk patients. The low cost and risk profile associated with a single standard dose of rectal indomethacin makes this an attractive prophylactic pharmacological agent in those patients in whom this medication is not otherwise contraindicated. While clinical judgment in selecting patients with appropriate indications for ERCP remains the most important measure in preventing PEP, rectal indomethacin is a safe, easily administered, widely available pharmacological prophylactic measure that could change how we address this serious ERCP-associated complication.

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Three-dimensional image reconstruction in capsule endoscopy

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Abstract

To date, limited research has been carried out in developing methods and materials that offer three-dimensional (3-D) representation of the digestive tract. In the field of capsule endoscopy (CE), hardware approaches have been developed that provide real time both 3-D information and texture using an infrared projector and a complementary metal oxide semiconductor camera. The major drawbacks of this system are its size, power consumption and packaging issues. A software approach to approximate a 3-D representation of digestive tract surface utilising current CE technology has been proposed. The algorithm utilizes the Shape from Shading technique and seem to provide promising results for polypoid structures and angioectasias. Further clinical evaluation is currently under way.

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Key words: Capsule endoscopy; Three-dimensional; Reconstruction; Angioectasias; Software

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INVITED COMMENTARY ON HOT ARTICLES

I read with great interest the recent paper by Karargyris *et al*^[1] on the use of elastic video interpolation in the three-dimensional (3-D) reconstruction of the digestive wall in capsule endoscopy (CE) videos. The article presents promising 3-D approaches in CE; due to its technical nature though it may be slightly difficult for the general gastroenterologist. Therefore, I invited its first author to help me present the clinically relevant points. These authors believe that proper consideration should be given for its further clinical application in CE.

Wireless CE, a milestone in minimally invasive investigation of the gastrointestinal tract, was made possible by the advent of power efficient/low cost image sensors based on complementary metal oxide semiconductor (CMOS) technology^[2,3]. Upon the 1950's debut of the world's first gastro-camera, few could have envisaged that, just half a century later, imaging of the human digestive tract would become wireless^[4-6]. However, this spectacular advent in medical endoscopy is not without its limitations. Let us not forget that a complete capsule platform



Figure 1 Various sensors which are commercially available^[12]. A: Miniature magnetometers offer orientation and acceleration information, [Bertda Services (SEA) Pte. Ltd.]; B: Three-dimensional (3-D) range camera used in a widely commercial product; C: 3-D guidance system used in endoscopy devices (inition).

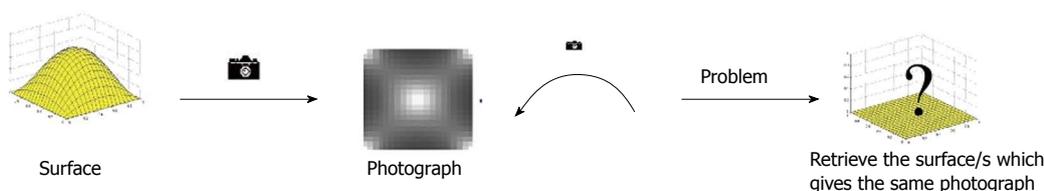


Figure 2 The Shape from Shading flow^[12,13]. Capturing a surface using a camera removes depth information. Shape from Shading techniques try to reproduce the missing depth information from a given image.

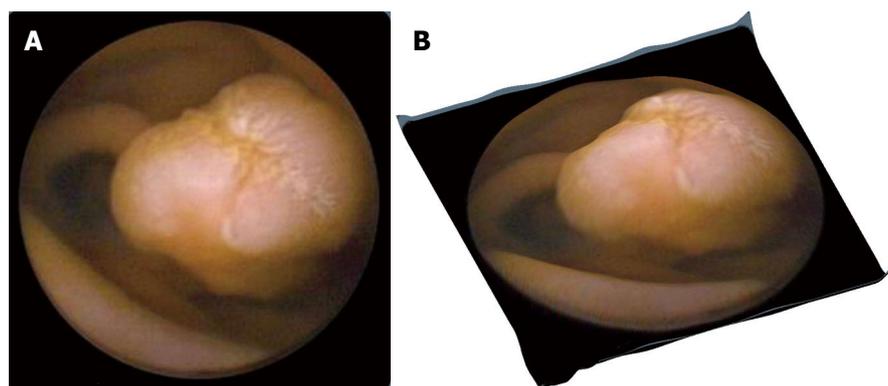


Figure 3 Original capsule endoscopy and 3-D represented image depicting a protrusion. A: Original capsule endoscopy image depicting a protrusion (polyp); B: Its 3-D represented image processed by the proposed Shape from Shading approach.

comprises six fundamental modules: locomotion, vision, telemetry, localisation, power and diagnostic/tissue manipulation tools^[7]. To present, due to space constraints, the majority of commercially available capsules include only a subset of the aforementioned modules^[7]. Furthermore, power limitation is a major hurdle which current CE technology has yet to overcome^[1,5,7].

Since the capsule needs 6-8 h to traverse through the small-bowel^[7,8], cameras within the currently marketed capsule endoscopes work at a capture rate of 2-3 frames per second (fps) in order to comply with power requirements^[7]. Nonetheless, this has an adverse effect on the smoothness of motion between consecutive frames and creates a visually unpleasant effect to the human eye^[1,7]. Furthermore, shape is an important element in human perception, yet CE suffers, unlike other diagnostic modalities i.e., Computed tomography, magnetic resonance imaging, from lack of 3-D information^[1]. Practically, this

can only be feasible with the use of new generation devices that have yet to be realised.

3-D technology is currently in use e.g., a magnetometer can provide not only acceleration values on the three axes but also the 3-D orientation of the device (Figure 1A)^[9]. Commercial time-of-flight range cameras (i.e., Microsoft's Kinect Project, Figure 1B)^[10] already exist in the market and in the near future this may be further improved and miniaturised for use inside a capsule endoscope. These cameras offer information on depth and colour. We should not forget that 3-D guidance systems are already used for endoscopic surgeries offering 3-D position information of the sensor (Figure 1C). Therefore, using the acquired information (orientation, acceleration, depth values, position *etc.*) from these miniature sensors in conjunction with sophisticated registration software algorithms, an accurate 3-D representation of the digestive tract could be created successfully.

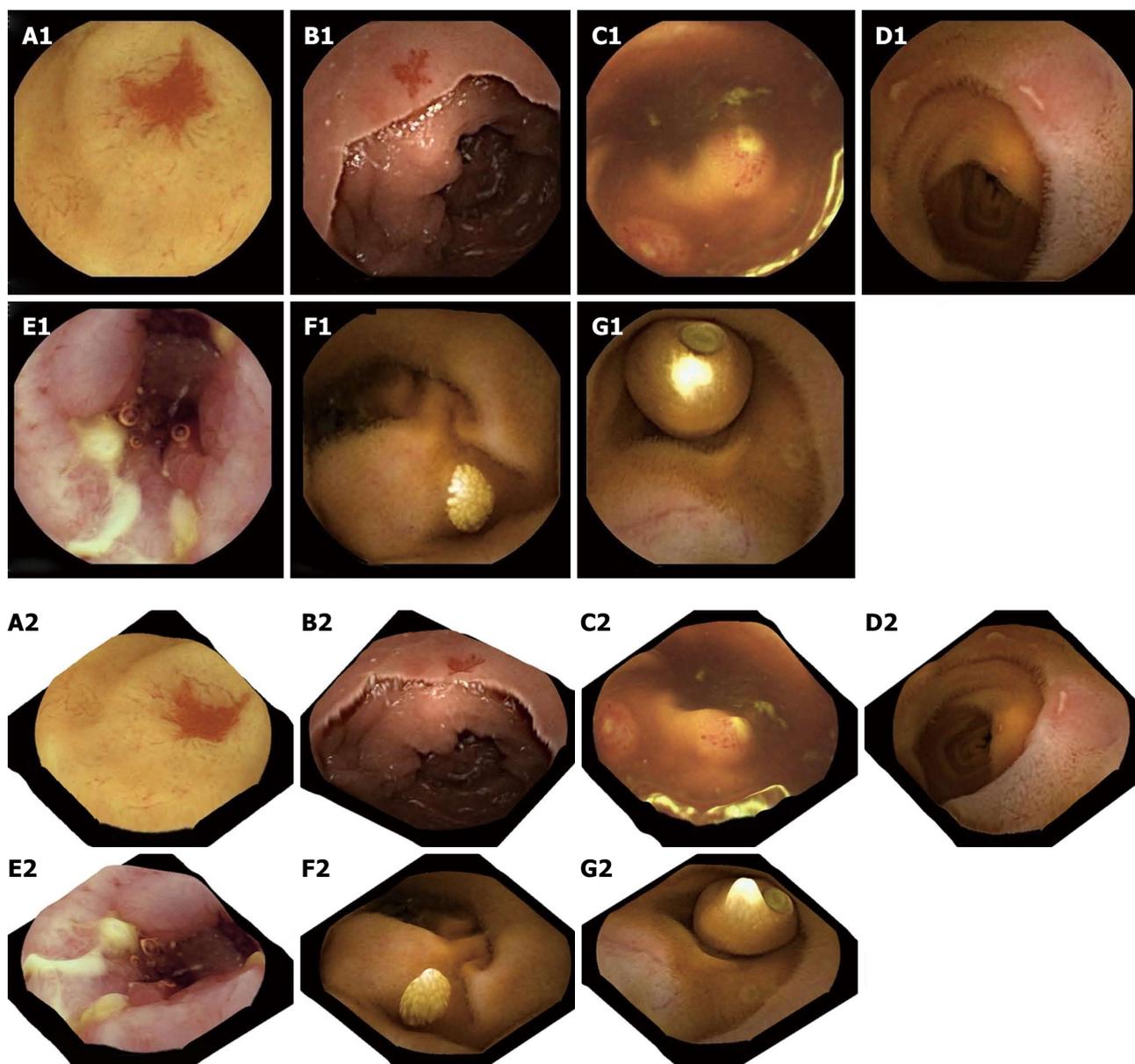


Figure 4 Two-dimensional capsule endoscopy images (upper panel) and three-dimensional representation of above structures is seen (lower panel). A1, A2: P2 angiectasia; B1, B2: P1 angiectasia; C1, C2: Lymphoid hyperplasia with superficial ulceration; D1, D2: Aphthous ulcers; E1, E2: Serpiginous ulcers; F1, F2: Nodular lymphangiectasia; G1, G2: Another capsule endoscope still inside the small-bowel.

Highlights removal

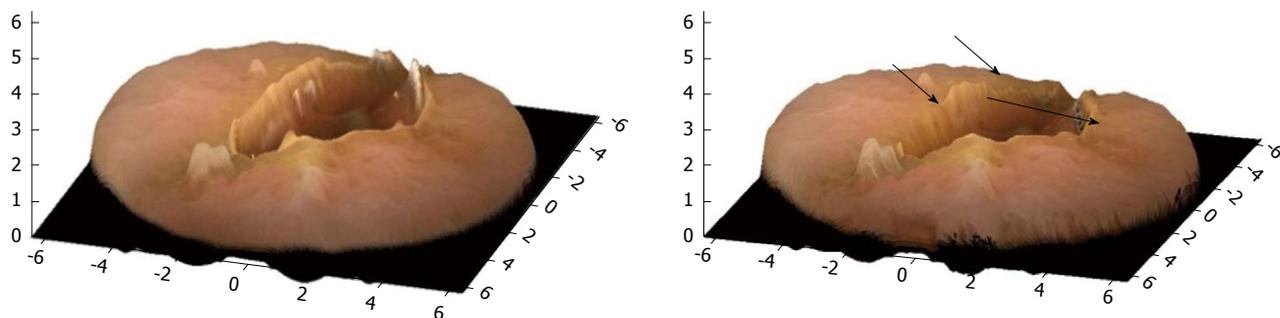


Figure 5 3-D representation of a single video capsule image with highlights removal (arrows).

3-D representation for endoscopy

To date, limited research has been carried out in developing methods and materials that offer 3-D representation of the digestive tract. For conventional endoscopy systems, stereo technology has been introduced to capture stereo images and to create depth information and therefore 3-D reconstruction of digestive structures. However, due to issues with size, such systems have not been widely accepted^[1,5]. Likewise, in CE there has been a hardware approach that provides in real time both 3-D information and texture using an infrared projector and a CMOS camera. The major drawbacks of this system are its size, power consumption and packaging issues^[11]. Therefore, in order to tackle the problem of the current hardware limitations, Karargyris *et al.*^[1,12] proposed the use of a software approach to approximate a 3-D representation of digestive tract surface utilising current CE technology.

Shape from shading

The Shape from Shading (SfS) technique is a member of a family of “shape recovery” algorithms called shape-from-X techniques^[1,13]. Essentially, SfS algorithms try to recover the shape of objects by using the gradual variation of shading (Figure 2)^[14]. SfS techniques can be divided into four groups or approaches: minimisation, propagation, local, and linear approaches^[13,14].

Karargyris *et al.*^[1] propose using a specific sub-category of SfS methods: the Tsai’s method (or linear approach)^[1,15] because (1) it produces good results for spherical surfaces, which in fact is the case with most shapes of digestive tract pathology e.g., polyps; (2) it is very fast; and (3) it “behaves” relatively well with specular surfaces (surfaces with mirror-like reflection of light). Of note, in CE the light source axis and the miniature camera are basically aligned. Tsai’s method works well on smooth objects and spherical structures^[15]. Additionally, Tsai’s method takes into consideration only the light direction, which in our case is 0° degrees (since the lighting direction and that of the camera are parallel).

A correction on the camera distortion is not performed because there is insufficient data on the capsule camera optics’ specifications. The result of applying Tsai’s method in a CE frame is given in Figure 3. Figure 3A shows a polyp in a conventional 2-D CE frame, whereas in Figure 3B the same polyp is presented using the 3-D software. The SfS approach gives promising outcomes, especially for visualizing polyps and vascular lesions, but less so for ulcers or lymphangiectasia (Figure 4). In the first 2 cases, the 3-D result is rather exciting, supporting the argument for further evaluation of this technique for use in clinical practice. Additionally, a 3-D representation may be helpful in the design of more accurate and robust computer-aided detection algorithms, incorporating other image enhancement tools e.g., virtual chromoendoscopy (FICE)^[16] or colour (blue) mode^[17] analysis of CE videos instead of still CE frames. However, prior to any software integration, further qualitative assessment and accuracy assessment should take place (work currently undertaken by

these authors).

Conversely, in cases of sudden large intensity change, Tsai’s method fails because it takes into consideration the preservation of intensity gradient. However, in non-artificial images such as CE frames, the brightness (image pixel values) transitions smoothly with no abrupt changes. Technically however, one can notice in Figure 5 the presence of highlights, hence false information about the shape. Highlights are essentially linear combinations of specular and diffuse reflection (light reflected at various angles) components of the surface.

Many objects in the real world are dielectric and homogeneous, hence displaying both types of reflections. Most digestive structures fall into this category^[18]. When the light beams fall on to such an object, some of them reflect back immediately creating the specular reflection, while the rest of the beams first penetrate the object and then reflect creating the diffuse reflection. Along with 3-D representation algorithm a highlight suppression scheme is applied to the original CE images to produce desired better results, whilst maintaining the shapes and structures of the digestive tract. Figure 5 shows the successful highlight removal without affecting the shapes of other objects. It has to be mentioned that highlights from light reflections on the surface of the digestive tract are still an open problem not only for a 3-D representation but also for traditional CE review.

In conclusion, the software by Karargyris *et al.*^[1] offers a new potential to reform and enhance the currently existing reading software in capsule endoscopy by improving lesion demarcation and highlighting the textural features of ulcers, angioectasias and polyps^[5,19]. Further work is needed to prove its clinical validity but the idea of a 3-D aid (with the present level of CE technology) seems, at least for this author, not only captivating but promising as well. However, it should not be forgotten that true 3-D capability requires dual video images, although the inclusion of two cameras within the shell of a capsule endoscopy might be unwieldy at present^[5].

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Colorectal cancer in patients with inflammatory bowel disease: Can we predict risk?

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Abstract

The inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), may be complicated by colorectal cancer (CRC). In a recent population-based cohort study of 47 347 Danish patients with IBD by Tine Jess and colleagues 268 patients with UC and 70 patients with CD developed CRC during 30 years of observation. The overall risk of CRC among patients with UC and CD was comparable with that of the general population. However, patients diagnosed with UC during childhood or as adolescents, patients with long duration of disease and those with concomitant primary sclerosing cholangitis were at increased risk. In this commentary, we discuss the mechanisms underlying carcinogenesis in IBD and current investigations of genetic susceptibility in IBD patients. Further advances will depend on the cooperative work by epidemiologist and molecular geneticists in order to identify genetic polymorphisms involved in IBD-associated CRC. The ultimate goal is to incorporate genotypes and clinical

parameters into a predictive model that will refine the prediction of risk for CRC in colonic IBD. The challenge will be to translate these new findings into clinical practice and to determine appropriate preventive strategies in order to avoid CRC in IBD patients. The achieved knowledge may also be relevant for other inflammation-associated cancers.

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Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Colorectal cancer; Inflammation-associated cancer; Genetics; Preventive strategies

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INVITED COMMENTARY ON HOT ARTICLES

Incidence of colorectal cancer in inflammatory bowel diseases

The inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), may be complicated by colorectal cancer (CRC). In a new population-based cohort study encompassing 47 347 Danish patients with IBD, 268 patients with UC and 70 patients with CD developed CRC during 30 years of observation^[1]. The authors concluded that the overall risk of CRC among patients with UC was comparable with that of the general

population [relative risk (RR), 1.07; 95% confidence interval (CI): 0.95-1.21]. The observed risk estimate persisted even after exclusion of patients with hemorrhagic proctitis (RR, 1.04; 95% CI: 0.91-1.18). When adjusted for age at diagnosis and duration of UC, a decreased risk of CRC among UC patients diagnosed in 1999-2008 was observed compared to UC patients diagnosed in 1989-1998 (RR, 0.59; 95% CI: 0.39-0.90). However, patients diagnosed with UC during childhood or adolescents (age 0-19 years; RR, 43.8; 95% CI: 27.2-70.7, age 20-39 years; RR, 2.65; 95% CI: 1.97-3.56) may be at increased risk as well as those with long duration of disease. Thirteen years after diagnosis, the CRC risk was significantly increased over background, and with longer follow-up the risk remained 50% above the risk in non-IBD individuals. The risk of CRC in patients with CD was similar to that of the non-IBD population. RR for CRC was 0.85 (95% CI: 0.67-1.07) among patients with CD and 0.80 (95% CI: 0.43-1.49) among patients with colonic CD.

Risk of CRC in IBD has been assessed previously in a Swedish population-based study^[2]. In agreement with the present study there was a trend towards lowered risk with shorter observation period^[2]. During 198 227 person-years follow-up for 7607 IBD cases, 188 cases of CRC were observed from 1954 to 2004^[2]. After adjusting for sex, age, duration of disease, type and extent of IBD, the authors found a decline in relative risk from a 5-fold increased risk in the 1960s to a doubled risk of CRC in the time-period 2000-2004 (*P* for trend 0.006). The overall risk of CRC among the patients with IBD, UC and CD colitis was found to be increased compared with the general population (standardized incidence ratio 2.3; 95% CI: 2.0-2.6, 2.7; 95% CI: 2.3-3.2, and 2.1; 95% CI: 1.2-3.4, respectively). The CRC risk among patients with colonic CD has also been estimated in hospital-based or community-based case-control studies^[3-5] and the overall relative risk estimate over the past 30 years was found to be 4.5 (range: 1.3-14.9) in a meta-analysis, with declining risk estimate over the past 30 years^[3]. On the other hand, a recent study by Herrington *et al*^[6] which was also published in *Gastroenterology* assessed time changes in risk of CRC within the Kaiser Permanente Medical Care Program, a community-based health care delivery system, from 1998 to 2010. The authors identified 29 and 53 CRC cases among CD and UC patients, respectively, corresponding to an incidence of CRC in patients with IBD which was 60% higher than in the general population. Furthermore, the incidence was found to be essentially constant over time. The study design may have some drawbacks such as missing detailed data on the study participants, selection bias, and too short observation period to detect the impact of optimizing IBD management on CRC risks which were discussed in the Editorials^[7].

There is general agreement that patients with UC diagnosed at a young age, with primary sclerosing cholangitis, and with long disease duration are at increased risk of CRC^[8-10]. Most cancers arise in extensive colitis and pancolitis and there is little or no increased risk associated

with proctitis while left sided colitis is associated with an intermediate cancer risk^[11,12]. The Danish study found no risk associated with having UC even after excluding cases with proctitis compared to the general population^[1].

According to the British Society of Gastroenterology (BSG) "It is now widely accepted that patients with ulcerative colitis have a similar risk to those with Crohn's colitis for a similar extent and duration of colonic involvement"^[13]. The Danish population-based study covered 30 years follow-up of all UC and CD patients and is by far the largest study to date on IBD and CRC risk^[1]. This study found no risk of CRC among patients with colonic CD compared to the general population which the authors speculated could be due to the medical treatment and follow-up leading to control of the intestinal inflammation^[1].

Surveillance colonoscopy is considered to be the gold standard in diagnosing early dysplastic alterations. The recent years new and emerging endoscopic imaging techniques have improved neoplasia detection rate^[14]. The existing recommendation is, however, based on evidence level IV: Evidence obtained from expert committee reports, opinions or clinical experiences of respected authorities^[13]. The study by Jess and colleagues constitutes a basis for future evidence-based guidelines.

Understanding the mechanism(s) of CRC in IBD

The relationship between inflammation and cancer has been well established in the gastro-intestinal system. Colitis-associated cancer has been investigated in mouse models^[15-17]. These studies have highlighted the role of toll-like receptors (TLR) and tumour necrosis factor- α (TNF- α) in the activation of nuclear factor κ B (NF κ B), which induces transcription of genes involved in tumorigenesis, including COX-2^[15-17]. Defect signalling via p53 may be an early event in the progression of colitis-induced dysplasia to cancer^[18]. Without p53-induced apoptosis, aberrant cells are not eliminated and cancer may ensue^[18]. Probiotic bacteria may prevent carcinogenesis in mouse models of cancer by producing conjugated linoleic acid which activates PPAR γ , inhibits COX-2 and induces apoptosis^[19,20].

Current strategies to identify genetic predictors of CRC in IBD

It is increasingly recognized that CRC consists of many entities having similar phenotypic appearance. This heterogeneity may at least in part be due to differences in genetic susceptibility which may act in combination with various environmental factors such as diet and intestinal microbes. Patients with IBD and CRC constitute 1%-2% of all cases of CRC^[21]. As a group, patients with IBD-associated CRC are characterized by intestinal inflammation and may thus represent a model with a relatively homogeneous mechanism for developing CRC. Indeed, distinct characteristics have been found for IBD-associated CRC. For example, a Norwegian study found that cancer was more often widespread than localized at diag-

nosis, age at diagnosis of CRC was lower, and prognosis poorer in IBD-associated CRC compared to CRC in the background population^[22]. Thus, "IBD-associated cancer serves as an excellent model of inflammation-associated cancer and might provide many important clues to understanding the pathogenesis of sporadic colorectal cancer"^[23]. On the other hand, a Swedish population-based study of more than 30 000 IBD cases identified 560 CRCs cases among first-degree relatives, giving no increased risk of CRC among first-degree relatives^[24]. The authors concluded that the study did not suggest a common cause of IBD and CRC in general and the risk of CRC in IBD seemed to be the result of the disease rather than genetic predisposition^[23].

The International IBD Genetics Consortium is a network of researchers working on the genetics of IBD^[25]. This group has initiated a case-control study to determine if genetic polymorphisms associated with IBD and other immune disorders related to IBD are more prevalent in patients with colonic IBD and CRC/dysplasia than in patients with colonic IBD alone. For each case with IBD-associated CRC/dysplasia, two IBD non-cancer cases from the same centre will be included. Analyses are carried out using the Immunochip, an Illumina Infinium genotyping chip containing 196 524 polymorphisms (718 small insertion deletions, 195 806 SNPs). The Immunochip was initiated by the Wellcome Trust Case-Control Consortium and designed to perform deep replication of major autoimmune and inflammatory pathways^[26]. Phenotypic information includes age at IBD and CRC/dysplasia diagnosis, gender, disease location, family history of CRC/dysplasia, medication, history of hospitalizations, and smoking habits.

Another approach may be based on utilizing existing pathological samples. For example, in Denmark, individual-based registration-systems have been developed along with the introduction of information technology and since 1978 nation-wide reporting of clinical diagnosis has been implemented^[27]. Due to the introduction of DNA changes (e.g., mutations and polyploidy) during carcinogenesis, pathological samples with signs of CRC should be avoided. Samples with signs of IBD which precede CRC development, may be used for DNA extraction and assessment of genetic polymorphisms^[28-31]. Furthermore, data may be linked to other registers such as Danish National Patient Register and The Danish Prescription Database for further investigations^[32]. The validity of the diagnoses of IBD and CRC, respectively, in the Danish National Patient Register are more than 90%^[33,34]. Thereby, candidate gene analyses of genetic polymorphisms in inflammatory pathways and associations found by genome-wide association studies may be performed and sought replicated.

Future research directions to predict the risk for CRC in IBD

A major challenge is now to identify the patients who would benefit from preventive strategies. Large and well-

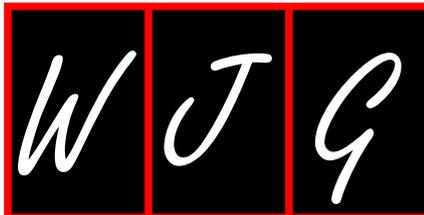
designed cohorts, such as population-based cohorts, with prospectively recorded data are required for the assessment of patients at risk. Provided that genetic susceptibility contributes to the risk of CRC in IBD, investigating genetic susceptibility in IBD patients may be particularly rewarding due to the expected relatively homogeneous biological mechanism(s) of action in this group. This may imply that associations may be identified in relatively small groups of well-characterized patients. The further advance will depend on the cooperative work of e.g., epidemiologists and molecular geneticists. The ultimate goal is to incorporate genotypes and clinical parameters into a predictive model that will refine the risk for CRC in colonic IBD. The challenge will be to translate these new findings into clinical practice and to determine appropriate preventive strategies in order to avoid CRC in IBD patients.

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Is enteroscopy necessary for diagnosis of celiac disease?

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Abstract

Celiac disease (CD) is an autoimmune inflammatory disease of the small intestine as a result of reaction to wheat protein, gluten. Exclusion of dietary gluten is the mainstay of the treatment that necessitates a precise diagnosis of the disease. Serological screening may aid in identifying patients with suspected CD, which should be confirmed by intestinal biopsy. It has been shown that duodenal biopsies are good for detection of the disease in most patients. However, there is a group of patients with positive serology and inconclusive pathology. As a result of the widespread use of serology, many patients with equivocal findings grow quickly. Unfortunately current endoscopic methods can only diagnose villous atrophy, which can be present in the later grades of disease (i.e., Marsh III). To diagnose CD correctly, going deeper in the intestine may be necessary. Enteroscopy can reveal changes in CD in the intestinal mucosa in 10%-17% of cases that have negative histology at initial workup. Invasiveness of the method limits its use. Capsule endoscopy may be a good substitute for enteroscopy. However, both techniques should be reserved for patients with suspected diagnosis of complications. This paper reviews the current literature in terms of the value of enteroscopy for diagnosis of CD.

INTRODUCTION

Celiac disease (CD) is the most common inflammatory disease of the small bowel with a prevalence of 1%-2.1% in different countries^[1]. CD was previously thought to be a pediatric malabsorption syndrome, but it is now primarily recognized as an adult disease that resembles a multisystem disorder with a range of clinical manifestations that vary according to age of presentation. The clinical presentation among adults has clearly changed over time. Typical presentation should not be expected in the adult population; fewer patients present with diarrhea or a malabsorption syndrome. Instead, silent symptoms such as anemia, osteoporosis or dyspepsia are the most common manifestations, and interestingly, patients are frequently overweight or even obese at presentation. Patients may also present with vague dyspeptic symptoms or esophageal reflux, irritable bowel syndrome, iron deficiency, or neurological disorders. In fact, over time there has been a substantial increase in prevalence of the disease, and serological testing for CD has affected the rate of diagnosis^[1-4].

Widespread use and availability of serology and awareness of the disease have led to a surge in the diagnosis of CD from a very rare disease to a common one.

In fact, screening of asymptomatic and at-risk individuals has contributed to this high prevalence^[3]. The majority of CD patients detected by screening (> 80%) are clinically silent or so called “oligosymptomatic”^[4]. Mainstay vehicles for screening are autoantibodies to tissue transglutaminase and endomysial antibody (EMA), which are highly sensitive and specific^[4-7].

Despite these effective tools, small bowel biopsy should be performed in suspected patients, and histopathological examination of the small intestine must show any of the following: villous atrophy, crypt hyperplasia and elevated intraepithelial lymphocytes (IELs). Although small bowel mucosal changes are not specific for CD, abnormal biopsy findings can confirm the diagnosis in the context of the clinical setting that includes symptoms, serology and exclusion of other disorders. The only current and effective treatment is dietary restriction of the gluten in affected individuals, which necessitates the correct diagnosis.

CD AT A GLANCE

Characteristic pathological changes of the small bowel found in CD have been classified by Marsh and further modified by Oberhuber^[8,9]. It is believed that small-bowel mucosal damage has three phases. In phase 1, the infiltrative phase, there are increased numbers of IELs. In phase 2, the hyperplastic phase, there is crypt hypertrophy. The destructive phase 3 of the disease is associated with varying degrees of villous atrophy that can be assessed during endoscopy^[8,9]. The mucosal changes associated with CD can be patchy with parts of the mucosa appearing normal and nearby parts severely affected in children and adults. This patchy villous atrophy or disease poses a significant sampling error that leads to the possibility of missing the diagnosis, which can be detrimental for a young patient with long life expectancy, because the course of untreated CD is not always benign. Delay in diagnosis in patients with severe presentation is associated with increased mortality, primarily because of malignancy. A major question is the ultimate outcome of undiagnosed, presumed silent CD? It has been suggested that there is a significantly increased risk of mortality in patients with undiagnosed CD. However, the association with increased mortality is not universal nor is the association with increased malignancy^[10-12]. Early diagnosis and treatment of CD has the potential to decrease risks of lymphoma, gastrointestinal cancer, bone disease, endocrine abnormalities, infertility and other autoimmune diseases^[13].

As a consequence of an intensified screening policy, individuals with positive antibodies but without diagnostic small-bowel mucosal villous atrophy frequently are found. The condition often is considered false-positive, but there also is evidence to suggest that such a finding is indicative of early stage CD. Randomized clinical trials on the natural history and treatment of CD patients with mild mucosal changes and positive antibodies are lacking, and there is no consensus whether these patients should be treated at all with a gluten-free diet before villous atro-

phy has developed^[2,10,13-15].

Despite recent advances in endoscopic imaging and serological tests, the accurate diagnosis of CD remains challenging. The site and number of biopsies to diagnose CD correctly have been the focus of recent research. Newly introduced technologies may carry a high yield but availability may limit their widespread use.

The gold standard of diagnosis relies on duodenal biopsy^[16]; however, the reliability of duodenal biopsy is not straightforward. Patients come to biopsy because of the result of positive serological tests, a high index of suspicion for a mucosal disease process, or because of routine duodenal biopsy at endoscopy^[17]. Biopsies from different sites of the duodenum in patients with positive celiac serology undergoing biopsy showed that none of the biopsies were considered normal. Moreover, in only 50% of patients was the degree of villous atrophy present in all sites the same; consistent with the patchy nature of the degree of villous atrophy. An interesting observation is that total villous atrophy significantly increased in a distal direction. Although more severe degrees of villous atrophy have been found distally, the diagnosis has mostly been confirmed in any location in the duodenum or jejunum^[2,9,10,15].

INTESTINAL INVOLVEMENT

CD involves the proximal small intestine including duodenum and upper jejunum and extends distally for a variable length into the ileum. Damaged small bowel mucosa heals in a distal to proximal direction. Mucosal atrophy is continuous in most patients as a diffuse proximal enteropathy, which can be seen by any means of endoscopy. Autopsy studies on CD patients have also confirmed the involvement of the duodenum and jejunum in most cases and occasional extension into the ileum. However, distribution and extent of the CD are variable^[13,18].

Dickey *et al*^[19] have evaluated terminal ileal biopsies of 30 patients with CD and control patients and found that IEL counts were significantly higher in the CD group. They concluded that increased IELs in the terminal ileum correlated with duodenal atrophy, and that this finding should alert physicians to consider CD.

Although evaluation of the extent of the bowel involvement is not possible by conventional methods, capsule endoscopy (CE) can give an estimation about whether the whole of the small bowel is affected. It has been reported that 66.6% of patients with CD had an extension of the mucosal changes beyond the proximal small intestine and 11.1% had entire small bowel involvement^[20]. According to Murray *et al*^[21], in the majority of patients, the abnormal findings seen in the CE started in the proximal duodenum and extended into the jejunum. Findings of atrophy were seen in a continuous pattern in the duodenum, but features of atrophy were seen less obviously and were patchy in the jejunum. Extensive enteropathy was seen in 59% of the patients, denoting that CD affected the small bowel more than we think.

Mucosal specimens can be obtained using radiographically guided suction capsule (Crosby capsule) biopsy, which has disadvantages such as long procedure time, high failure rate, discomfort and radiation exposure during the procedure, although it is possible to take large biopsy specimens. Perforation, intramural hematoma of the small bowel and pancreatitis are reported complications^[22]. Nowadays, Crosby capsule biopsy is not performed due to comparable efficacy of the duodenal biopsy to detect villous atrophy. Another important fact to take into account is the patchy nature of CD, which necessitates multiple biopsy approach to minimize sampling errors^[23].

There are no clear-cut recommendations for the exact number of biopsy specimens to confirm or exclude diagnosis of CD, although the American Gastroenterological Association technical review recommends 4-6 biopsies^[24]. Unfortunately, there is a gap between evidence-based data and real-life practice. A survey has shown that 63% of patients had fewer than four duodenum biopsies, which may indicate the reluctance of the endoscopists to take an adequate number of biopsies^[25].

Site of the biopsy is another object of the debate. Pais *et al*^[26] have found that duodenal biopsy specimens show some variability in terms of histological changes, and a minority of patients may have a discrepancy of more than two Marsh grades between biopsy sites. Ravelli *et al*^[15] have found no cases of normal histology and coexisting villous atrophy in the same patient. This observation was supported by another study by Thijs *et al*^[22] in which no discrepancy between jejunal and duodenal biopsies was found. Biopsies from the duodenum have been demonstrated to be useful for the diagnosis of CD and almost replaced the need for the jejunal biopsy.

Unfortunately, biopsy specimens from the duodenum harbor some problems and may not be a good place for the diagnosis of CD, given the nature of the disease and necessity of a strict diet. Not only is there more natural irregularity of the proximal duodenal mucosa, but specifically, the influence of nutrients mixed with gastric acid from the stomach and digestive fluids released into the duodenum in reaction to a meal may induce a chronic mild inflammatory response^[27,28]. This may alter the appearance of mucosal inflammation and villous architectural changes, and therefore, disqualify duodenal biopsies for diagnostic use, especially when minor architectural changes and intraepithelial lymphocytosis must be considered^[29].

All of the current endoscopic imaging techniques rely on the morphological changes of the mucosa associated with CD, which may direct the endoscopist for sampling. The value of endoscopy in the diagnosis of CD is limited to villous atrophy (Marsh grade 3). Celiac patients with villous atrophy are easily diagnosed, and most of them have positive serology, thus making this group of patients non-challenging. Histological changes in this group are so characteristic that they cannot be mistaken for other diseases. In contrast, patients with milder enteropathy,

which is the most prevalent form of the disease at present, may show increased IELs that cannot be identified under white light or even with narrow band imaging or magnification endoscopy.

Diagnostic accuracy of biopsy specimens can be improved with advanced endoscopic technologies. Magnification endoscopy with narrow band imaging is a useful tool for obtaining biopsies at diseased sites^[30]. These white light or blue-green light endoscopies are not capable of detecting increased IELs, which in turn limits us to the advanced stage of the disease with apparent atrophy and changes, but the problem is to detect the patients with subtle changes (i.e., Marsh grades 1 and 2). Confocal endomicroscopy (CEM) may aid in diagnosis in theory. However, fact is a little different from theory; CEM is good at detecting atrophy, although it cannot differentiate subgrades, and increased IELs, but falls short at detecting crypt hyperplasia, topical acriflavine use is helpful for quantification of IELs but fluorescein is not helpful. Very limited availability and safety issues on acriflavine use are the major drawbacks of CEM, and some imaging improvements should be done before its prime time use in CD^[31].

ENDOSCOPIC FEATURES OF CD

The opportunity to make a correct diagnosis of CD might, therefore, also depend on the endoscopic appearance of the small bowel mucosa. Several endoscopic markers related but not specific to CD have been identified. These endoscopic markers are useful to determine whether duodenal biopsies are indicated and possibly to target from where biopsies should be taken. Endoscopic markers of CD are as follows: a reduction or absence of duodenal folds; scalloping, which is a notched appearance of the duodenal folds; visible submucosal vasculature; a mosaic pattern, which is the micronodular or cobblestone appearance of the mucosal surface; and mucosal fissures, crevices or grooves^[17] (Figure 1). These indirect signs of villous atrophy have been helpful for predicting the presence or absence of duodenal villi and for targeting duodenal biopsies during upper endoscopy for diagnosing CD. Nevertheless, the sensitivity of these signs has been demonstrated to be variable in the different studies, and therefore, multiple endoscopic biopsies from descending duodenum and bulbar mucosa are recommended to ameliorate the diagnostic accuracy and to avoid underdiagnosis of patchy forms of CD^[32,33].

Contradictory results concerning the value of these endoscopic markers of villous atrophy have been reported. Among several studies, the overall sensitivity and specificity of endoscopic markers of CD vary from 6% to 94% and from 83% to 100%, respectively. Several possible explanations exist for the absence of endoscopic markers in patients with CD. For example, such markers might actually be absent for degrees of enteropathy milder than subtotal or total villous atrophy (e.g., partial villous atrophy) and absent in cases in which the histo-

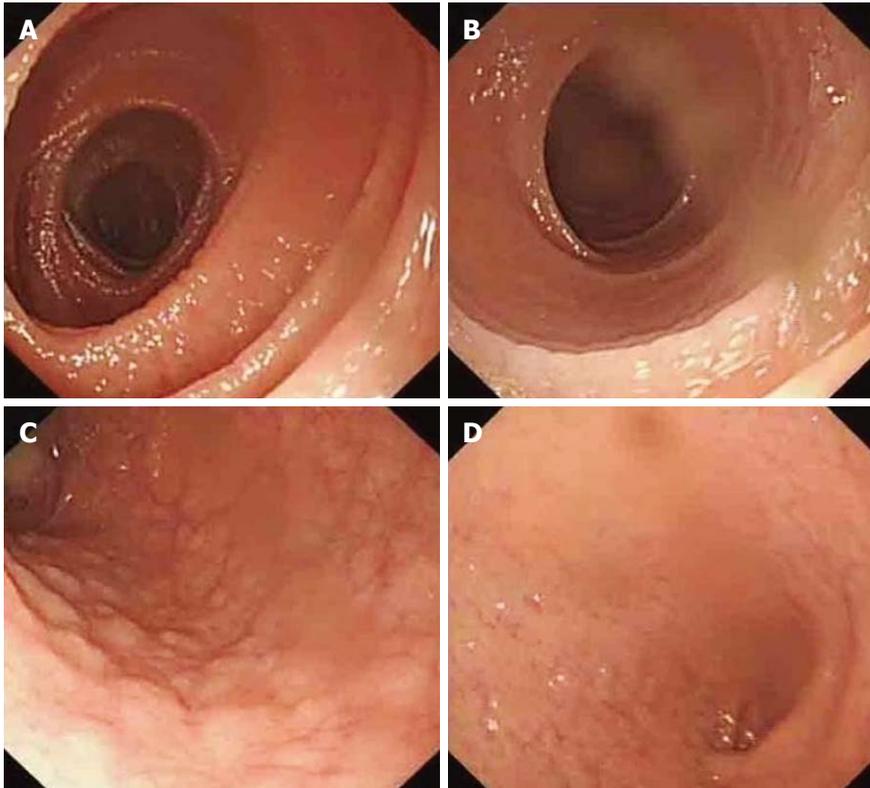


Figure 1 Proximal jejunum mucosa showing scalloping (A), reduced loss of mucosal folds (B), nodularity and mosaic appearance (C) and total mucosal atrophy (D), seen in patients with celiac disease during enteroscopy.

pathological involvement of the duodenum is patchy. In contrast, scalloping of duodenal folds has been reported in some patients who have moderate-to-severe enteropathy that is unrelated to CD; scalloping has a positive predictive value of 69% for CD and 96% for any duodenal mucosal pathology^[34]. Scalloping is not specific for CD but rather a predictor of mucosal disease as evidenced by villous atrophy, widening, and edema^[35].

It is possible to augment the villous changes by a simple procedure of underwater examination of the mucosa, which is called the water-immersion technique (WIT), which consists of the instillation of water into the duodenum after removal of air and adds only a few seconds to the examination time. WIT-assisted duodenoscopy has been demonstrated as reliable in distinguishing accurately the presence or absence of villi in the duodenal bulb and the descending duodenum^[32,33]. However, no study has specifically addressed the value of WIT during enteroscopy. We usually perform WIT to assess the villi structure in the jejunum during the enteroscopy examination of patients with diarrhea and malabsorption and find it useful for diagnosis of CD.

MAKING THE CASE FOR ENTEROSCOPY

CD is a gluten-dependent enteropathy characterized by chronic small intestinal inflammation and villous atrophy. However, CD is not the only cause of an inflammatory cell infiltrate with or without villous atrophy in duodenal

mucosa. Other causes include postviral enteritis, cow or soy milk enteritis, Crohn's disease, common variable immunodeficiency, autoimmune enteropathy, nonsteroidal anti-inflammatory drugs, giardiasis, tropical sprue, and tuberculosis. It is more likely that the normal state of the bulb mucosa is not as free of inflammation as is mucosa of the second or third part of the duodenum, nor does it have a villous/crypt ratio the same as these zones. It has been proposed that the anatomical location of the bulb makes it more vulnerable than the more distal duodenum to injury by gluten. However, similar reasoning applies also to potential injury of the bulbar mucosa by aforementioned causes and gastric acid. In addition, Brunner's glands and lymphoid nodules can give a common endoscopic finding of nodularity in the duodenal bulb, which can also distort the overlying architecture. On biopsy, lymphoid aggregates are also commonly found in the duodenal bulb of younger children. That is why some findings in the bulb may be a part of life rather than disease. Biopsy samples from the duodenal bulb may be difficult to interpret, in fact, the duodenal bulb is not considered a useful site for the diagnosis of CD, even though this site has rarely been reported to be the only one showing reliable histological changes in adults and children with CD. Taking biopsy samples more distally may decrease the likelihood of confusing histological findings^[34].

CD has many atypical manifestations, and endoscopic findings alone are not considered sensitive or specific for the diagnosis of CD. Pais *et al*^[20] examined 247 patients

to determine how many duodenal biopsy specimens were needed to diagnose CD. They concluded that only two specimens led to confirmation of CD in 90% of cases and that four descending duodenal biopsy specimens led to 100% confidence in the diagnosis. Comparison of biopsy specimens from the second, third, and fourth parts of the duodenum, the ligament of Treitz, and the proximal jejunum has shown that each site is suitable for diagnosing CD^[7]. Mucosal specimens taken from the distal duodenal or jejunal mucosa are strongly correlated, therefore, biopsy samples from the second or third part of the duodenum are considered adequate to obtain material for histological interpretation^[29]. Thus, biopsy of the other parts of the small intestine may be needed for precise diagnosis of CD, which is an indication for enteroscopy.

ENTEROSCOPY

We have long been aware that complete examination of the small bowel is crucial for evaluation of refractory disease or its complications. However, conventional endoscopy has limited value for evaluation of complications like ulcerative jejunoileitis and lymphoma that may be located deep in the small bowel, which necessitates deep enteroscopy techniques such as push enteroscopy (PE), balloon-assisted enteroscopy (BAE) and CE. Invasiveness of the enteroscopy technique limits its use. Two studies have explored the value of PE for the diagnosis of complicated CD.

Höroldt *et al*^[36] have searched the possible role of PE with jejunal biopsies. They prospectively recruited 31 patients who had symptoms suggestive of CD and positive serology, but non-diagnostic duodenal biopsies that were either normal or showed increased IELs. Enteroscopy with duodenal and additional jejunal biopsies was performed in all of the patients, who continued a normal gluten containing diet, 6-12 wk after index endoscopy. Repeat biopsies confirmed CD in five of the eight patients who were positive for EMA. Moreover, in 60% of cases, these changes were diagnostic in the jejunal biopsies only. De Vitis *et al*^[37] also studied a similar group of 23 patients, and in their group, only four patients were diagnosed with CD according to jejunal biopsies alone. According to these enteroscopy studies, CD can be further diagnosed in 10%-17% of patients with equivocal findings in the previous studies of patients who were presumed to have CD. A limitation of PE is that it evaluates a fraction of the small bowel, leaving the majority of the small bowel uninvestigated.

Cellier *et al*^[38] demonstrated that PE detected jejunal ulcerations in 62.5% of CD patients, in whom no duodenal lesions were observed. They found that PE with jejunal biopsies has diagnostic value only in patients with refractory CD but not in those with uncomplicated CD. However PE requires expertise and takes longer than standard esophagogastroduodenoscopy (EGD). Nowadays, it has mostly been replaced by BAE, which enables concise investigation of the small bowel. However, is it

worth digging deeper? BAE makes it possible to evaluate the entire small bowel with biopsy capability. After the introduction of BAE into clinical practice, it has been used to evaluate the small bowel in various diseases, with a high success rate for complete bowel examination^[39]. Unfortunately, there is no study specially addressing the value of BAE in the diagnosis of uncomplicated CD. According to a report by Hadithi *et al*^[40], who performed double balloon enteroscopy (DBE) in refractory CD, DBE had a significant diagnostic yield and revealed complications such as ulcerative jejunoileitis and lymphoma in 30% of patients. A further important result of the study was that DBE successfully ruled out T cell lymphoma in 25% of patients. Potential risks of BAE limit its use in every patient, which makes it difficult to recommend the procedure to every single CD patient, and it should be reserved for those patients with unequivocal findings or abnormal imaging results. Enteroscopy should be considered in patients with refractory CD and in those with a high clinical or serological suspicion of CD but inconclusive duodenal biopsies. Although this group of patients will always remain small, it is important to bear in mind that enteroscopy can sometimes be of value to diagnose CD^[41]. It should be emphasized that BAE is an effective way for the evaluation of the complications of CD and should be utilized early in the diagnostic algorithm.

Another way of examining the small intestine is CE, which may be a possible substitute for EGD because of its minimal invasiveness, however, its cost and limited availability make it insufficient to replace EGD. CE has an eightfold magnification capacity and therefore is able to assess the small bowel mucosa. For this reason, CE could offer an alternative in patients who are unable or unwilling to undergo endoscopic examination. CE is done without air inflation, with the round dome-shaped edge housing the optical system close to the mucosa. It allows examination of the entire small bowel and facilitates diagnosis of complications^[42]. CE studies have failed to demonstrate any correlation between clinical presentation and the length of involvement. Diagnostic yield of CE increases significantly when a CD patient is under the risk of having a complication or malignancy, such as patients with iron deficiency anemia or refractory disease. In these high probability patients, ulcerations or other positive findings can be revealed in up to half the patients^[43]. CE is not superior to the conventional EGD in the case of new diagnosis of CD patients^[44]. Another point to remember is that CE is a poor modality for examination of the duodenum due to rapid transfer of the capsule in this area. That is why, if there is limited proximal enteropathy, CE may miss the mucosal changes; even Marsh grade 3c changes can be missed. Addition of Fuji Intelligent Color Enhancement - capability or post-processing of the acquired images may aid discrimination of villous atrophy^[45]. When should we use CE for the diagnosis of uncomplicated CD? Patients who have less than four duodenal biopsies should be directed for repeat endoscopy with at least four biopsies and CE should be performed in those patients

who still have normal duodenal histology and are positive for celiac serology and HLA-DQ2 or HLA-DQ8^[46]. Similar reasoning may also apply to BAE with biopsy of the jejunum or deep intestine, and in this case, BAE with biopsy could be superior to CE in terms of biopsy and histological confirmation of the disease. Otherwise, both methods rely on white light visible morphological changes. Therefore, it should be stressed that CE is not a substitute for histological examination, however, CE can detect complications missed by routine EGD^[44]. That is why the use of both BAEs and CE to evaluate the entire small bowel at the time of initial diagnosis does not seem to be justified^[47].

CONCLUSION

Is enteroscopy needed for the diagnosis of the CD? We can answer “yes” to this question, with reserve. Based on these findings, enteroscopy examination for CD should be reserved for patients with positive serology and negative histopathology at initial EGD, and in the search for complications during follow-up. Enteroscopy cannot be recommended at the initial work-up of CD patients. CE may find a place for the diagnosis of complications of CD because of its noninvasiveness and ease of use. The main problem is to diagnose the early forms of the disease by simple examination, for which the current endoscopy methods have failed in terms of detection, because these methods successfully detect atrophy, which is the only visible sign of the disease. We believe that addition of water immersion, even during enteroscopy, is helpful, easy and incurs no cost, and should be performed in every suspected patient to minimize unnecessary biopsies.

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Diffusion-weighted imaging of biliopancreatic disorders: Correlation with conventional magnetic resonance imaging

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Abstract

Diffusion-weighted magnetic resonance imaging (DWI)

is a well established method for the evaluation of intracranial diseases, such as acute stroke. DWI for extracranial application is more difficult due to physiological motion artifacts and the heterogeneous composition of the organs. However, thanks to the newer technical development of DWI, DWI has become increasingly used over the past few years in extracranial organs including the abdomen and pelvis. Most previous studies of DWI have been limited to the evaluation of diffuse parenchymal abnormalities and focal lesions in abdominal organs, whereas there are few studies about DWI for the evaluation of the biliopancreatic tract. Although further studies are needed to determine its performance in evaluating bile duct, gallbladder and pancreas diseases, DWI has potential in the assessment of the functional information on the biliopancreatic tract concerning the status of tissue cellularity, because increased cellularity is associated with impeded diffusion, as indicated by a reduction in the apparent diffusion coefficient. The detection of malignant lesions and their differentiation from benign tumor-like lesions in the biliopancreatic tract could be improved using DWI in conjunction with findings obtained with conventional magnetic resonance cholangiopancreatography. Additionally, DWI can be useful for the assessment of the biliopancreatic tract in patients with renal impairment because contrast-enhanced computed tomography or magnetic resonance scans should be avoided in these patients.

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Key words: Magnetic resonance imaging; Diffusion-weighted imaging; Biliary tract; Gallbladder; Pancreas

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INTRODUCTION

Diffusion-weighted magnetic resonance imaging (DWI) provides information on the random (Brownian) motion of water molecules in the body. It is well established that DWI is a useful tool for the evaluation of intracranial diseases, such as acute stroke. The development of phased-array surface coils, high-gradient amplitudes, and rapid imaging techniques such as echo planar imaging (EPI) and parallel imaging have been instrumental in allowing the extracranial application of DWI^[1-3].

The degree of restriction to water diffusion in biological tissues is directly proportional to tissue cellularity and the integrity of cell membranes. In tissues with a high cell density and associated with many intact cell membranes, such as malignant tumors, the motion of water molecules is more restricted than in less cellular tissue. The degree of water motion is found to be proportional to the degree of signal attenuation in DWI. Thus, more cellular solid tumors show relatively higher signal intensities and exhibit lower apparent diffusion coefficient (ADC, expressed in mm^2/s) values on DWI using two or more b values than do less cellular tissues. Recently, this utility of DWI has been expanded to examination of abdominal organs; the ADC values of malignant masses are significantly lower than those of benign masses in the liver, pancreas, kidney, and prostate, although there is a small degree of overlap^[4-9]. Most previous studies on DWI have been limited to the evaluation of diffuse parenchymal abnormalities and focal lesions in abdominal organs, whereas there are few studies about DWI for the evaluation of the biliopancreatic tract^[10].

To date, contrast-enhanced magnetic resonance imaging (MRI) combined with magnetic resonance cholangiopancreatography (MRCP) has been found to be accurate for diagnosis of biliopancreatic diseases. The role of DWI for the evaluation of biliopancreatic diseases is not yet well established. However, because DWI yields qualitative and quantitative information reflecting cell membrane integrity and tissue cellularity, DWI can be used to differentiate normal and abnormal structures of tissues better, and thus may help in the characterization of various abnormalities in the biliopancreatic tract when added to conventional MRI.

In this article, we briefly review the basic concepts for the biological basis of DWI and its technical considerations. Additionally, we illustrate clinical applications of DWI for the following: evaluation of the biliopancreatic tract, including stone-related complications such as

acute cholangitis, hepatic abscesses, and acute gallstone pancreatitis; characterization and diagnosis of gallbladder lesions, including cholecystitis, gallbladder empyema, and gallbladder carcinoma; characterization of intrahepatic biliary lesions and diagnosis of malignant lesions in the intrahepatic bile ducts; and characterization of extrahepatic biliary lesions and diagnosis of malignant lesions in the extrahepatic bile ducts, including extrahepatic cholangiocarcinoma, pancreatic carcinoma, and focal pancreatitis. We also describe pitfalls of DWI misinterpretation.

BASIC CONCEPTS

In biological tissue, the diffusion of water molecules is impeded or restricted by natural barriers, such as cell membranes, large protein molecules, and tissue cellularity. Pathological conditions, such as tumors, cytotoxic edema, abscesses, and fibrosis, in which the physical nature of the intracellular and extracellular spaces changes, result in increased restriction of the diffusion of water molecules. In tissues of low cellularity or where the cellular membranes have been disrupted, the diffusion of water molecules is relatively free or less restricted (Figure 1)^[1-3,8,11].

DWI is typically obtained using an ultrafast T2-weighted single-shot spin-echo EPI sequence by applying a symmetric pair diffusion-sensitizing gradient, known as the Stejskal-Tanner sequence. It provides information on the random (Brownian) motion of water molecules in tissues between the first dephasing (diffusion-sensitizing) gradient and the second rephasing gradient on either side of the 180° refocusing pulse. Static molecules acquire phase information from the first diffusion-sensitizing gradient, but information will be cancelled out by the second gradient. As a result, signal intensity is preserved, with the exception of T2 decay. In contrast, less restricted water molecules move a considerable distance between the first dephasing (diffusion-sensitizing) and second rephasing gradients. The moving water molecules are not entirely rephased, resulting in reduction of the overall T2 signal intensity (Figure 2)^[1-3].

The sensitivity of a DWI sequence to water diffusion can be altered by changing the parameter known as the b value, which represents the diffusion factor (measured in s/mm^2) and the strength of the diffusion gradients. DWI is obtained with at least two different b values. DWI using lower b values ($50\text{-}100 \text{ s}/\text{mm}^2$) is sensitive only to fast motion of water molecules. On DWI using lower b values, water molecules in vessels are depicted as dark blood flow (black-blood images). DWI using higher b values ($> 500 \text{ s}/\text{mm}^2$) is sensitive to both fast and slow motion of water molecules. Water movements in highly cellular tissue are restricted and retain their signals even at higher b values. The images obtained at different b values allow the quantification of the ADC of tissues, which is usually displayed as a parametric (ADC) map. The mean or median ADC value can be measured by drawing regions of interest (ROIs) on the ADC map. Tissues with restricted

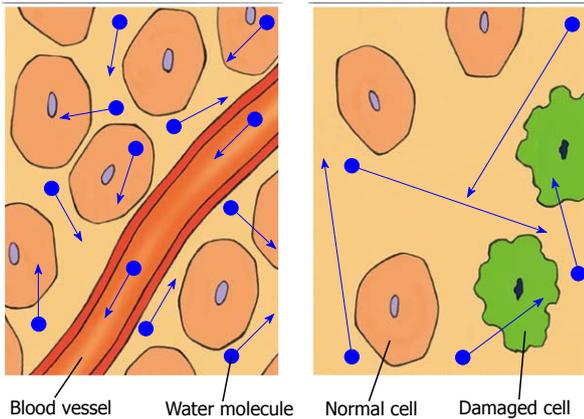


Figure 1 Diffusion of water molecules. Highly cellular tissues with intact cell membrane restrict the movement of water molecules within intravascular, intracellular, and extracellular space. In contrast, relatively less cellular tissues or damaged cells with defective cellular membrane increase extracellular space, which allow greater water molecule movement.

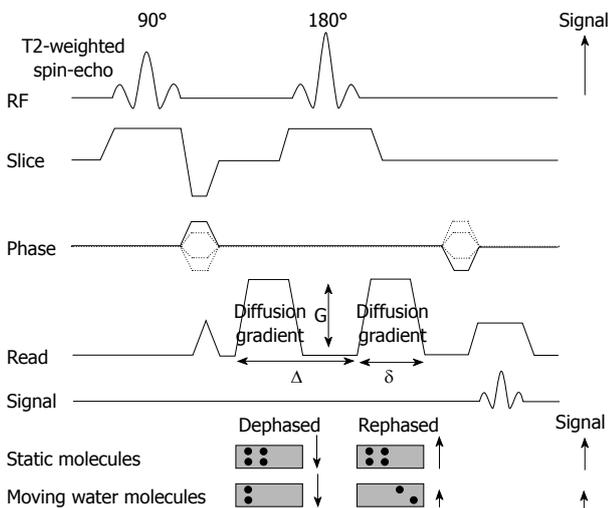


Figure 2 Diagram of diffusion-weighted sequence. DWI is based on T2-weighted spin-echo sequencing with application of two equal gradient pulses (a dephasing gradient and a rephasing gradient) on each side of the 180° radiofrequency pulse. Static molecules are dephased by the first diffusion gradient and rephased perfectly by the second diffusion gradient; therefore measured high signal intensity is preserved. In contrast, moving molecules undergo dephasing but are not entirely rephased by the second gradient because of their motion, thereby resulting in signal loss. DWI: Diffusion-weighted magnetic resonance imaging; RF: Radiofrequency.

diffusion and high cellular density show low ADC values, whereas tissues with less cellular density show higher ADC values^[1,2].

TECHNICAL CONSIDERATIONS

The signal intensity on DWI is dependent on diffusion of water molecules and T2 relaxation time. Thus, an area with a very long T2 relaxation time (e.g., bile in the biliary tract) maintains high signals on high-b-value DWI, and can be mistaken for restricted diffusion. This phenomenon is known as the “T2 shine-through” effect (Figure 3). The T2 shine-through effect inevitably

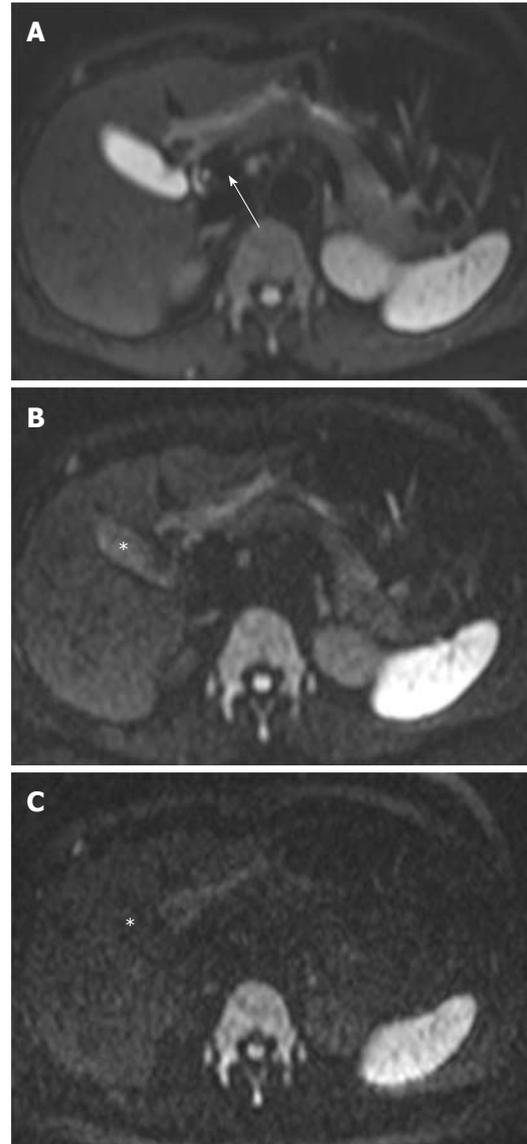


Figure 3 DWI of the normal liver, pancreas, and biliary tract. A: DWI at $b = 50 \text{ s/mm}^2$ shows that the liver is hypointense compared to the kidney and spleen, and isointense compared to the pancreas; there is a signal void within the portal vein (arrow). DWI using low b values results in decreasing signals of fast motion of water molecules, such as that occurring within vessels. Such images are referred to as black-blood images; B: DWI at $b = 500 \text{ s/mm}^2$ shows that the signal intensity of bile is decreased (asterisk) and the wall of the gallbladder is not identified. The liver is isointense compared to the pancreas; C: DWI at $b = 1000 \text{ s/mm}^2$ shows a significant reduction in the signal intensity of bile in the gallbladder (asterisk). DWI: Diffusion-weighted magnetic resonance imaging.

confounds DWI images, and the ADC map eliminates this effect. In clinical practice, higher-b-value DWI (usually $800\text{-}1000 \text{ s/mm}^2$) results in a reduction of the signal from moving protons in the bile ducts, cyst, vessels, and fluid in the bowel. This leads to a reduction in the T2 shine-through effect, resulting in increased contrast between lesions and visceral organs such as the liver or gallbladder. However, the tradeoffs of higher-b-value DWI are a lower signal-to-noise ratio, the possibility of ADC error, and increased image distortion due to the longer echo time required^[1,2,8,12].

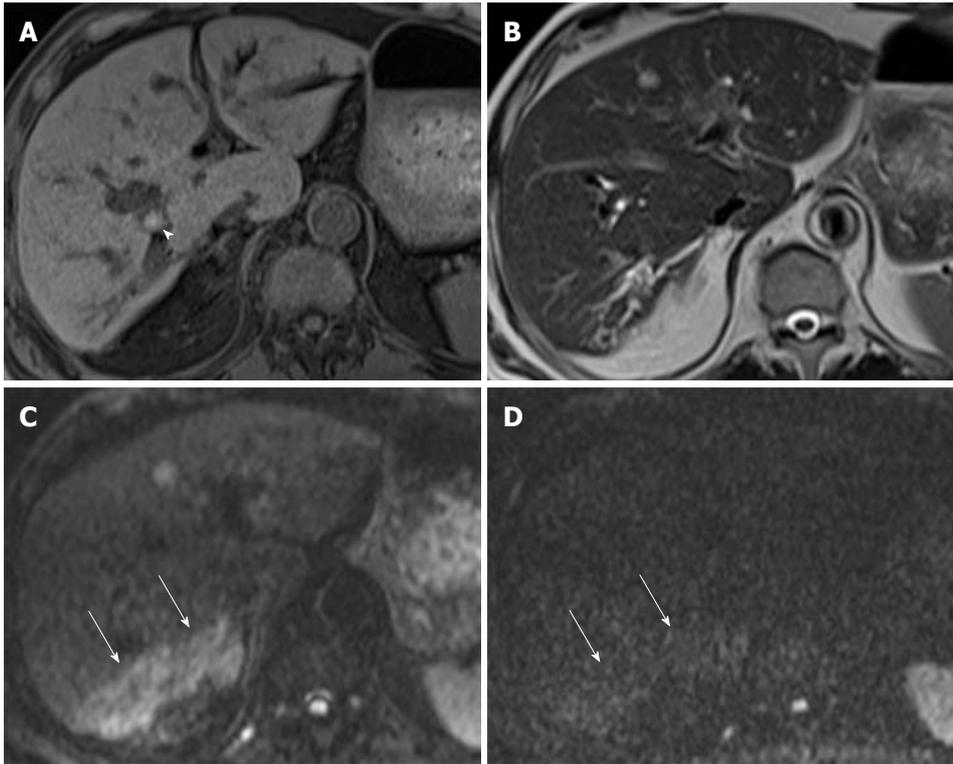


Figure 4 Parenchymal changes in acute cholangitis in a 76-year-old man. A: Axial fat-saturated T1-weighted image shows a hyperintense stone (arrowhead) with upstream bile duct dilation; B: Axial T2-weighted rapid acquisition relaxation enhancement image shows mild bile duct dilatation in the right lobe of the liver, but no definite area of increased parenchymal signal intensity; C: DWI at $b = 50 \text{ s/mm}^2$ shows wedge-shaped areas of increased parenchymal signal intensity in segment 6 (arrows). Parenchymal changes are more conspicuous on black-blood images than on routine T2-weighted images; D: DWI at $b = 800 \text{ s/mm}^2$ shows that most areas of increased parenchymal signal intensity usually return to isointensity (arrows). Such a finding can be a clue for differentiating parenchymal changes due to cholangitis from abscesses. DWI: Diffusion-weighted magnetic resonance imaging.

DWI performed during free breathing can lead to substantial signal loss. Thus, breath-hold or respiration-triggered DWI would be necessary to prevent signal loss as a result of respiratory movement. Breath-hold DWI is generally used because of its short acquisition time. However, only a limited number of image sections of relatively large section thicknesses can be acquired. Moreover, because this technique is usually performed using a single-shot EPI technique, the signal-to-noise ratio and lesion conspicuity is reduced. In contrast, respiration-triggered DWI provides higher signal-to-noise and contrast-to-noise ratios, although its acquisition time is longer than that of breath-hold DWI^[13,14].

Fat suppression is necessary to increase the dynamic range of DWI and reduce the chemical shift artifacts that are prevalent in EPI. Inversion recovery sequence is preferred when performing DWI over a large area of the body, because it is likely to produce more uniform fat suppression^[13].

Unlike brain imaging where b values are well established, b values in other parts of the body vary widely between investigators. Thus, b values for body imaging require optimization. Biexponential signal intensity in the abdominal organs has been shown in DWI with increasing b values; the initial rapid decrease in signal intensity is noted with a small increase in b value, and is followed by a more gradual decrease of signal intensity

beyond approximately $b = 100 \text{ s/mm}^2$. ADC measurement using at least three b values adequately reflects this biexponential behavior, compared with the use of only two b values. Low b values are sensitive to capillary perfusion, which can increase the amount of perfusion contamination in ADC measurement. In contrast, high b values enable less perfusion contamination in the ADC measurement and reflect tissue sensitivity^[13].

In our institution, MRI is performed with a superconductive 1.5-T imaging unit (Magnetom Avanto; Siemens Medical Solutions, Erlangen, Germany), and a 3.0-T imaging unit (Magnetom Trio; Siemens Medical Solutions) by using a phased-array multicoil. For DWI, respiration-triggered, fat-suppressed, single-shot EPI is performed in the transverse plane with a parallel imaging (generalized autocalibrating partially parallel acquisition) acceleration factor of two. DWI is performed prior to the intravenous injection of gadolinium chelates. Pre-contrast DWI is usually preferred because gadolinium chelates may affect the ADC values by decreasing the signal intensity on these T2-sequences due to shortening of the T1 and T2 relaxation time. However, it has been reported that DWI after administration of gadolinium chelates does not appear to affect ADC values significantly^[15]. The imaging parameters of DWI in the 1.5-T unit are as follows: 5000/103 (repetition time ms/echo time ms), 90° flip angle, 4-mm section thickness, 1-mm

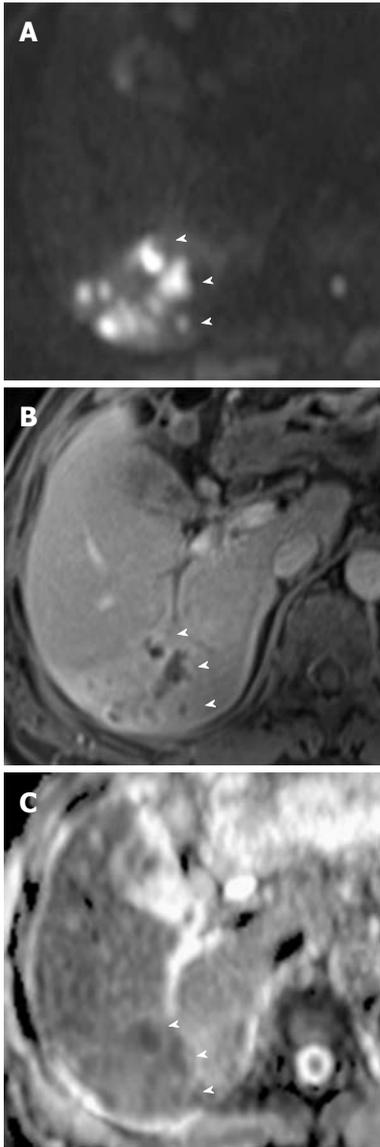


Figure 5 Liver abscesses complicating acute cholangitis in a 79-year-old man. A: DWI at $b = 1000 \text{ s/mm}^2$ shows multiple liver abscesses with high signal intensity (arrowheads); B: Multiple abscesses with peripheral rim enhancement (arrowheads) are less conspicuous on contrast-enhanced fat-saturated T1-weighted images (B) than on DWI (A); C: On an ADC map, multiple abscesses appear as low signal intensity (arrowheads) due to restriction of diffusion. DWI: Diffusion-weighted magnetic resonance imaging; ADC: Apparent diffusion coefficient.

intersection gap, 964-Hz/pixel bandwidth, 20 slices, 230-mm field of view, 192×192 matrix, and 85-s acquisition time. Imaging parameters in the 3.0-T unit are as follows: 3900/92 (repetition time ms/echo time ms), 90° flip angle, 5-mm section thickness, 0.5-mm intersection gap, 1184-Hz/pixel bandwidth, 24 slices, 230-mm field of view, 192×192 matrix, and 85-s acquisition time. Each acquisition is obtained using five different b values including low values (0 s/mm^2 and 50 s/mm^2) and high values (500 s/mm^2 , 800 s/mm^2 and 1000 s/mm^2)^[16,17]. The ADC map is generated automatically with the built-in software of the MRI unit.

The signal intensity of the normal liver on DWI is

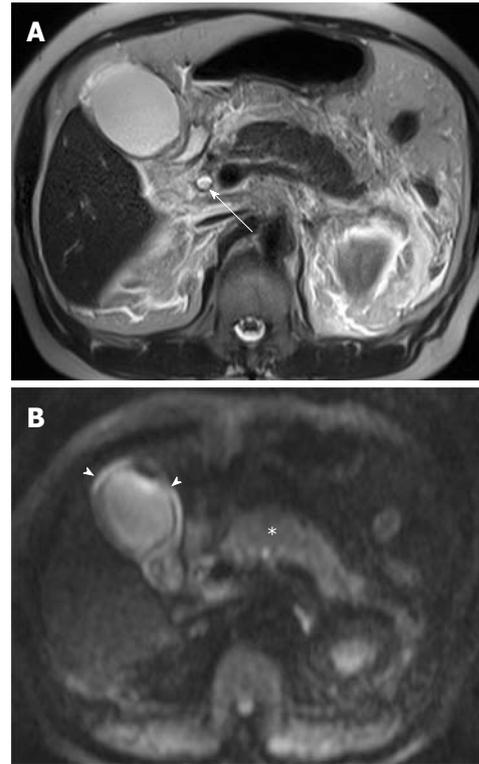


Figure 6 Acute pancreatitis and cholecystitis due to a bile duct stone in a 72-year-old woman. A: Axial T2-weighted rapid acquisition relaxation enhancement image demonstrates stones in the distal common bile duct (arrow), a distended gallbladder with pericholecystic fluid, and pancreatic edema with peripancreatic fluid, findings suggestive of cholecystitis and pancreatitis; B: DWI at $b = 800 \text{ s/mm}^2$ shows that the pancreas is slightly hyperintense (asterisk) compared to the liver, and the presence of a peripancreatic fluid collection; findings indicative of pancreatitis. A distended gallbladder with diffuse and symmetric high signal intensity in the wall (arrowheads), due to the restriction of water diffusion in the inflamed gallbladder wall, is also seen. DWI: Diffusion-weighted magnetic resonance imaging.

the same as on T2-weighted images. The liver is hypointense compared to the kidney and spleen, and isointense or hypointense compared to the pancreas (Figure 3). The reported ADC value of the normal liver ranges from $1.02 \times 10^{-3} \text{ mm}^2/\text{s}$ to $1.83 \times 10^{-3} \text{ mm}^2/\text{s}$ ^[4,18-20]. Yoshikawa *et al.*^[18] have reported that the mean ADC value of the pancreas ranges from $1.02 \times 10^{-3} \text{ mm}^2/\text{s}$ to $1.94 \times 10^{-3} \text{ mm}^2/\text{s}$ using DWI with two values (0 s/mm^2 and 600 s/mm^2). On higher DWI (usually $800\text{-}1000 \text{ s/mm}^2$), the walls of the bile duct and gallbladder are not identified (Figure 3).

CLINICAL APPLICATIONS OF DWI FOR EVALUATION OF THE BILIOPANCREATIC TRACT

DWI for evaluation of gallstone-related complications

Acute cholangitis is the most frequently encountered benign inflammatory lesion and is usually related to bile duct stones. Acute cholangitis usually manifests as a dilated bile duct, due to bile duct obstruction, mild and diffuse bile duct wall thickening, and hepatic paren-

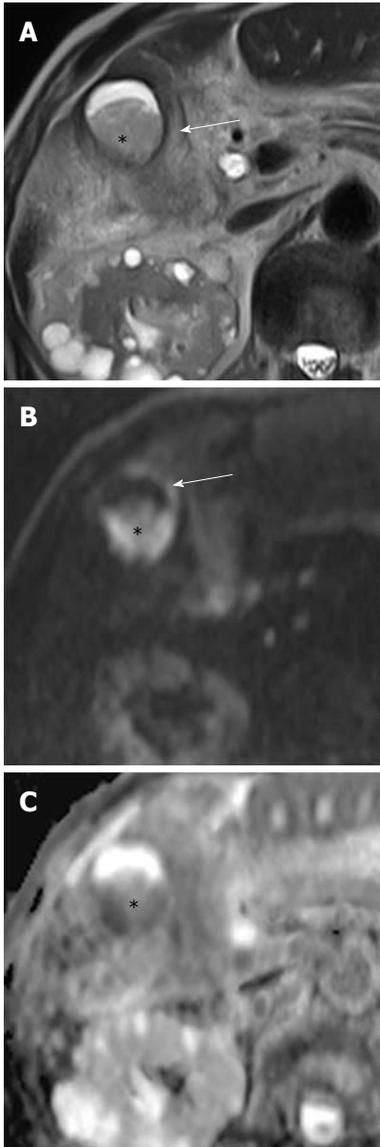


Figure 7 Gallbladder empyema in a 92-year-old man. A: Axial T2-weighted rapid acquisition relaxation enhancement image demonstrates a fluid-fluid level with low signal intensity in the dependent portion (asterisk) of an inflamed gallbladder (arrow); B: DWI at $b = 1000 \text{ s/mm}^2$ shows purulent bile with high signal intensity (asterisk), and diffuse, symmetric high signal intensity in the wall of the gallbladder (arrow); C: On an ADC map, pus in the dependent portion of the gallbladder appears with low signal intensity (asterisk) due to restriction of diffusion. Empyema was confirmed by aspiration of pus during percutaneous cholecystostomy. DWI: Diffusion-weighted magnetic resonance imaging; ADC: Apparent diffusion coefficient.

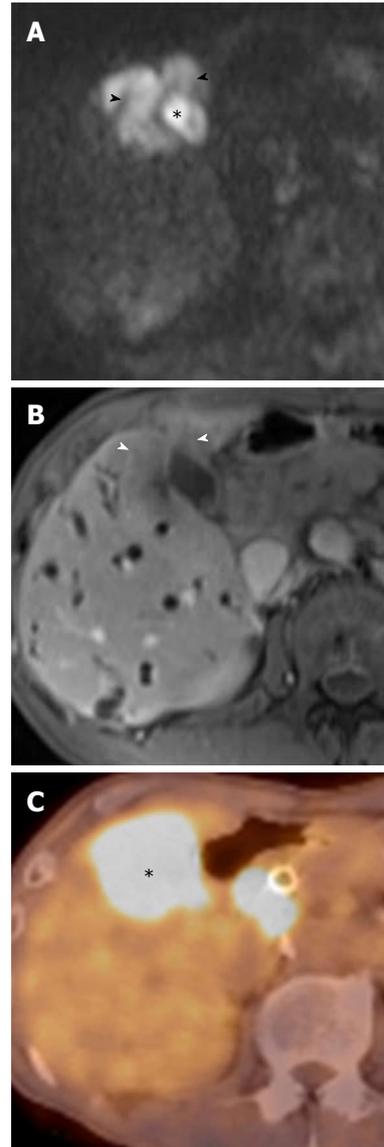


Figure 8 Gallbladder carcinoma in a 70-year-old man. A: DWI at $b = 1000 \text{ s/mm}^2$ shows high signal intensity in the mass occupying the entire gallbladder (asterisk), which invades the hepatic parenchyma adjacent to the gallbladder (arrowheads); B: Gallbladder carcinoma with direct liver invasion (arrowheads) is less conspicuous on contrast-enhanced, fat-saturated T1-weighted images than on DWI; C: Fused positron emission tomography-CT image confirms intense hypermetabolism (asterisk) in the gallbladder carcinoma with direct liver invasion adjacent to the gallbladder. DWI: Diffusion-weighted magnetic resonance imaging; CT: Computed tomography.

chymal changes on MRI. Hepatic parenchymal changes appear as patchy or wedge-shaped areas of increased parenchymal signal intensity on T2-weighted images and transient inhomogeneous parenchymal enhancement in arterial dominant phase imaging^[21,22].

In a recent study, DWI using low b values ($< 100 \text{ s/mm}^2$), giving black-blood images, detected focal hepatic lesions more clearly than routine turbo spin-echo T2-weighted images, and can potentially replace the routine turbo spin-echo T2-weighted images for lesion detection^[23]. In our experience, the increased parenchymal signal inten-

sity is more conspicuous on black-blood images than on routine T2-weighted images in patients with acute cholangitis. On DWI using high b values, areas of increased parenchymal signal intensity usually return to signal isointensity, and infrequently remain at high signal intensity (Figure 4). This may suggest the differentiation of parenchymal changes due to cholangitis from abscesses, which remain at very high signal intensity on DWI using high b values. The causes for signal changes on DWI in the setting of acute inflammation have not been studied extensively. On DWI using low b values, signal changes in cholangitis are influenced by both perfusion and dif-

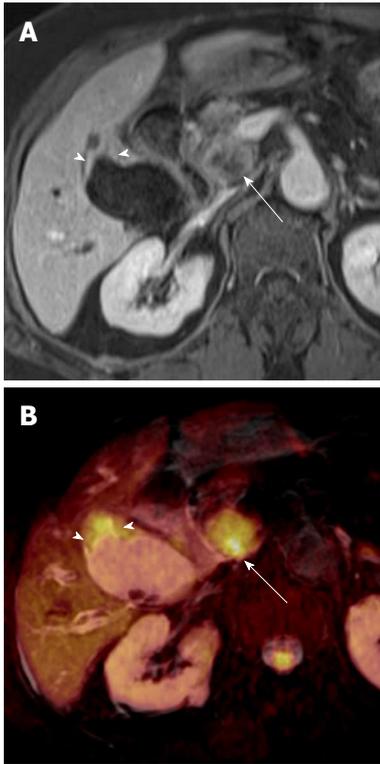


Figure 9 Gallbladder carcinoma (focal wall-thickening type) in a 77-year-old woman. A: Axial contrast-enhanced, fat-saturated, T1-weighted image shows focal wall thickening (arrowheads) in the gallbladder with a metastatic lymph node in the portocaval space (arrow); B: Fusion image of T2-weighted image and DWI at $b = 800 \text{ s/mm}^2$ shows focal, asymmetric high signal intensity in the fundal portion of the gallbladder (arrowheads) with a hyperintense metastatic lymph node in the portocaval space (arrow). DWI: Diffusion-weighted magnetic resonance imaging.

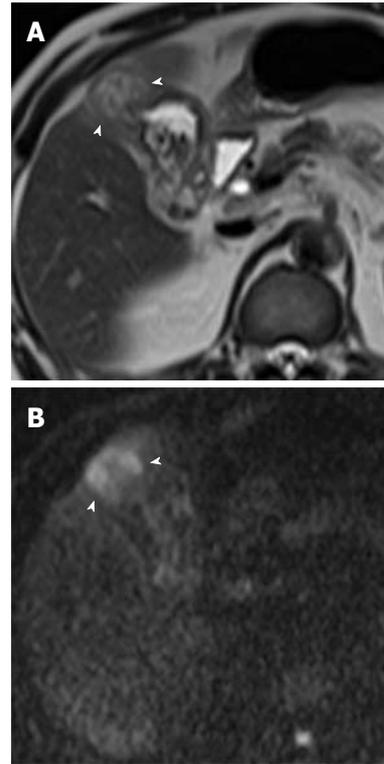


Figure 10 Xanthogranulomatous cholecystitis in a 55-year-old man. A: Axial T2-weighted rapid acquisition relaxation enhancement image shows focal wall thickening with a fundal mass (arrowheads). There is focal high signal intensity within the thickened wall of the gallbladder; a finding that is consistent with an intramural collection; B: DWI at $b = 800 \text{ s/mm}^2$ shows focal high signal intensity in the fundal portion of the gallbladder (arrowheads). Xanthogranulomatous cholecystitis was confirmed by laparoscopic cholecystectomy. DWI: Diffusion-weighted magnetic resonance imaging.

fusion; perfusion effects may be attributed to vasodilatation involving both arterioles and capillary beds, leading to increase blood flow, and diffusion effects may be explained by increases in the size and numbers of inflammatory cells, leading to restriction of water molecules. However, when going to higher b values, the perfusion effect is decreased, leading to decreased signal intensity on DWI. Increased parenchymal signal intensity on DWI may be reversible after treatment, although further studies are needed to confirm this.

Hepatic abscesses are defined as intrahepatic single or multiple collections of pus within the liver. They result from infectious cholangitis and are usually seen in patients with suppurative cholangitis. Pyogenic abscesses have variable signal intensities on T1- and T2-weighted images. However, most lesions appear hypointense on T1-weighted images and hyperintense on T2-weighted images. Peripheral rim enhancement and transient hepatic intensity differences associated with abscesses can be seen after administration of gadolinium. Abscesses can be a life-threatening emergency if not recognized and treated promptly^[24].

DWI is helpful in the early detection of abscesses and their differentiation from cystic or necrotic tumors. On DWI using high b values, abscesses appear hyperin-

tense with low ADC values (Figure 5). This appearance is explained by the dense viscous content of the abscess and the presence of cellular infiltrates within the abscess. In comparison, cystic or necrotic tumors have a greater degree of signal attenuation on higher-b-value DWI and return higher ADC values^[25].

In a recent study, there was no difference in the ability to detect acute pancreatitis between DWI and computed tomography (CT)^[26]. However, DWI can be useful to detect gallstone pancreatitis more clearly than can nonenhanced CT in the setting of contrast contraindications. Acute pancreatitis has restricted diffusion because of acute inflammation, and thus may appear as hyperintense on high-b-value DWI (Figure 6). Peripancreatic fluid usually has no restricted diffusion on high-b-value DWI, but infected fluid may have restricted diffusion. Signal changes in pancreatitis on DWI may be also reversible after treatment.

DWI for characterization and diagnosis of gallbladder lesions

Cholecystitis: Cholecystitis is defined as inflammation of the gallbladder and is usually related to the presence of gallstones. In patients clinically suspected to have acute cholecystitis, ultrasonography (US) is usually favored

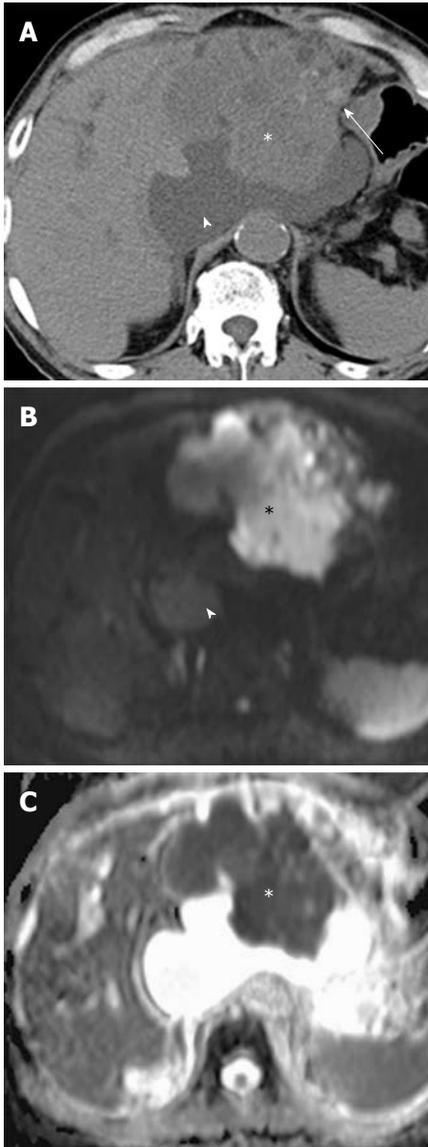


Figure 11 Intrahepatic cholangiocarcinoma (mass-forming type) in a 71-year-old man. A: Nonenhanced CT image shows intrahepatic cholangiocarcinoma in the left lobe of the liver (asterisk), stones in the dilated left bile ducts (arrow), and a fluid collection in the lesser sac (arrowhead); B: DWI at $b = 1000 \text{ s/mm}^2$ shows intrahepatic cholangiocarcinoma with high signal intensity (asterisk). Note a fluid collection (arrowhead) in the lesser sac, which appears as significant attenuation of the signal intensity reduction at a high b value; C: On the ADC map, cholangiocarcinoma appears as low signal intensity (asterisk) due to restriction of diffusion. DWI: Diffusion-weighted magnetic resonance imaging; CT: Computed tomography; ADC: Apparent diffusion coefficient.

as the initial imaging technique. CT and MRI usually provide morphological information similar to that provided by US, and can be performed initially if the clinical presentation is atypical. Increased wall enhancement of the gallbladder and increased pericholecystic hepatic parenchymal enhancement are frequent and specific CT and MRI findings of acute cholecystitis. In acute cholecystitis, the wall of the distended gallbladder is typically thickened due to inflammatory edema, inflammatory exudates, and hemorrhage^[21]. DWI can provide additional important information in cholecystitis when

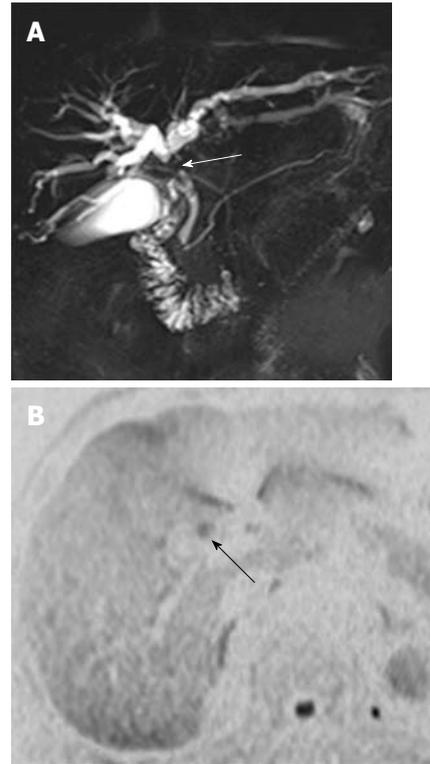


Figure 12 Cholangiocarcinoma (periductal-infiltrating type) in a 70-year-old man. A: Coronal image from thick-slab, single-shot MRCP shows marked dilatation of the intrahepatic ducts and abrupt narrowing at the confluence of the hepatic duct (arrow); B: DWI at $b = 800 \text{ s/mm}^2$ with inverted black-and-white image contrast clearly depicts the mass (arrow) at the confluence of the hepatic duct. DWI: Diffusion-weighted magnetic resonance imaging; MRCP: Magnetic resonance cholangiopancreatography.

added to conventional MRI or MRCP. On DWI using high b values, diffuse and symmetric hyperintensity in the wall of the gallbladder is seen in acute cholecystitis, because of restricted diffusion in the inflamed gallbladder wall (Figure 6). However, restricted diffusion in the gallbladder wall can be also seen in malignant lesions of the gallbladder.

Pus in the gallbladder (empyema) occurs in approximately 2%-3% of patients with acute cholecystitis. Empyema carries a high risk of sepsis and perforation. Thus, urgent drainage or surgical resection with systemic antibiotic coverage is required as soon as the diagnosis is suspected. A diagnosis of empyema may be suggested by the identification of pus as a fluid-fluid level in the dependent portion of the gallbladder on T2-weighted images. However, a fluid-fluid level of the bile in the gallbladder is not specific for empyema; this can be seen in other conditions, such as concentrated bile, sludge, or hemobilia^[21]. It has been reported that DWI is a reliable imaging technique for differentiating pyonephrosis from hydronephrosis. The pus in pyonephrosis has high viscosity and cellularity, thus leading to impeded diffusion and a low ADC value^[27]. DWI findings of gallbladder empyema on DWI may be similar to those of pyonephrosis (Figure 7). Thus, DWI may be helpful

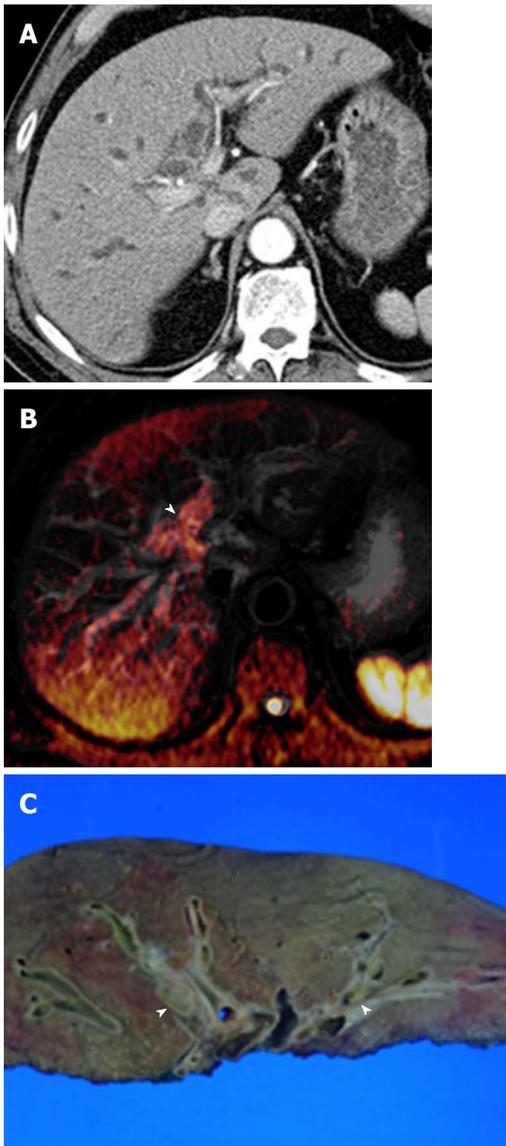


Figure 13 Hilar cholangiocarcinoma (intraductal-growing type) in a 67-year-old man. A: Contrast-enhanced CT shows mild intrahepatic duct dilatation. However, intraductal masses are not clearly depicted on CT; B: Fusion image of T2-weighted and diffusion-weighted images at $b = 800 \text{ s/mm}^2$ shows high signal intensity (arrowhead) at the first branch of the intrahepatic duct; C: Photograph of the gross specimen shows intraductal growing masses (arrowheads) in the bile duct. Histological analysis revealed biliary intraepithelial neoplasia with high grade dysplasia. CT: Computed tomography.

in differentiating pus from concentrated bile, sludge, or hemobilia in the gallbladder.

Gallbladder carcinoma: Imaging findings of focal or diffuse wall thickening or a mass replacing the gallbladder mimics the appearance of acute cholecystitis. DWI can be helpful in the diagnosis of gallbladder carcinoma, the detection of liver and lymph node metastasis, and differentiation from inflammatory conditions of the gallbladder. A hyperintense mass occupying the entire gallbladder lumen or focal and asymmetric high signal intensity in or around the gallbladder wall is more common in malignant lesions of the gallbladder (Figures 8 and 9)^[28]. In contrast, diffuse and smooth high signal intensity in

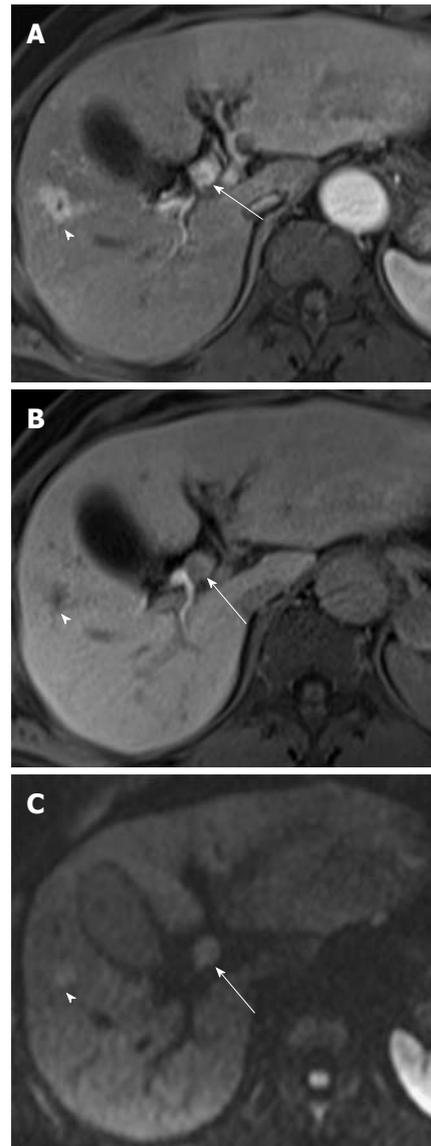


Figure 14 Hepatocellular carcinoma with tumor thrombus in bile duct in a 62-year-old man. A: Gadoteric acid-enhanced T1-weighted image obtained during the arterial phase shows a hypervascular HCC in the right lobe of the liver (arrowhead) and an intraluminal enhancing mass (arrow) in the common hepatic duct; B: On gadoteric acid-enhanced T1-weighted image obtained 20 min after injection, a small HCC (arrowhead) is hypointense relative to the surrounding liver. Contrast material filling the left intrahepatic duct is delayed owing to a partial bile duct obstruction caused by tumor thrombus in the bile duct (arrow); C: DWI at $b = 800 \text{ s/mm}^2$ shows a hyperintense small HCC (arrowhead) and tumor thrombus in the bile duct (arrow). HCC: Hepatocellular carcinoma.

the wall of the gallbladder due to acute inflammation is more common in cholecystitis on DWI using high b values. In our experience, however, focal and irregular high signal intensities in the gallbladder wall on DWI are also seen in xanthogranulomatous cholecystitis and papillary adenomatous polyps (Figure 10). Thus, differentiating gallbladder carcinoma from some adenomatous polyps or xanthogranulomatous cholecystitis may be difficult, even with DWI. Additionally, the presence of associated findings on DWI, such as direct invasion of the liver or adjacent structures, hematogenous liver metastasis, and nodal

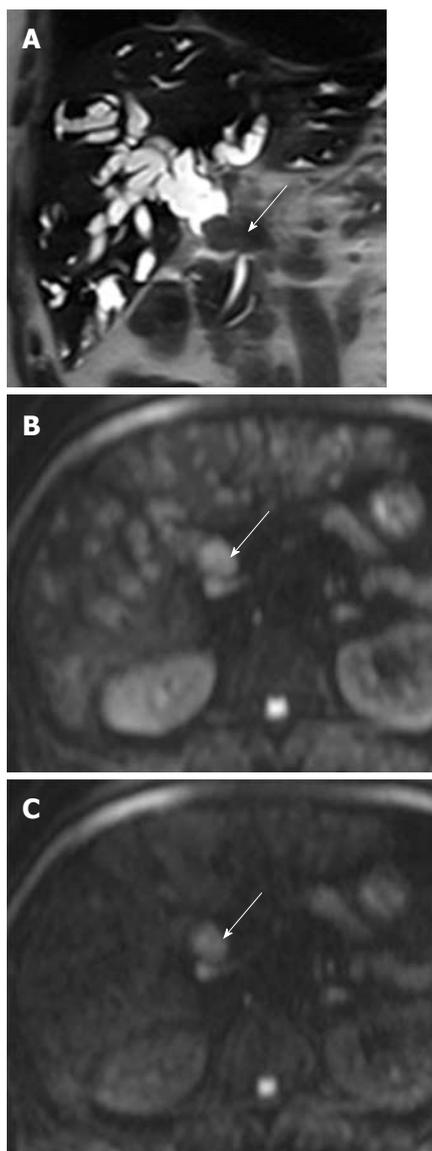


Figure 15 Extrahepatic cholangiocarcinoma in a 62-year-old man. A: Coronal T2-weighted rapid acquisition relaxation enhancement image shows a mass (arrow) in the distal common bile duct and marked upstream bile duct dilatation; B: DWI at $b = 800 \text{ s/mm}^2$ shows a mass with high signal intensity (arrow), but the signal intensity of bile is also increased; C: DWI at $b = 1000 \text{ s/mm}^2$ shows a mass with high signal intensity (arrow). Note that the signal intensity of bile in the bile duct is significantly reduced. DWI: Diffusion-weighted magnetic resonance imaging.

metastasis, favor the diagnosis of gallbladder carcinoma rather than benign gallbladder lesions (Figures 8 and 9).

DWI for characterization of intrahepatic biliary lesions and diagnosis of malignant lesions in the intrahepatic bile duct

Depending on their sites of origin, cholangiocarcinoma is classified as intrahepatic or extrahepatic. Based on its growth pattern, it is also classified as mass-forming, periductal-infiltrating, or intraductal-growing type. Intrahepatic cholangiocarcinoma originates from second-order or greater peripheral branches of the intrahepatic bile duct. The mass-forming cholangiocarcinoma is the

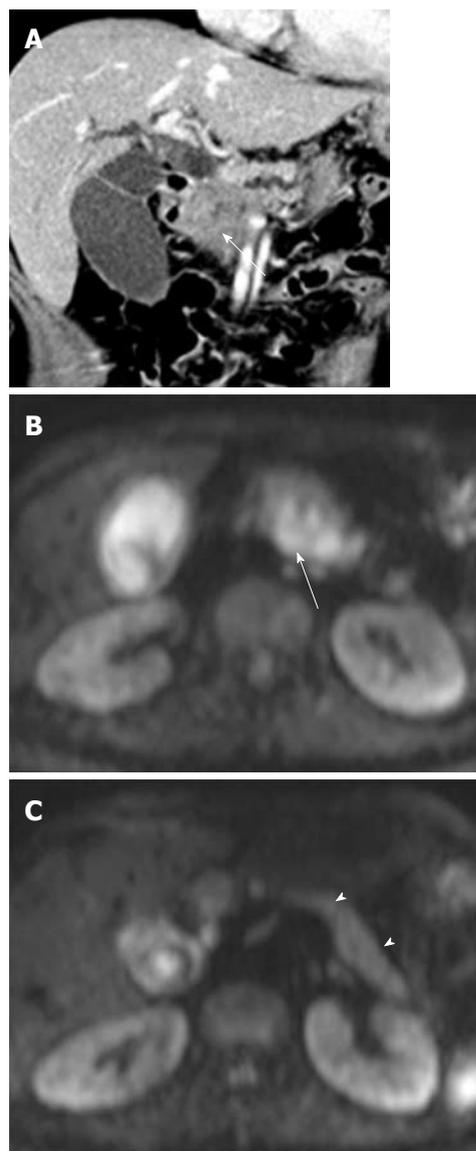


Figure 16 Pancreatic adenocarcinoma in a 50-year-old woman. A: Coronal reformatted contrast-enhanced CT image shows a double duct sign secondary to a pancreatic head cancer (arrow); B: On DWI at $b = 800 \text{ s/mm}^2$, pancreatic adenocarcinoma (arrow) shows hyperintensity; C: DWI at $b = 800 \text{ s/mm}^2$ superior to (B) shows high signal intensity of the remaining pancreas (arrowheads) due to obstructive pancreatitis. However, the remaining pancreas is less hyperintense relative to pancreatic adenocarcinoma. The ADC value of pancreatic cancer ($1.23 \pm 0.32 \text{ mm}^2/\text{s}$) is significantly lower than that of the remaining pancreas ($1.85 \pm 0.45 \text{ mm}^2/\text{s}$). DWI: Diffusion-weighted magnetic resonance imaging; CT: Computed tomography; ADC: Apparent diffusion coefficient.

most common type of intrahepatic cholangiocarcinoma, but periductal-infiltrating and intraductal-growing intrahepatic cholangiocarcinoma is also seen^[29,30].

Mass-forming intrahepatic cholangiocarcinoma appears as a single, predominantly homogeneous mass with well-circumscribed lobulated margins. Satellite nodules are frequent and vary in size. They usually appear as noncapsulated tumors, hypointense on T1-weighted images, and mild-to-moderately hyperintense on T2-weighted images, depending on the amount of fibrous tissue, necrosis, and mucin content. Dynamic-enhanced

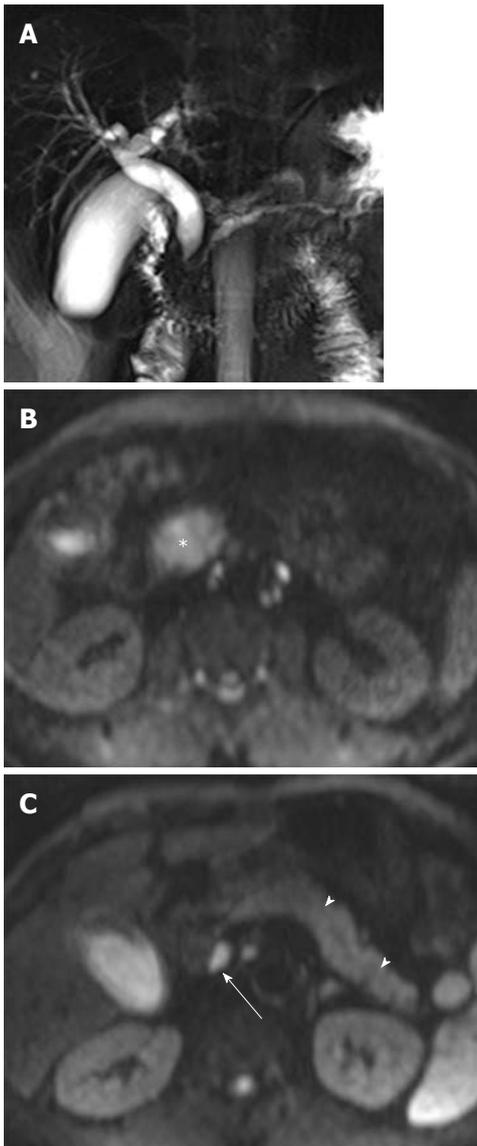


Figure 17 Focal pancreatitis in a 52-year-old man. A: Coronal image from thick-slab single-shot MRCP shows a double duct sign with tapered narrowing of pancreatic duct in the head of the pancreas; B: On DWI at $b = 800 \text{ s/mm}^2$ focal pancreatitis (asterisk) shows hyperintensity; C: DWI at $b = 800 \text{ s/mm}^2$ superior to (B) shows high signal intensity of the remaining pancreas due to obstructive pancreatitis. The remaining pancreas is slightly hypointense (arrowheads) relative to pancreatic adenocarcinoma. The ADC value of focal pancreatitis ($1.48 \pm 0.16 \text{ mm}^2/\text{s}$) is similar to that of the remaining pancreas ($1.54 \pm 0.28 \text{ mm}^2/\text{s}$). Note a hyperintense reactive lymph node (arrow). DWI: Diffusion-weighted magnetic resonance imaging; MRCP: Magnetic resonance cholangiopancreatography; ADC: Apparent diffusion coefficient.

MRI reveals minimal-to-moderate initial peripheral rim enhancement followed by progressive and concentric incomplete filling of the tumor with contrast material^[29].

In several studies, DWI is useful for detection of focal hepatic lesions, differentiation of cystic and solid hepatic lesions, and differentiation of benign and malignant focal hepatic lesions^[4,19,31]. In one investigation using two b values (0 and 500 s/mm^2), there were significant differences between the ADCs of benign and malignant focal hepatic lesions ($2.45 \pm 0.96 \times 10^{-3}$ and $1.08 \pm 0.50 \times 10^{-3} \text{ mm}^2/\text{s}$ for $b = 0$ and 500 s/mm^2 , respectively; $P < 0.001$),

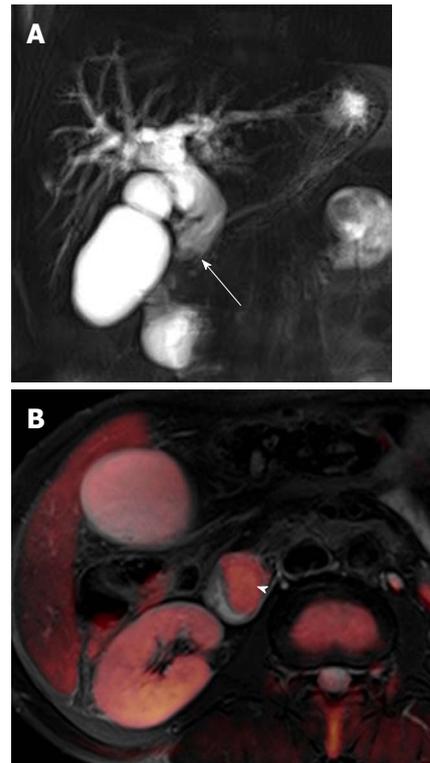


Figure 18 Ampullary carcinoma in a 63-year-old man. A: Coronal image from thick-slab single-shot MRCP shows marked bile duct dilatation with abrupt narrowing (arrow) at the distal common bile duct; B: Fusion image of T2-weighted and diffusion-weighted imaging at $b = 800 \text{ s/mm}^2$ shows an ampullary mass with hyperintensity (arrowhead). MRCP: Magnetic resonance cholangiopancreatography.

although there was some overlap^[4].

Mass-forming intrahepatic cholangiocarcinoma is usually large, achieving a diameter of up to 15 cm because early symptoms are rarely present. Thus, it is readily depicted on DWI and remains hyperintense on DWI using high b values with low ADC values; similar to values of other malignant focal hepatic lesions (Figure 11). Central hypointensity of the mass lesion may be seen on T2-weighted images and DWI using high b values, which may reflect fibrotic tissue in the central portion of the tumor. Peripheral hyperintensity can be demonstrated on DWI using high b values, corresponding to the more highly cellular region. As mentioned earlier, it is, however, difficult to distinguish mass-forming intrahepatic cholangiocarcinoma, hepatocellular carcinoma (HCC), and metastasis. Thus, DWI should be used along with conventional MRI sequences, such as dynamic-enhanced MRI, to differentiate mass-forming intrahepatic cholangiocarcinoma from other hepatic malignancies.

Cholangiocarcinoma tends to occur in atrophied or heavily stone-burdened segments. On cross-sectional images, it is difficult to diagnose cholangiocarcinoma associated with hepatolithiasis. A diagnosis of cholangiocarcinoma associated with hepatolithiasis should be considered when a mass involving or surrounding the bile duct is seen, or when focal nodular biliary wall thickening with increased enhancement is present in the

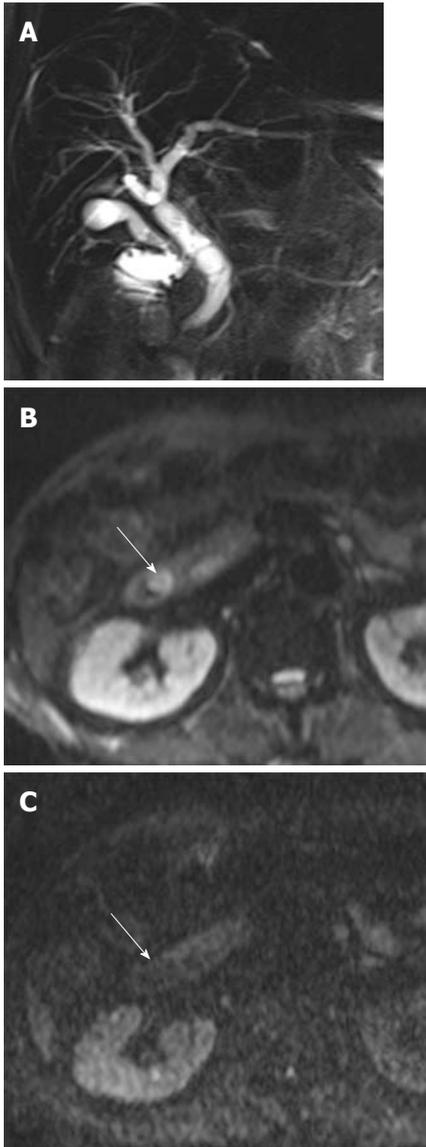


Figure 19 Papillitis due to a recently passed stone in a 75-year-old man. A: Coronal image from thick-slab single-shot MRCP shows mild bile duct dilatation; B: DWI at $b = 50 \text{ s/mm}^2$ shows a papilla with high signal intensity (arrow); C: On DWI at $b = 800 \text{ s/mm}^2$, the signal intensity of the papilla returns to the isosignal intensity (arrow). DWI: Diffusion-weighted magnetic resonance imaging; MRCP: Magnetic resonance cholangiopancreatography.

region of the stricture^[32]. DWI using high b values can be useful for the detection of cholangiocarcinoma associated with hepatolithiasis (Figure 11).

Diffuse periductal thickening along the bile duct in the periductal-infiltrating type, causing mild-to-marked bile duct dilatation, is seen in intrahepatic cholangiocarcinoma^[29]. DWI may be helpful for the detection of malignant bile duct lesions causing a variable degree of intrahepatic bile duct dilatation, as well as for differentiating between intrahepatic malignant and benign bile duct lesions (Figure 12).

Intraductal-growing cholangiocarcinoma appears as hyperintense intraluminal filling defects on DWI using high b values with low ADC values (Figure 13). However, detection of small malignant bile duct lesions is

limited on DWI because tumor detection with DWI is affected by variable causes, such as low spatial resolution of higher- b -value DWI, tumor cellularity, bowel peristalsis, and artifacts.

Invasion of the intrahepatic bile duct is infrequent in HCC, and seen in approximately 3.3%-9.0% of cases^[33]. Obstruction of the bile duct in patients with HCC is caused by invasion of the intrahepatic bile duct, blood clots from the tumor, and tumor fragments. HCC with bile duct tumor thrombi can be distinguished from hematomas in the bile duct based on the presence of mass enhancement on dynamic-enhanced CT or MRI^[34,35]. DWI can be useful for the differentiation of HCC and bile duct tumor thrombi from blood clots within the bile on the basis of identification of restricted diffusion of the mass lesion in the bile duct. They appear as hyperintense intraluminal filling defects on DWI using high b values with low ADC values (Figure 14). However, because imaging findings of HCC with bile duct thrombi are similar to those of intraductal-growing cholangiocarcinoma, conventional MRI findings of a mass outside the ductal system, hypervascularity on dynamic imaging, and underlying cirrhosis favor the diagnosis of HCC with bile duct tumor thrombi rather than intraductal cholangiocarcinoma.

DWI for characterization of extrahepatic biliary lesions and diagnosis of malignant lesions in the extrahepatic bile duct

Approximately two-thirds of extrahepatic bile duct cancer arises at the hepatic hilum (known as hilar cholangiocarcinoma or Klatskin tumor), and approximately one-third originates from the distal common bile duct. The most common pattern of tumor growth is focal infiltration of the ductal wall or the periductal-infiltrating type, resulting in focal strictures. Other tumor growth patterns include the mass-forming and intraductal-growing types^[29].

Extrahepatic bile duct carcinoma causes varying degrees of upstream bile duct dilatation. MRCP is highly accurate in identifying the presence and level of bile duct obstruction. Although differentiation between benign and malignant biliary lesions by MRCP is sometimes difficult, inspection of the bile duct lumen and wall on thin-section images helps to identify malignancies, which often cause an eccentric, abrupt change in the bile duct caliber, with irregular shouldering at the transition point from large obstructed to small-caliber decompressed ducts. Benign strictures tend to show short-segment involvement with smooth, gradual, and concentric narrowing^[36].

It has been reported that DWI is useful for the detection of extrahepatic cholangiocarcinoma and differentiation between malignant and benign biliary lesions. In a study using two b values (0 s/mm^2 and 500 s/mm^2), the sensitivity, specificity and accuracy of the detection of extrahepatic carcinoma were 94.3%, 100% and 96.4%, respectively^[10]. On DWI using high b values, a high signal intensity with a low ADC value at the transition point

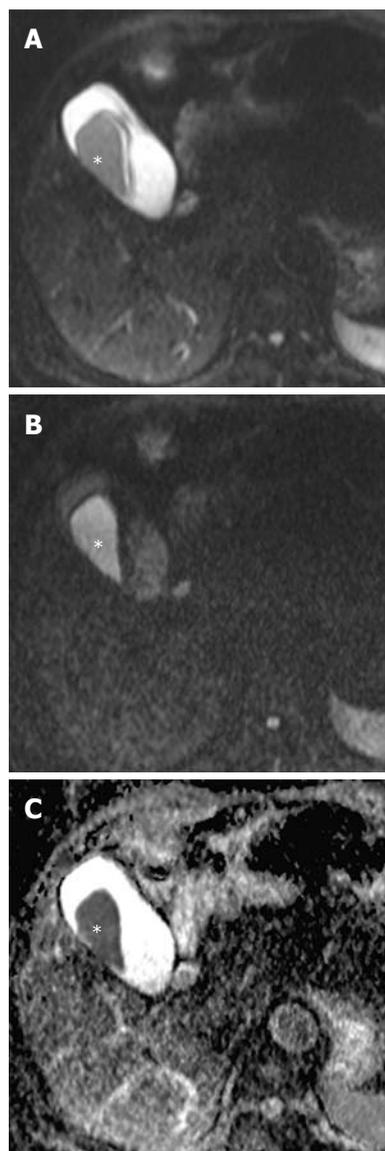


Figure 20 Hemobilia secondary to percutaneous liver biopsy in a 55-year-old man. A: DWI at $b = 50 \text{ s/mm}^2$ shows a hypointense hematoma in the gallbladder (asterisk); B: On DWI at $b = 800 \text{ s/mm}^2$, signal intensity of the hematoma changes to high signal (asterisk). C: On the ADC map, the hematoma in the gallbladder appears as low signal intensity (asterisk), which is associated with intact RBC membranes (i.e., hyperacute, acute, and early subacute hematomas). DWI: Diffusion-weighted magnetic resonance imaging; ADC: Apparent diffusion coefficient; RBC: Red blood cell.

from large obstructed to small-caliber decompressed ducts is a highly suggestive finding for malignant, rather than benign, biliary lesions because benign lesions usually show no hyper- or isointensity to the surrounding structures in the transitional area (Figure 15). Thus, this finding can be a clue for differentiating between malignant and benign biliary lesions. However, there is some degree of overlap; benign active inflammatory conditions rarely have hyperintensity, and some malignant lesions are not demonstrated on DWI using high b values^[10].

Pancreatic carcinoma, cholangiocarcinoma, or chronic pancreatitis should be considered when a bile duct stricture is limited to the distal (intrapancreatic) common

bile duct. Pancreatitis remains difficult to distinguish from pancreatic carcinoma on the basis of MRI findings, particularly in cases of acute or chronic mass-forming pancreatitis, because both appear as hypointense masses or mass-like lesions in the pancreas on T1-weighted images and are associated with ductal obstruction^[37].

In a study using b values (0 s/mm^2 and 600 s/mm^2), DWI is helpful in detecting focal pancreatic lesions and differentiating between pancreatic carcinoma and mass-forming pancreatitis. Mass-forming pancreatitis has either lower or higher ADC values than pancreatic carcinoma, but ultimately follows the ADC values of the remaining pancreatic parenchyma, whereas the focal lesion in pancreatic carcinoma is invariably lower than the remaining parenchyma (Figures 16 and 17)^[7]. In a study using a high b value (1000 s/mm^2), the sensitivity and specificity for the detection of pancreatic adenocarcinoma was 96.2% and 98.6%, respectively^[38]. ADC values in pancreatic carcinoma tend to be lower than in the normal pancreas in most studies, although there is some degree of overlap^[7,38]. Pancreatic carcinoma invokes a fibrotic response similar to desmoplastic reaction, therefore, ADC values are correlated with the degree of fibrosis; the ADC value of pancreatic carcinoma with loose fibrosis is higher than that of pancreatic carcinoma with dense fibrosis^[39]. In a recent study, ADC at lower b values ($< 500 \text{ s/mm}^2$) was higher in mass-forming pancreatitis than in pancreatic carcinoma, whereas ADC at high b values was not significantly different between mass-forming pancreatitis and pancreatic carcinoma^[17]. This was attributed to increasing perfusion effects at lower b values, which was correlated with high vascularity in chronic pancreatitis. Thus, DWI alone is suboptimal for the differentiation of pancreatic masses and mass-like lesions, and should be interpreted in conjunction with conventional MRI.

Periampullary lesions have one or more of the following MRI features: the presence of biliary dilatation at the level of the ampulla of Vater with or without a dilated pancreatic duct, bulging of the papilla, a mass lesion in or around the ampulla of Vater, and abnormal enhancement of the papilla. It is not always easy to distinguish between neoplastic and non-neoplastic conditions in or around the ampulla of Vater using conventional MRI, because of the confusing or overlapping findings^[40].

In our experience, ampullary or periampullary carcinoma displays mild-to-moderately high signal intensity on DWI using high b values with low ADC values, reflecting the high cellularity of tumors, whereas benign lesions, such as sphincter of Oddi dysfunction or papillary stenosis, show no high signal intensity in or around the ampulla of Vater (Figures 18 and 19). Thus, DWI can be helpful for detecting highly cellular malignant lesions and distinguishing between malignant and benign conditions in or around the ampulla of Vater. However, as mentioned earlier, some small malignant lesions in or around the ampulla of Vater are not identified on DWI using high b values.

Table 1 Characteristic diffusion-weighted magnetic resonance imaging features in the various biliopancreatic tract disorders

Categories	Diagnosis	Characteristic DWI features
DWI for the evaluation of gallstone-related complications	Acute cholangitis	Hyperintense parenchyma on low b values usually returns to isointense on high b values
	Hepatic abscess	Hyperintense on high b values with low ADC values
	Acute pancreatitis	Hyperintense on high b values
DWI for the characterization and diagnosis of gallbladder lesions	Cholecystitis	Diffuse and symmetric hyperintensity in the gallbladder wall on high b values Pus in the dependent portion: hyperintense on high b values with low ADC values
	Gallbladder carcinoma	Hyperintense mass occupying the entire gallbladder lumen or focal and asymmetric hyperintensity in or around the gallbladder wall on high b values
DWI for the characterization of intrahepatic biliary lesions and diagnosis of malignant lesions in the intrahepatic bile duct	Mass-forming cholangiocarcinoma	Hyperintense on high b values with low ADC values, similar to that of other malignant hepatic masses
	Periductal-infiltrative cholangiocarcinoma	Hyperintense periductal thickening along the bile duct on high b values with low ADC values
	Intraductal-growing cholangiocarcinoma	Hyperintense intraluminal filling defect on high b values with low ADC values
DWI for characterization of extrahepatic biliary lesions and diagnosis of malignant lesions in the extrahepatic bile duct	HCC with bile duct thrombi	Similar to that of intraductal-growing cholangiocarcinoma
	Extrahepatic cholangiocarcinoma	Hyperintense on high b values with low ADC values, at the transition point from large obstructed to small-caliber decompressed ducts
	Pancreatic carcinoma	Lower ADC values than those of the remaining pancreas
	Mass-forming pancreatitis	Similar ADC values to those of remaining pancreas
	Ampullary or periampullary carcinoma	Mild to moderate hyperintensity in or around ampulla of Vater on high b values with low ADC values
	Sphincter Oddi dysfunction or papillary stenosis	Isointensity in or around ampulla of vater on high b values

DWI: Diffusion-weighted magnetic resonance imaging; HCC: Hepatocellular carcinoma; ADC: Apparent diffusion coefficient.

PITFALLS

DWI is susceptible to a variety of artifacts that arise from motion, use of strong gradient pulses, and EPI technique. Physiological motion artifacts such as respiratory motion, cardiac pulsation, movement of the diaphragm, and motility of the bowel lead to ghosting images and blurring. The pulsatile motion of the heart and aorta obscure or diminish visualization of and increase ADC in the left lobe of the liver. These artifacts can be overcome using respiratory or electrocardiographic triggering. The EPI technique produces a low spatial resolution and signal-to-noise ratio. The rapid on-and-off switching of the high-intensity gradient field easily produce eddy currents, leading to geometrical distortion and image shearing artifacts that may become more pronounced with increased b values. DWI is also highly sensitive to magnetic field inhomogeneity. Susceptibility artifacts caused by field inhomogeneity are prevalent at air-tissue interfaces or around tissue-metal interfaces^[13,41].

Although restricted diffusion is generally considered to be associated with malignant lesions, some malignant lesions may not be detected on DWI. Some malignant tumors are too small for the DWI signal change to be obvious, or restriction to water diffusion is likely to be limited in malignant tumors with low cellularity, such as tumors with large cystic components. In contrast, some benign lesions sometimes exhibit restricted diffusion on imaging with high b values. The highly cellular tissue in reactive lymph nodes may show restricted diffusion (Figure 17). Thus, the role of DWI and ADC in distinguish-

ing between benign and malignant lymph nodes remains unclear^[42]. Additionally, diffusion of water in hematoma may be significantly restricted. Decreased ADC values in hemorrhage with intact red blood cell (RBC) membranes (i.e., hyperacute, acute, and early subacute hematoma) and increased ADCs after lysis of RBC membranes (i.e., "free" methemoglobin in subacute-to-chronic hematoma) have been reported (Figure 20). This may be mistaken for malignant lesions on DWI or the ADC map, causing erroneous detection or characterization of lesions^[43].

Thus, the radiologists have to be aware of potential pitfalls and limitation of the technique, and it should be kept in mind that DWI should be interpreted in conjunction with other conventional MRI.

CONCLUSION

DWI results for evaluating biliopancreatic diseases are still preliminary, and further studies are needed to determine its performance in the biliopancreatic tract. Additionally, correlation of DWI with pathological findings is required to define better the pathophysiology of various biliopancreatic diseases. Nevertheless, DWI can complement morphological information obtained by conventional MRCP by providing additional functional information concerning the alteration of tissue cellularity due to pathological processes. The detection of abnormal lesions and the differentiation of malignant from benign tumor-like lesions in the biliopancreatic tract can be improved by combined evaluation using both DWI and conventional MRI (Table 1). Moreover, DWI can

be a reasonable alternative technique for the assessment of the biliopancreatic tract in the setting of a contraindication to contrast agents such as renal insufficiency or contrast allergy.

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Efficacy of MK615 for the treatment of patients with liver disorders

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Abstract

AIM: To investigate the hepatoprotective effect of MK615, a Japanese apricot extract, in an animal model, and its clinical therapeutic effect.

METHODS: Wistar rats were administered physiological saline (4 mL/kg) or MK615 solution (4 mL/kg) for 7 d. On the sixth d, acute hepatic injury was induced by

administering a single intraperitoneal injection (*ip*) of D-galactosamine hydrochloride (D-GalN) (600 mg/kg). Plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined, and liver tissues were used for histopathological analysis. Fifty-eight patients with liver disorders [hepatitis C ($n = 40$), non-alcoholic fatty liver disease ($n = 15$), and autoimmune liver disease ($n = 3$)] were orally administered commercially available Misatol ME-containing MK615 (13 g/d) daily for 12 wk. Blood and urine were sampled immediately before and 6 wk, 12 wk, and 16 wk after the start of intake to measure various biochemical parameters. The percentage change in ALT and AST levels after 12 wk from the pre-intake baseline served as a primary endpoint.

RESULTS: D-GalN effectively induced acute hepatic injury in the rats. At 48 h after the *ip* injection of D-GalN, the plasma levels of ALT (475.6 ± 191.5 IU/L *vs* 225.3 ± 194.2 IU/L, $P < 0.05$) and AST (1253.9 ± 223.4 IU/L *vs* 621.9 ± 478.2 IU/L, $P < 0.05$) in the MK615 group were significantly lower than the control group. Scattered single cell necrosis, loss of hepatocytes, and extensive inflammatory cell infiltration were observed in hepatic tissue samples collected from the control group. However, these findings were less pronounced in the group receiving MK615. At the end of the clinical study, serum ALT and AST levels were significantly decreased compared with pre-intake baseline levels from 103.5 ± 58.8 IU/L to 71.8 ± 39.3 IU/L ($P < 0.05$) and from 93.5 ± 55.6 IU/L to 65.5 ± 34.8 IU/L ($P < 0.05$), respectively. A reduction of $\geq 30\%$ from the pre-study baseline ALT level was observed in 26 (45%) of the 58 patients, while 25 (43%) patients exhibited similar AST level reductions. The chronic hepatitis C group exhibited significant ALT and AST level reductions from 93.4 ± 51.1 IU/L to 64.6 ± 35.1 IU/L ($P < 0.05$) and from 94.2 ± 55.5 IU/L to 67.2 ± 35.6 IU/L ($P < 0.05$), respectively. A reduction of $\geq 30\%$ from the pre-study baseline ALT level was observed in 20 (50%) of the 40 patients.

ALT levels in both the combined ursodeoxycholic acid (UDCA) treatment and the UDCA uncombined groups were significantly lower after Misatol ME administration. MK615 protected hepatocytes from D-GalN-induced cytotoxicity in rats. Misatol ME decreased elevated ALT and AST levels in patients with liver disorders.

CONCLUSION: These results suggest that MK615 and Misatol ME are promising hepatoprotective agents for patients with liver disorders.

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Key words: *Prunus mume*; MK615; Liver damage; Hepatitis C; Non-alcoholic fatty liver disease

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INTRODUCTION

Japanese apricot (*Prunus mume* Sieb. et Zucc.), hereinafter referred to as *ume*, was brought to Japan from China around the eighth century. The flesh of this fruit has been used not only as food but also as medicine. *Ishinbo*, the oldest medical monograph in Japan, which was written in AD 984, indicates that both *umeboshi* (pickled *ume*) and *ubai* (smoke-dried *ume*) were used as medicines (e.g., as anti-diarrheal agents and for detoxification in food or drug poisoning). *Shokokukodenbiho*, published in 1817, also refers to the effectiveness of *ume* extracts. It is thus evident that *ume* was used extensively as a folk remedy in Japan. Syringaresinol, a lignan in *ume*, was recently shown to control infection by inhibiting the migration of *Helicobacter pylori*^[1]. MK615, an extract from Japanese apricot, contains triterpenoids such as ursolic acid (UA)^[2], oleanolic acid (OA)^[2,4], lupeol^[2,4], α -amyrin^[2], and β -sitosterol^[4]. These substances have been shown to exert various biological actions. Reports have described diverse effects, including anti-tumor activity (against tumor cell lines such as those of gastric cancer^[5], leukemia^[5], breast cancer^[4,6], hepatocellular carcinoma^[7,8], colon cancer^[9], pancreatic cancer^[10], and malignant melanoma^[11]) and immunopotentialiation in experimental animals exposed to X-rays^[4]. MK615 was previously reported to inhibit the release of high-mobility group box 1 (HMGB1) from lipopolysaccharide (LPS)-stimulated macrophage-like RAW264.7 cells and to activate the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), resulting in the

induction of heme oxygenase-1 (HO-1). MK615 was also shown to suppress the formation of inflammation-inducing cytokines [tumor necrosis factor α (TNF- α) and interleukin-6 (IL-6)] by inactivating mitogen-activated protein kinases (MAPKs) and the transcription factor nuclear factor- κ B (NF- κ B)^[3,12]. It is thus evident that *ume* extracts exert anti-inflammatory and antioxidative actions. However, the significance of these actions in the liver has not been adequately clarified.

Given the anti-inflammatory and antioxidative actions of MK615, we investigated the hepatoprotective effects of MK615. In addition, the effects of Misatol ME, a beverage containing MK615 that is approved as a health food product in Japan, were clinically evaluated in patients with liver disorders that included hepatitis C, chronic inflammation of the liver, as well as fatty liver disease, which is closely involved in oxidative stress.

MATERIALS AND METHODS

Effect of MK615 on D-galactosamine hydrochloride-induced acute hepatic injury in rats

Preparation of MK615 solution: MK615 solution was prepared from a condensed extract of *ume*. In brief, *ume* were squeezed using a press, and the *ume* juice was then heated and concentrated 20-fold^[5]. The condensed extract was neutralized using NaOH and was then heat-sterilized. The MK615 solution contained the neutral, condensed *ume* extract.

D-galactosamine hydrochloride-induced hepatic injury in rats: Seven-week-old male Wistar rats (Crj:WI) weighing 200-240 g were purchased from Charles River Laboratories Japan (Yokohama, Japan). All rats were maintained under controlled temperature and lighting conditions (12/12-h dark/light cycle), and water and standard diet were provided ad libitum in accordance with the institute's guidelines for care and use of laboratory animals in research.

Acute hepatic injury was induced by administering a single intraperitoneal (*ip*) injection of D-galactosamine hydrochloride (D-GalN) (600 mg/kg; Wako Pure Chemical Industries, Osaka, Japan). In this study, rats were divided into 3 experimental groups. In group I (the vehicle control group), rats were administered physiological saline (4 mL/kg per day) *via* gavage for 7 d and injected with D-GalN (*ip*) 2 h after the sixth oral administration of saline (6 d from the first oral administration). In group II (the MK615 group), rats received MK615 solution (4 mL/kg per day) *via* gavage for 7 d and were injected with D-GalN (*ip*) 2 h after the sixth oral administration of MK615 solution. In group III, rats were administered the neutral MK615 solution (4 mL/kg per day) *via* gavage for 7 d and were injected with saline (*ip*) 2 h after the sixth oral administration of MK615 solution. group III served as a negative experimental control without D-GalN-induced hepatic injury (Figure 1). Treatments involving oral administration by gavage were conducted between

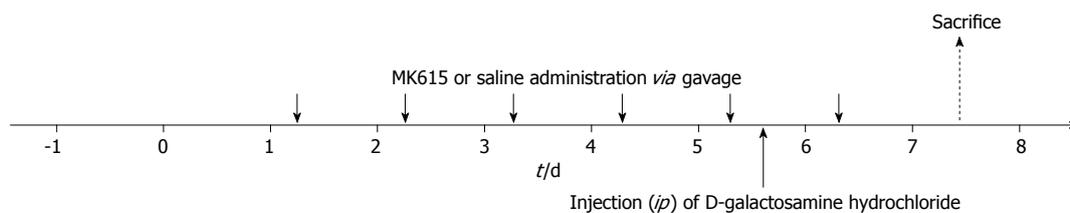


Figure 1 Experiment protocol of D-galactosamine hydrochloride-induced acute hepatic injury in rats.

9:00 and 10:00 AM and *ip* injections were administered between 11:00 AM and 12:00 noon. All rats were sacrificed by exsanguination under anesthesia 48 h after the *ip* injection of D-GalN or saline (8 d after the first oral administration). Blood samples from the abdominal aorta were immediately heparinized, and plasma samples were isolated by centrifugation. Plasma samples were frozen and stored at -80°C until used, and subsequently analyzed to determine the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Liver tissue samples were also obtained from each rat and used for histopathological analysis. The plasma levels of ALT and AST were determined using a commercially available analytical kit (Transaminase CII-Test; Wako Pure Chemical Industries).

Evaluation of the effects of MK615 in patients with liver disorder

Subjects: This study involved patients who were definitively diagnosed with a liver disorder at the Jikei University School of Medicine Hospital, the St. Marianna University School of Medicine Hospital, or the Kurihara Clinic between December 2007 and December 2009 and who met the following requirements: (1) ALT level exceeding reference limits when tested within 3 mo before the start of this study, indicating the presence of hepatopathy; (2) serum hepatitis C virus (HCV)-RNA positivity (determined by real-time polymerase chain reaction) in patients with chronic hepatitis C; and (3) presence of fatty liver confirmed by diagnostic imaging in cases of non-alcoholic fatty liver disease (NAFLD). The following patients were excluded from the study: (1) those receiving treatment for liver cirrhosis, hepatocellular carcinoma, or other malignant tumors; (2) patients receiving treatment with Stronger Neo-Minophagen C; (3) those receiving treatment with interferon (IFN); and (4) habitual drinkers (alcohol consumption, > 30 g/d) or occasional heavy drinkers. Concomitant use of drugs or any treatment with antiviral, immunomodulating, or marrow-suppressive activity was prohibited during the study period, but continued use of drugs that had been initiated before the study was permitted. No patients were heavy drinkers. The ethics committee of each participating facility approved the study protocol. Informed consent to participate in the study was obtained in writing from all patients.

Methods: In Japan, MK615 solution is commercially available as Misatol ME (AdaBio Co. Ltd., Takasaki, Japan). For the clinical study, Misatol ME was used as the

MK615 solution and was ingested orally every d (2×6.5 g packs/d) for 12 wk. Blood and urine were sampled immediately before and 6 wk, 12 wk, and 16 wk after the start of MK615 intake to measure the following parameters: white blood cell (WBC) count, differential leukocyte count, red blood cell (RBC) count, hemoglobin, hematocrit, platelet count, ALT, AST, γ -glutamyl transpeptidase (γ -GTP), alkaline phosphatase (ALP), total protein, albumin, total cholesterol, cholinesterase, and total bilirubin, as well as urinalysis parameters. The percentage change in ALT and AST levels after 12 wk of intake from the pre-intake baseline served as primary and secondary endpoints, respectively. In the analysis of these endpoints, an improvement of $\geq 50\%$ from the pre-intake baseline was regarded “markedly effective”, $\geq 30\%$ was regarded “effective”, $\leq 30\%$ as “ineffective”, and an aggravation of $\geq 30\%$ as “worsened”. The response rate was defined as the percentage of “markedly effective” plus “effective” cases.

Statistical analysis

Data are expressed as mean \pm SD. Statistical analyses were performed using Stat View for Windows Version 5.0 (SAS Institute Inc., North Carolina, United States). Differences between 2 groups were analyzed using the Mann-Whitney *U* test. Comparisons between baseline and each time point were performed using Dunnett’s test. $P < 0.05$ was considered significant.

RESULTS

The effect of MK615 on D-galactosamine hydrochloride-induced acute hepatic injury in rats

ALT and AST plasma levels in control rats were elevated 48 h after D-GalN induction, with mean values of 475.6 ± 191.5 IU/L ($n = 8$) and 1253.9 ± 223.4 IU/L ($n = 8$), respectively. In the MK615 group, the ALT and AST levels were 225.3 ± 194.2 IU/L ($n = 9$) and 621.9 ± 478.2 IU/L ($n = 9$), respectively. The levels of ALT and AST in the MK615 group rats were significantly lower than in those of the control group ($P = 0.0433$ for ALT, $P = 0.0124$ for AST by Mann-Whitney *U* test) (Figure 2A and B).

Liver tissues were obtained from both control group rats and MK615 group rats at 48 h after D-GalN injection. Scattered single cell necrosis (swollen eosinophilic hepatocytes) and loss of hepatocytes was observed in hepatic tissue samples from the control group. Extensive inflammatory cell infiltration was also noted (Figure 2C). Figure 2D shows that these features of D-GalN-induced

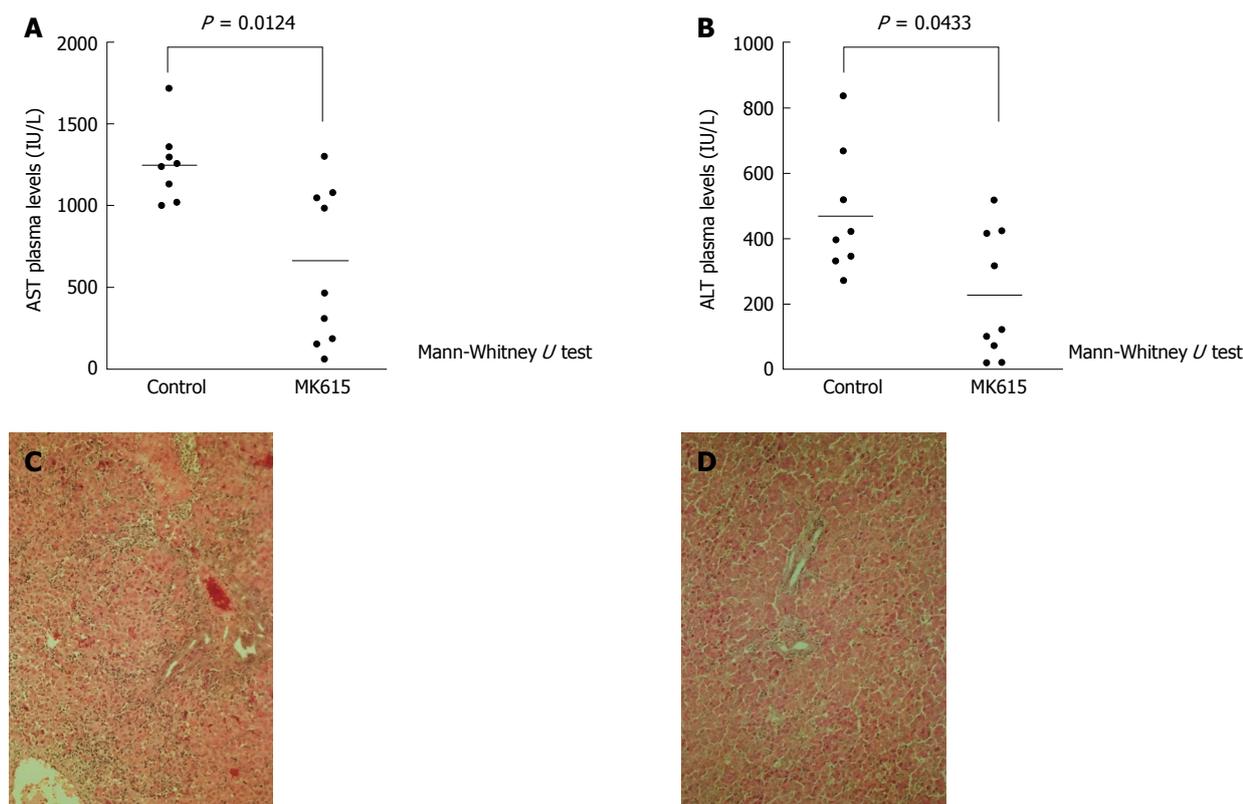


Figure 2 Effect of MK615 in D-galactosamine hydrochloride-induced acute hepatic injury in rats. A: AST plasma levels; B: ALT plasma levels; C: Control group (liver); D: MK615 group (liver). AST:Aspartate aminotransferase; ALT: Alanine aminotransferase.

Table 1 Background of patients with liver disorders

	Chronic hepatitis C	NAFLD	Autoimmune liver disease
Number	40	15	3
Gender (M/F)	25/15	14/1	1/2
Age (yr)	64.4 ± 11.3	52.5 ± 13.7	65.7 ± 4.0
HCV viral load(10 ⁶ /mL)	6.2 ± 0.8		
≥ 5log/< 5log/ND	35/3/2		
WBC count (/μL)	4153 ± 994	6800 ± 1578	3967 ± 723
RBC count (10 ⁴ /μL)	415 ± 59	490 ± 51	448 ± 48
Hemoglobin (g/dL)	13.1 ± 1.8	15.5 ± 1.1	12.5 ± 1.7
Platelet count (10 ⁴ /μL)	13.8 ± 5.7	20.3 ± 8.4	17.2 ± 5.1
AST (IU/L)	94.2 ± 55.5	84.5 ± 50.0	129.3 ± 90.5
ALT (IU/L)	93.4 ± 51.1	131.9 ± 72.5	96.7 ± 50.1
γ-GTP (IU/L)	72.9 ± 60.5	181.9 ± 197.5	120.3 ± 74.2
LDH (IU/L)	237.8 ± 54.8	228.9 ± 44.4	270 ± 44.3
ALP (IU/L)	318.1 ± 116.8	303.4 ± 106.8	391 ± 293.1
Total bilirubin (mg/dL)	0.84 ± 0.29	0.8 ± 0.44	0.67 ± 0.15
Total cholesterol (mg/dL)	162 ± 35.2	188.5 ± 49.1	174.7 ± 40.1
Total protein (g/dL)	7.5 ± 0.6	7.7 ± 0.3	7.9 ± 0.9
Albumin (g/dL)	3.9 ± 0.4	4.4 ± 0.3	3.9 ± 0.9
BUN (mg/dL)	16.2 ± 4.0	13.6 ± 3.2	15.3 ± 3.1
Creatinine (mg/dL)	0.76 ± 0.16	0.75 ± 0.1	0.62 ± 0.06

Data are expressed as the mean ± standard deviation. NAFLD: Non-alcoholic fatty liver disease; ND: Not done; M: Male; F: Female; HCV: Hepatitis C virus; WBC: White blood cell; RBC: Red blood cell; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γ-GTP: γ guanosine triphosphate; LDH: Lactate dehydrogenase; ALP: Alkaline phosphatase; BUN: Blood urea nitrogen.

hepatic injury were reduced in the treatment group re-

ceiving the MK615 solution.

The effects of MK615 in patients with liver disorders

We enrolled 58 patients in this clinical study (mean age, 61.4 ± 12.7 years; range: 29-82 years; 40 men and 18 women). The diagnosis was chronic hepatitis C in 40 patients, NAFLD in 15 patients, and autoimmune liver disease in 3 patients (2 with autoimmune hepatitis and 1 with primary sclerosing cholangitis). Table 1 lists the background variables in relation to the diseases diagnosed.

Analysis of the entire study population determined that ALT levels had decreased significantly from 103.5 ± 58.8 IU/L before the start of the study to 81.3 ± 45.7 IU/L ($P < 0.05$) at 6 wk, 71.8 ± 39.3 IU/L ($P < 0.05$) at 12 wk, and 72.3 ± 40.3 IU/L ($P < 0.05$) at 16 wk (Figure 3A). AST levels decreased significantly from 93.5 ± 55.6 IU/L before the start of the study to 77.6 ± 47.1 IU/L ($P < 0.05$) at 6 wk, 65.5 ± 34.8 IU/L ($P < 0.05$) at 12 wk, and 68.3 ± 37.8 IU/L ($P < 0.05$) at 16 wk (Figure 3B). A reduction of ≥ 30% from pre-study baseline ALT levels was observed in 26 (45%) of the 58 patients, whereas 25 (43%) patients exhibited a similar reduction in AST levels (Table 2).

When the effects of Misatol ME were analyzed in relation to the disease diagnosed, the chronic hepatitis C group exhibited significant ALT level reductions from the pre-study baseline of 93.4 ± 51.1 IU/L to 75.3 ± 46.6 IU/L ($P < 0.05$) at 6 wk, 64.6 ± 35.1 IU/L ($P < 0.05$) at 12 wk, and 64.6 ± 33.8 IU/L ($P < 0.05$) at 16 wk (Figure

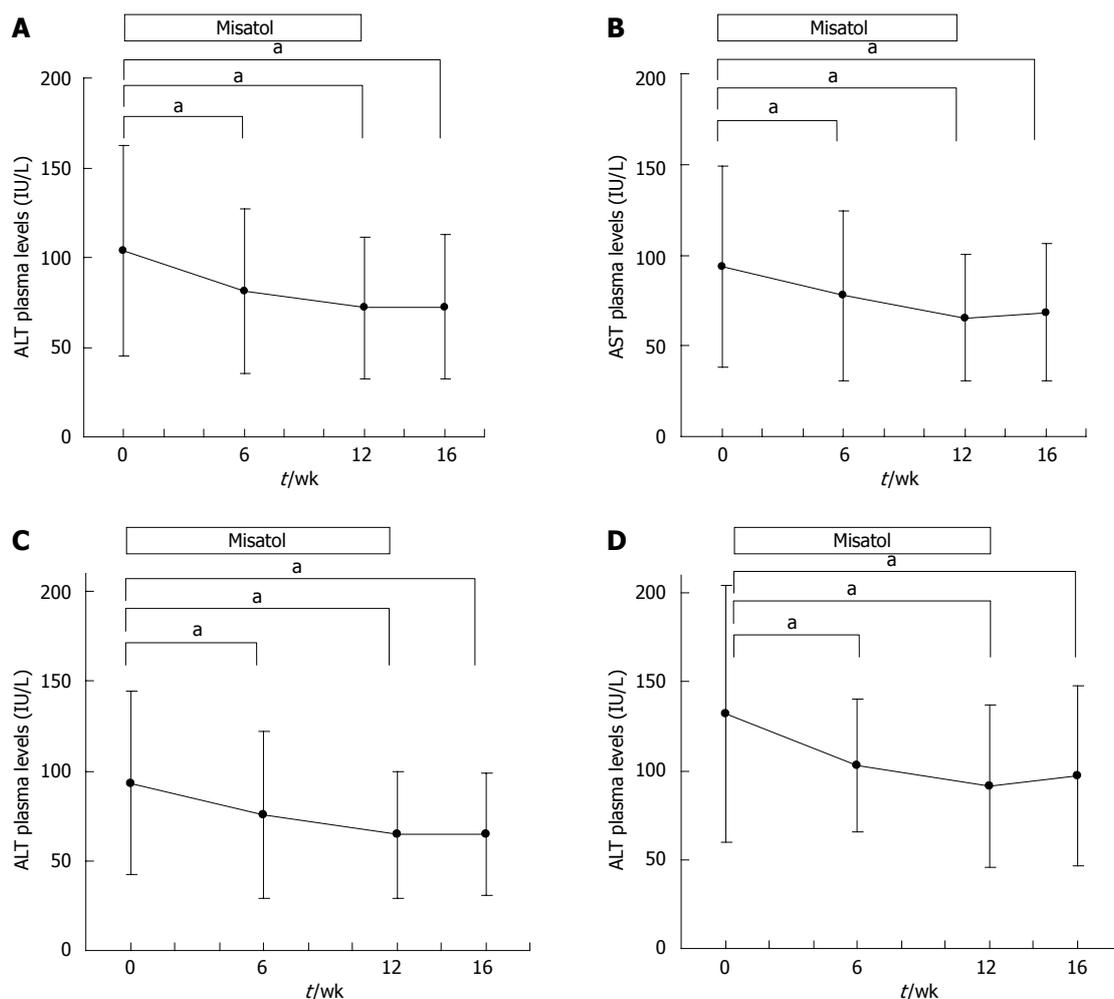


Figure 3 Effects of MK615 in patients with liver disorder, chronic hepatitis C and non-alcoholic fatty liver disease. A: Alanine aminotransferase (ALT); B: Aspartate aminotransferase (AST); C: Chronic hepatitis C group (ALT); D: Non-alcoholic fatty liver disease group (ALT). ^a*P* < 0.05 vs 0 wk group. Dunnett's test.

Table 2 Response rate of MK615 therapy in patients with liver disorder (%)		
	ALT	AST
Chronic hepatitis C	20/40 (50)	16/40 (40)
NAFLD	5/15 (33)	6/15 (40)
Autoimmune liver disease	1/3 (33)	3/3 (100)
Total	26/58 (45)	25/58 (43)

NAFLD: Non-alcoholic fatty liver disease; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

3C). This same group of patients exhibited significant AST level reductions from the pre-study baseline of 94.2 ± 55.5 IU/L to 78.8 ± 49.5 IU/L (*P* < 0.05) at 6 wk, 67.2 ± 35.6 IU/L (*P* < 0.05) at 12 wk, and 66.6 ± 33.7 IU/L (*P* < 0.05) at 16 wk. In the chronic hepatitis C group, a reduction of $\geq 30\%$ from the pre-study baseline ALT level was observed in 20 (50%) of the 40 patients, while 16 (40%) patients exhibited similar AST level reductions (Table 2). Among the patients with chronic hepatitis C, ALT data before the start of test beverage intake (24 wk before starting intake) were available for 32 patients. These patients were subdivided into combined ursode-

oxycholic acid (UDCA) treatment (*n* = 20) (Figure 4A) and UDCA uncombined (*n* = 12) groups (Figure 4B). In both the combined UDCA treatment and UDCA uncombined groups, ALT levels were significantly lower after the intake of Misatol ME compared with those before intake.

The NAFLD group exhibited significant ALT level reductions from 131.9 ± 72.5 IU/L before the start of the study to 102.8 ± 37.6 IU/L (*P* < 0.05) at 6 wk, 90.9 ± 45.6 IU/L (*P* < 0.05) at 12 wk, and 96.9 ± 50.8 IU/L (*P* < 0.05) at 16 wk (Figure 3D). This group also exhibited significant AST level reductions during the Misatol ME intake period compared with the pre-start baseline level; levels were 84.5 ± 50.0 IU/L before the start of the study, 66.7 ± 24.2 IU/L (*P* < 0.05) at 6 wk, 58.1 ± 26.0 IU/L (*P* < 0.05) at 12 wk, and 69.8 ± 41.9 IU/L (NS) at 16 wk. In the NAFLD group, a reduction of $\geq 30\%$ from the pre-study baseline ALT level was observed in 5 (33%) of the 15 patients, whereas similar AST level reductions were observed in 6 (40%) patients (Table 2).

The levels of γ -GTP in the entire study population also decreased significantly after Misatol ME intake compared with pre-intake baseline levels (data not shown).

Table 3 presents the hematological and biochemical

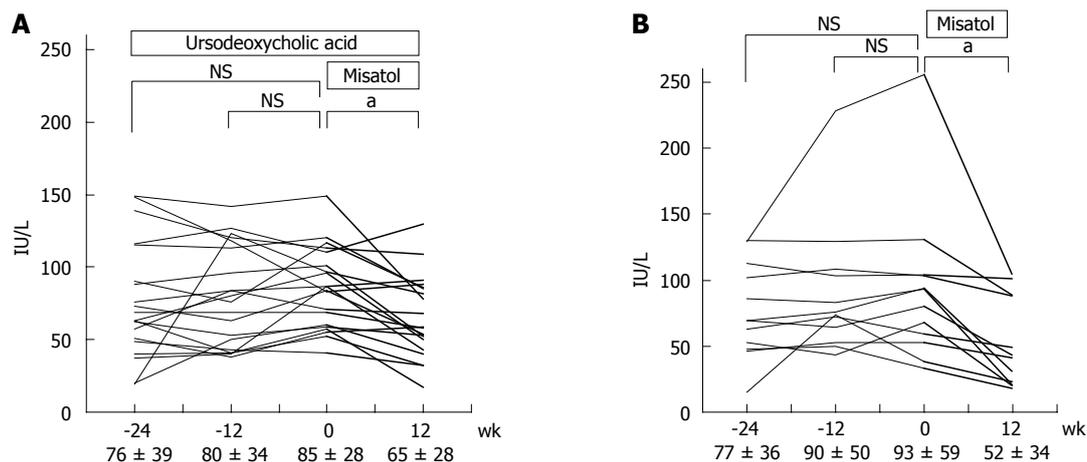


Figure 4 Effects of MK615 in patients with chronic hepatitis C (alanine aminotransferase). A: Misatol was added on ursodeoxycholic acid; B: Only Misatol was used. ^a $P < 0.05$ vs 0 wk group. Dunnett's test. NS: Not significant.

Table 3 Changes of serum level during MK615 therapy in patients with liver disorders

	Before therapy	During therapy 12 wk	P value ¹
WBC count (/ μ L)	4828 \pm 1640	4977 \pm 1855	NS
RBC count (10^4 / μ L)	436 \pm 65	435 \pm 65	NS
Hemoglobin (g/dL)	13.7 \pm 2.0	13.8 \pm 1.9	NS
Platelet count (10^3 / μ L)	15.7 \pm 7.0	15.6 \pm 6.6	NS
AST (IU/L)	94 \pm 56	66 \pm 35	< 0.05
ALT (IU/L)	104 \pm 59	72 \pm 39	< 0.05
γ -GTP (IU/L)	104 \pm 121	74 \pm 93	< 0.05
LDH (IU/L)	237 \pm 52	227 \pm 52	< 0.05
ALP (IU/L)	318 \pm 124	298 \pm 126	< 0.05
Total bilirubin (mg/dL)	0.8 \pm 0.3	0.8 \pm 0.3	NS
Total cholesterol (mg/dL)	170 \pm 40	171 \pm 43	NS
Total protein (g/dL)	7.6 \pm 0.6	7.6 \pm 0.5	NS
Albumin (g/dL)	4.0 \pm 0.5	4.1 \pm 0.4	NS
BUN (mg/dL)	15.5 \pm 3.9	14.7 \pm 3.5	NS
Creatinine (mg/dL)	0.75 \pm 0.15	0.74 \pm 0.15	NS

Data are expressed as the mean \pm standard deviation. ¹Dunnett's test. NS: Not significant; WBC: White blood cell; RBC: Red blood cell; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γ -GTP: γ -guanosine triphosphate; LDH: Lactate dehydrogenase; ALP: Alkaline phosphatase; BUN: Blood urea nitrogen; NS: Not significant.

data obtained for the clinical study. No change associated with Misatol ME intake was noted in any hematological or biochemical parameter other than in the indicators of liver function, which improved after MK615 intake. An unexplained eruption was observed in 1 patient with NAFLD, which was the only adverse event observed during this study, and was not found to have a causal relationship with the intake of Misatol ME.

DISCUSSION

This is the first study demonstrating that Misatol ME (a beverage containing MK615, an *ume* extract) lowers blood transaminase levels in patients with liver disorders such as chronic hepatitis C and NAFLD. *ume* has been used as traditional medicine and food in Japan since ancient

times^[5]. The clinical effects of *ume* have been attributed to the biological activity of MK615. MK615 contains triterpenoids such as OA, UA, lupeol, α -amyrin, and β -sitarosterol^[2-4], and has been shown to exert anti-tumor activity against various tumor cell lines, including those of gastric cancer^[5], leukemia^[5], breast cancer^[4,6], hepatocellular carcinoma^[7,8], colorectal cancer^[9], pancreatic cancer^[10], esophageal cancer^[2], and malignant melanoma^[11]. The possible mechanisms underlying the anti-tumor activity of MK615 include induction of apoptosis^[2,6,9,11], induction of autophagy^[9], cell cycle arrest^[2,6,7,10], reduced expression of receptors for advanced glycation end products (RAGE) on membrane surfaces of cancer cells^[8,11], and immunopotentialiation following exposure to X-rays^[4]. MK615 inhibits the release of HMGB1 from mouse macrophage-like RAW264.7 cells^[5]. This inhibitory activity is mediated by Nrf2 activation and HO-1 induction, suggesting that MK615 possesses antioxidative activity^[3]. The authors also previously demonstrated that MK615 suppressed the release of the inflammatory cytokines TNF- α and IL-6 in RAW264.7 cells^[12]. This suppression was mediated by the inactivation of MAPKs and NF- κ B, thus indicating an anti-inflammatory effect of MK615^[12].

The present study reveals that MK615 also exerts hepatoprotective activity in a rat model of D-GalN-induced hepatopathy, given that treatment with MK615 resulted in lower plasma ALT and AST levels accompanied by histological evidence of suppressed destruction of hepatic parenchymal cells when compared with untreated controls. Therefore, MK615 protected the rats from D-GalN-induced hepatopathy.

Previous studies using animal models of D-GalN-induced hepatopathy revealed the activation of MAPKs in the liver^[13], suggesting that liver protection might be achieved by the induction of HO-1^[14] or by the inhibition of NF- κ B in Kupffer cells^[15]. In the present study, the effects of MK615 in suppressing MAPK phosphorylation, inducing HO-1, and inhibiting NF- κ B activation may have protected the rats from D-GalN-induced hepatopathy.

Additionally, it was shown that the intake of Misatol

ME, which contains MK615, lowered the elevated levels of AST and ALT in patients with hepatic impairment. This effect was observed in patients with etiologically different hepatic diseases, i.e., those with hepatitis C and those with NAFLD. No adverse event was associated with the intake of Misatol ME during this study. Furthermore, add-on Misatol ME in combination with UDCA, which had been initiated before the start of Misatol ME intake, resulted in further AST and ALT level reductions in patients with hepatitis C. Moreover, the reduction in ALT levels was also noted in patients who were previously resistant to UDCA therapy.

A major approach to treating HCV infection is antiviral therapy using a combination of IFN and ribavirin^[16]. In cases in which the virus cannot be eradicated or IFN is not indicated, it is important to prevent the progression of HCV infection to liver cirrhosis or liver cancer^[17]. In practice, the progression of HCV infection to liver fibrosis is accelerated by higher levels of ALT^[18-21]. Therefore, when dealing with cases in which virus eradication is difficult, therapeutic interventions that result in lower ALT levels are important for delaying disease progression. In the present study, Misatol ME was shown to significantly reduce ALT levels in patients with chronic hepatitis C, and further reductions in ALT levels were also observed in patients refractory or poorly responsive to UDCA. Given the significance of these findings, Misatol ME warrants further evaluation as a potential treatment for liver disease, including an evaluation of its efficacy during prolonged use. Because Misatol ME is a functional food, conducting the same controlled study to investigate its potential as a medicine was difficult. Nevertheless, the usefulness of Misatol ME as a functional food was clarified. A future investigation is required in which a detailed analysis of the active principal component of Misatol ME should be conducted to elucidate the mechanism underlying its effectiveness as a functional food.

The mechanism underlying the hepatoprotective activity of Misatol ME in patients with chronic hepatitis C appears to involve the anti-inflammatory and antioxidative actions of the MK615 component of Misatol ME. Patients with chronic hepatitis C have high levels of inflammatory cytokines such as TNF- α and IL-6^[22-25]. MK615 inhibits the phosphorylation of MAPKs in LPS-stimulated macrophage-like RAW264.7 cells and suppresses the formation of TNF- α and IL-6 by inhibiting NF- κ B activation^[12]; these findings suggest that the effect of MK615 in suppressing cytokine formation contributes to the suppression of hepatocyte damage in patients with hepatic impairment. Given that Nrf2 activation^[26-29] and HO-1 induction^[14,30-32] are known to be hepatoprotective, the authors previously demonstrated that MK615 and its component OA activate the transcription factor Nrf2 in LPS-stimulated macrophage-like RAW264.7 cells and induce HO-1, one of the target genes^[3]. Whether MK615 also activates Nrf2 and induces HO-1 in clinical cases is unknown. However, it appears highly probable that the antioxidative action of MK615 protects the liver.

MK615 was also effective in patients with NAFLD,

reducing serum AST and ALT levels in these patients, as well as in those with hepatitis C. The involvement of factors such as oxidative stress, insulin resistance, and TNF- α in the progression of NAFLD into non-alcoholic steatohepatitis (NASH) has been suggested^[33-35]. Diet and exercise are the standard therapies for the treatment of such cases^[36,37]. However, the outcomes of these treatments are often unsatisfactory. The effects of MK615 on oxidative stress and insulin resistance in patients with NAFLD are most likely based on the antioxidative effect and the inflammatory cytokine-suppressive action of MK615. Therefore, MK615 therapy may be a promising new means of treating such cases clinically. Obesity is considered a major factor associated with NAFLD. The livers of obese individuals display disturbances in autophagy, with upregulation of autophagy reducing insulin resistance^[38]. Since MK615 has been demonstrated to induce autophagy in colorectal carcinoma cell lines^[9], this effect is also expected to be useful for treatment^[39]. More recently, it was reported that a rat model of NASH exhibited increased expression of RAGE in the liver, suggesting that inhibiting RAGE expression can protect the liver^[40]. MK615 reduces the expression of RAGE on the cell membranes of the high-RAGE expression hepatocellular carcinoma cell line HuH7^[8]. This RAGE suppression may also play a role in the hepatoprotective effects of Misatol ME.

In the present study, MK615 and Misatol ME, which contains MK615, were shown to potentially alleviate various types of hepatic impairment caused by different factors. MK615 contains multiple triterpenoids (OA, UA, lupeol, *etc.*); previous *in vitro* and *in vivo* studies have shown that these triterpenoids protect the liver from various hepatotoxic substances, such as D-galactosamine, acetaminophen, carbon tetrachloride, and ethanol^[27-29,41-47]. As a result of these diverse actions, Misatol ME may exert extensive hepatoprotective effects in patients with hepatic impairments of differing etiologies. Therefore, further studies are required to elucidate the diverse actions of Misatol ME and to assess the significance of its long-term use and its clinical efficacy in suppressing the onset and progression of cancer, as previously demonstrated at experimental level.

COMMENTS

Background

MK615, an extract from Japanese apricot, contains triterpenoids. These substances have been shown to exert various biological actions. In the present study, MK615 (a beverage containing MK615, an *ume* extract) was found to protect hepatocytes from D-galactosamine hydrochloride-induced cytotoxicity in rats. MK615 decreased the elevated alanine aminotransferase (ALT) and aspartate aminotransferase levels in the patients with liver disorder.

Research frontiers

The mechanism underlying the hepatoprotective activity of MK615 in patients with chronic hepatitis C appears to involve the anti-inflammatory and antioxidative actions of the MK615 component of MK615.

Innovations and breakthroughs

This is the first study to indicate that MK615 lowers blood transaminase levels in patients with liver disorders such as chronic hepatitis C and non-alcoholic

fatty liver disease.

Applications

In treating hepatitis C virus infection, therapeutic interventions that result in lower ALT levels are important for delaying disease progression. In the present study, MK615 was shown to significantly reduce the ALT levels in the patients with chronic hepatitis C, and further reductions in ALT levels were observed in the patients refractory or poorly responsive to ursodeoxycholic acid. Given the significance of these findings, MK615 warrants further evaluation as a potential treatment for liver disease, including an evaluation of its efficacy during prolonged use.

Terminology

MK615, an extract from Japanese apricot, contains triterpenoids such as ursolic acid, oleanolic acid, lupeol, α -amyrin, and -sitosterol. Ume extracts exert anti-inflammatory and antioxidative actions.

Peer review

The strongest point of this study should be the histological comparison of the rat livers with galactosamine-induced injury pretreated with MK615 and those not pretreated with MK615. The result is interesting and suggest that MK615 are promising hepatoprotective agents for patients with liver disorders.

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siRNA-mediated downregulation of TC21 sensitizes esophageal cancer cells to cisplatin

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We demonstrated the effect of TC21 downregulation of cell signaling in esophageal cancer cells by assessing the phosphorylation status of its downstream targets, phosphoinositide 3-kinase (PI3K), phosphatase and tensin homolog (PTEN), protein kinase B (pAkt), nuclear factor- κ B (NF- κ B) and cyclinD1 using specific antibodies. Cell survival analysis after cisplatin treatment was carried out by cell viability assay and cell cycle analysis using flow cytometry.

RESULTS: TC21 knockdown in human ESCC cell line TE13 cells, showed only a marginal increase (14.2%) in cell death compared with control cells. The expressions of the signaling proteins PI3K and pAkt, transcription factor NF- κ B, and cell cycle protein cyclin D1 were markedly decreased in response to TC21 downregulation, whereas the level of pPTEN, an antagonist of PI3K, was increased. In addition, we evaluated the potential of TC21 as a putative target for sensitizing ESCC cells to the chemotherapeutic agent cisplatin. Increased cell death (38.4%) was observed in cells treated with cisplatin after TC21 knockdown compared with cells which were treated with cisplatin alone (20% cell death).

CONCLUSION: Results suggest that TC21 mediates its effects *via* the PI3K-Akt pathway, NF- κ B and cyclin D1, and enhances chemoresistance in esophageal cancer cells.

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Key words: TC21; Esophageal squamous cell carcinoma; siRNA; Cisplatin; Chemosensitivity

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Abstract

AIM: To determine the functional significance of TC21 in esophageal squamous cell carcinoma (ESCC).

METHODS: TC21 siRNA transfection was carried out using Hyperfectamine to knock down TC21, and transcripts were analyzed by reverse transcription-polymerase chain reaction and protein by Western blotting.

INTRODUCTION

Esophageal cancer is one of the most aggressive malignancies of the gastrointestinal tract, ranking as the sixth most common cancer among males and ninth most common cancer among females globally. Esophageal squamous cell carcinoma (ESCC) is the predominant histological subtype of esophageal cancer in India, characterized by a high mortality rate and strong association with dietary habits and lifestyle^[1,2]. It is the second most common cancer among males and fourth most common cancer among females in India^[3]. Despite advances in multimodality therapy, because of its insidious symptomatology, late stage of diagnosis and poor efficacy of treatment, the prognosis of patients with ESCC still remains poor, with an average 5-year survival of < 10% globally^[4,5] and 12% in India^[6,7].

TC21/R-Ras2 is the only member of the R-Ras subfamily for which overexpression or mutants were detected in human tumor cells, including cells derived from uterine sarcoma^[8], ovarian cancer^[9] and mammary tumors^[10]. Increased expression of TC21 was found in breast cancer cells^[11-13]. Our laboratory identified TC21 overexpression in human oral squamous cell carcinomas using differential display and verified its increased expressions independently in oral cancer^[14,15], as well as in esophageal carcinomas^[16]. These clinical studies suggested that deregulated TC21 activity might contribute to human oncogenesis, however like R-Ras, the functions of TC21 are not completely understood.

The R-Ras family of Ras-related proteins, including R-Ras, TC21 (R-Ras2), and M-Ras (R-Ras3), share 55% amino acid identity with H-Ras, including identical core effector regions^[17]. Besides H-, K-, and N-Ras, TC21 is the only Ras superfamily member known to transform epithelial and fibroblast cell lines^[18], and induce tumor formation *in vivo*^[9]. Despite their similarities, R-Ras and TC21/R-Ras2 exhibited differential transforming properties in a variety of cell lines. In NIH 3T3 fibroblasts, TC21/R-Ras2 induced foci formation and tumor growth more efficiently than R-Ras^[18]. TC21/R-Ras2 also potently transformed Rat-1 fibroblasts and various epithelial cell lines, including MCF-10A, RIE-1, and EpH4^[19,20]. In comparison, R-Ras was unable to transform Rat-1 fibroblasts, but promoted tumor growth in cervical epithelial cells^[21,22]. Phosphoinositide 3-kinase (PI3K) is the predominant effector of R-Ras and TC21/R-Ras2 transforming activity; however, these oncogenes also activate Raf1, Ral-GDS, extracellular signal-regulated kinase (ERK) 1/2, c-Jun NH2-terminal kinase, and p38 mitogen-activated protein kinase (MAPK) in a cell type-specific manner^[23-25]. Previous reports suggested that TC21 induces its effects in multiple ways in different cell types, for example TC21 has been shown to activate p38 MAPK in Cos7 cells^[18], and p38 MAPK activation is important for TC21-induced ureteric bud cell proliferation^[26], therefore, TC21-mediated signaling is tissue specific^[20-22,26,27]. We have earlier identified TC21 to be overexpressed in ESCC^[16], but its role in ESCC is not well understood. This study was designed to determine the

functional significance of TC21 protein in esophageal cancer. Further, we aimed to analyze the effect of TC21 downregulation on the sensitivity of ESCC cells to chemotherapeutic agents using cisplatin as a prototype.

MATERIALS AND METHODS

Reagents

Cisplatin, propidium iodide and protease inhibitor cocktail were procured from Sigma-Aldrich (St. Louis, MO, United States); protein assay reagent was obtained from Bio-Rad Laboratories (Hercules, CA, United States); lipofectamine, TC21 small interfering RNA (siRNA) and scrambled sequence siRNA from Qiagen, RPMI 1640 (Invitrogen); primary antibodies directed against protein kinase B [pAkt, (Ser 473)], pAkt (Thr 308), protein Glycogen synthase kinase 3β [pGSK3β (Ser9)], total Akt, phosphatase and tensin homolog [pPTEN, (Ser 380)], and protein Phosphoinositide-dependent kinase-1 [pPDK1 (Ser 241)] were procured from Cell Signaling Technology (Beverly, MA, United States); cyclin D1 and GSK3β were obtained from Santa Cruz Biotechnology Inc., (California, United States), β-actin (AC-15), GAPDH (Abcam Inc. Cambridge, MA, United States). Secondary antibodies, alkaline phosphatase conjugated goat anti-mouse IgG, goat anti rabbit IgG or rabbit anti-goat IgG, were from Cell Signaling Technology (Beverly, MA, United States). Enhanced chemiluminescence (ECL) Western blotting detection reagents were obtained from Santa Cruz Biotechnology Inc., CA, United States.

Cells and cell culture

A human ESCC line, TE13, were grown in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were incubated at 37 °C in 5% humidified CO₂ enriched atmosphere and routinely sub-cultured every 2 d by trypsinization.

Cisplatin treatment and cell viability assay

Cells were seeded and grown to 60%-70% confluence in triplicates prior to addition of cisplatin. The cells were treated with varying doses of cisplatin (0-10 μg/mL) for 24 h. Thereafter, the cells were harvested and LD50 was determined by measuring the cell viability using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent according to the manufacturer's instructions. The conversion of MTT to formazan by mitochondrial dehydrogenases was measured at wavelength 570 nm in a microtiter plate reader. The percentage cell death was calculated individually for each dose as follows: $(A_{\text{control}} - A_{\text{treated}}) / A_{\text{control}} \times 100$, as described earlier^[28]. For further experiments, the cisplatin concentration required to inhibit cell growth by 50% (LD50) was determined by interpolation from dose-response curves.

RNA interference

TE13 cells were seeded and grown to 70% confluence

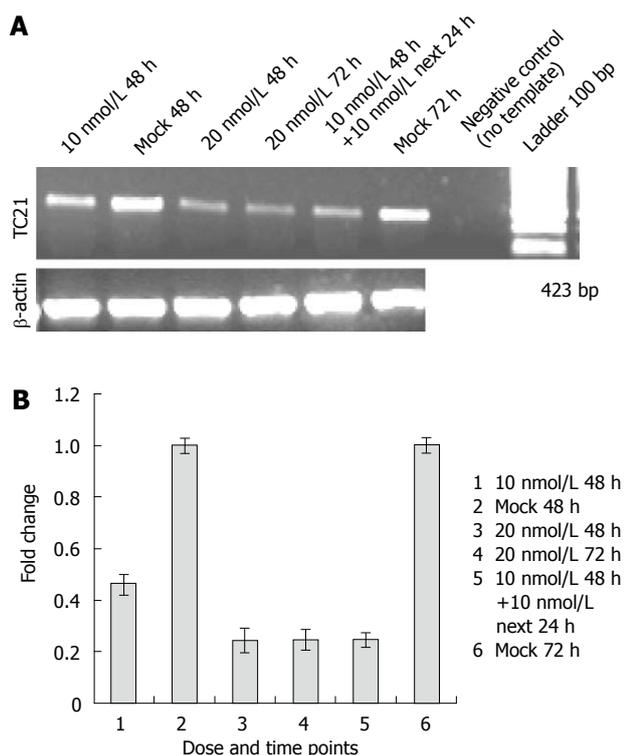


Figure 1 Small interfering RNA-targeting TC21/R-Ras2 was transfected in TE13 cells. A: Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis: Small interfering RNA (siRNA)-targeting TC21/R-Ras2 was transfected in TE13 cells in a dose-dependent manner. After 48 h and 72 h, cells were lysed, RNA was isolated and the mRNA level was determined semiquantitatively by RT-PCR using TC21-specific primers; B: Bar diagram showing fold change in level of TC21 transcripts after TC21 knockdown.

in growth medium without antibiotics. Transfection was carried out using Hyperfectamine according to the manufacturer's instructions, using TC21 siRNA or scrambled sequence siRNA at final concentrations of 20 nmol/L and 10 nmol/L, respectively. After 24 h, 48 h and 72 h cells were harvested for reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting analysis for detection of TC21. For cisplatin treatment the cells were transfected with TC21 siRNA for 48 h, followed by cisplatin treatment for 24 h and compared to untreated cells as controls, transfected with a scrambled sequence siRNA (negative control), or with the transfection reagent alone (mock). At least three independent experiments were performed with reproducible results. MAPK siRNA was used as a positive control and was detected by Western blotting for ERK.

RNA isolation

Total RNA was isolated from TE13 cells (untreated and TC21 siRNA transfected cells) using Qiagen kit following the manufacturer's instructions or the standard protocol. DNase I treatment of RNA was carried out using the Message Clean Kit (Gen Hunter Corp., Brookline, MASS, United States). RNA was quantified using formaldehyde agarose gel and by measuring spectrophotometrically the absorbance at 260 nm and 280 nm (ND-1000 UV-Vis Spectrophotometer from NanoDrop Technologies).

RT-PCR analysis

Briefly, cDNA was prepared using 1 μ g of total RNA and Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Life Technologies Inc., Gaithersburg, MD, United States) with oligo dT as the primer. Primers used for amplification of TC21-specific sequences were forward 5'-CCTTAGACCAAGAAGCTGGC-3' and reverse 5'-CAGGCATTTGGTATTTTGGC-3'. The PCR cycling parameters were initial denaturation at 94 $^{\circ}$ C for 5 min; 30 cycles of 94 $^{\circ}$ C for 1 min, 54 $^{\circ}$ C for 1 min and 72 $^{\circ}$ C for 2 min and final extension at 72 $^{\circ}$ C for 10 min. PCR for β -actin was reverse transcribed for all the samples to check for the quality and quantity of the initial RNA used. The PCR-amplified products were electrophoresed on 1.2% agarose gels and bands were visualized by ethidium bromide staining.

Protein extraction and Western blotting

After the above described TC21 siRNA treatment with or without cisplatin, TE13 cells were harvested and protein extracts were made by lysing cells in buffer, containing 50 mmol Tris-HCl, pH 7.5, 150 mmol NaCl, 1% Triton X-100, 10% glycerol, 1 mmol DTT, 10 mmol NaF, supplemented with 1 mmol activated Na_3VO_4 and 1 \times protease inhibitor cocktail. Protein concentration was measured using Bradford reagent (Sigma) and bovine serum albumin as standard. Proteins were separated by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes by electroblotting. Membranes were blocked with phosphate-buffered saline containing 1% bovine serum albumin, followed by incubation with primary and secondary antibodies. Detection of antibody-protein complexes was done using an ECL Western blotting kit according to the manufacturer's instructions.

Flow cytometry

TE13 cells were transfected with TC21 siRNA, cisplatin alone, or a combination of siRNA and cisplatin for specific time intervals as described above, cells were harvested and resuspended in phosphate-buffered saline. For cell cycle analysis, cells were fixed in 70% ethanol overnight at -20 $^{\circ}$ C and stained with propidium iodide (20 μ g/mL) and RNaseA (10 μ g/mL). All flow cytometric analyses were done using a FACS Caliber flow cytometer (San Jose, CA, United States). The acquired data were analyzed using BD FACS Diva software. For each measurement 10 000 cells were analyzed.

Statistical analysis

The Western blotting data were subjected to statistical analyses using SPSS 10.0 software (Chicago). Densitometry analysis of Western blotting was carried out using ChemiImager 4400 software. The integrated density value was compared with the loading control. The protein expression of 4 different groups (mock, scrambled sequence, TC21siRNA 48 h treatment and TC21siRNA 72 h treatment) were compared using one-way analysis

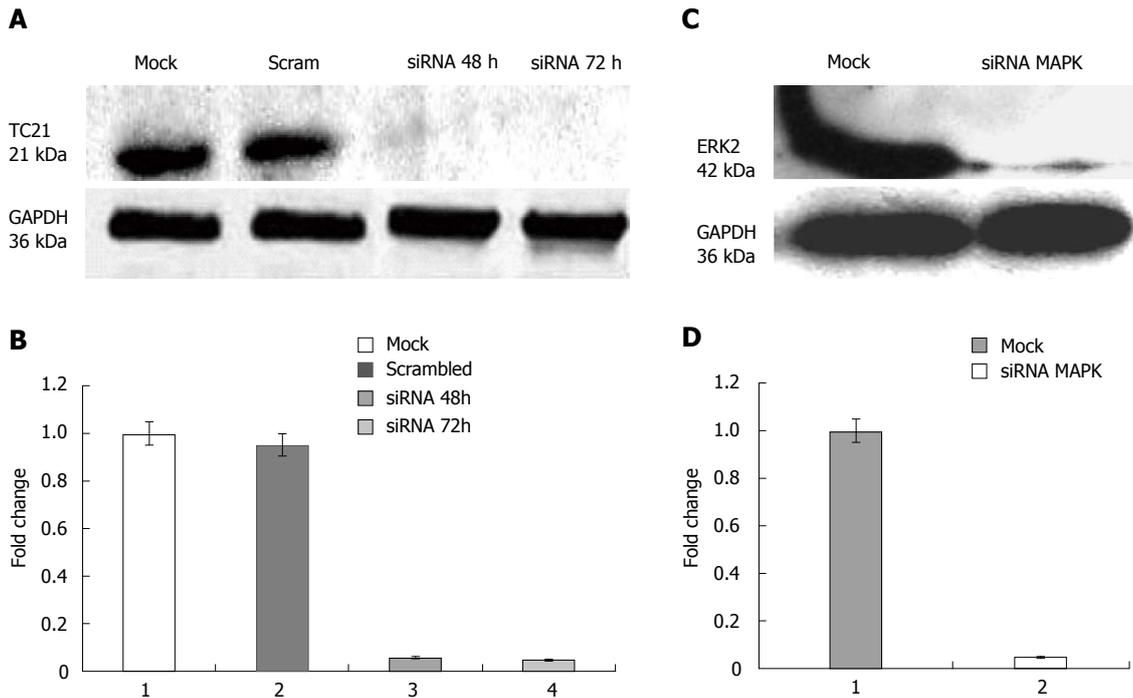


Figure 2 TC21 protein and extracellular signal-regulated kinase 2 level. A: Western blotting was carried out using specific antibody; the panel shows inhibition of TC21 protein compared with cells without transfection or transfection with a negative control siRNA (scrambled sequence); B: Bar diagram showing more than 95% decrease in TC21 protein level after TC21 knockdown compared with untreated mock and nonspecific scrambled sequence; C: Western blotting analyses show inhibition of ERK2 protein by targeted siRNA as positive control; D: Bar diagram showing more than 98% decrease in ERK2 level after MAPK knockdown compared with untreated mock and nonspecific scrambled siRNA sequence. ERK: Extracellular signal-regulated kinase; MAPK: Mitogen-activated protein kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

of variance (ANOVA). ANOVA was applied for statistical analysis with the *post hoc* (Bonferroni) multiple range test. $P < 0.05$ was considered to be significant.

RESULTS

TC21/R-Ras2 gene silencing using siRNA

Evaluation of TC21 mRNA and protein levels 48 h and 72 h post-transfection of TC21 siRNA revealed an 80% reduction in mRNA levels and a 95% reduction in TC21 protein levels as compared with the respective controls transfected with scrambled siRNA (negative control) or with transfection reagent alone (mock) as shown in Figure 1A, B and Figure 2A, B. MAPK siRNA was used as a positive control and its detection was carried out by Western blotting for ERK (Figure 2C, D).

TC21 activates the Akt pathway in ESCC

Since TC21 has been shown to activate PI3K, we investigated the role of the PI3K pathway in TC21-mediated esophageal tumorigenesis. siRNA-mediated TC21 down-regulation resulted in a significant decrease in the expression of phosphorylated Akt/PKB with $P < 0.001$ (Ser 473, Thr 308 showed equal reduction) and phosphorylated glycogen synthase kinase-3 β , pGSK3 β (Ser 9), without any change in the levels of total Akt (Figure 3A, B). A significant increase in expression of PTEN was observed in TC21 siRNA-treated TE13 cells compared with con-

trols ($P < 0.001$). Since PTEN is a PI3K antagonist and inhibits downstream signaling through Akt, its upregulation in siRNA-treated cells suggests the involvement of PI3K in TC21-mediated esophageal tumorigenesis. Moreover, knockdown of TC21 resulted in a significant decrease in PDK1 expression which may be responsible for the decrease in the expression of pAkt/PKB, resulting in reduced levels of pGSK3 β (Figure 3A, B).

TC21 activates the anti-apoptotic factor nuclear factor- κ B and cyclin D1

Western blotting analysis of whole cell lysates from TC21-knockdown TE13 cells probed with antibodies specific for the p65 subunit of nuclear factor- κ B (NF- κ B) and cyclin D1 showed significant decrease in NF- κ B and cyclin D1 proteins compared with untransfected controls (Figure 3A, B). Our results suggest that NF- κ B targeting the growth promoting protein cyclin D1, may be the downstream targets of TC21 signaling through the PI3-K/Akt pathway, thereby increasing survival of esophageal cancer cells.

TC21 knockdown does not affect P-Raf protein

There was no significant change in phosphorylated Raf protein expression observed in TC21-knockdown esophageal cancer cells for 72 h of transfection in comparison with control cells, but there was a decreased P-Raf protein level observed in TC21 siRNA treated for 48 h (Figure 3A, B).

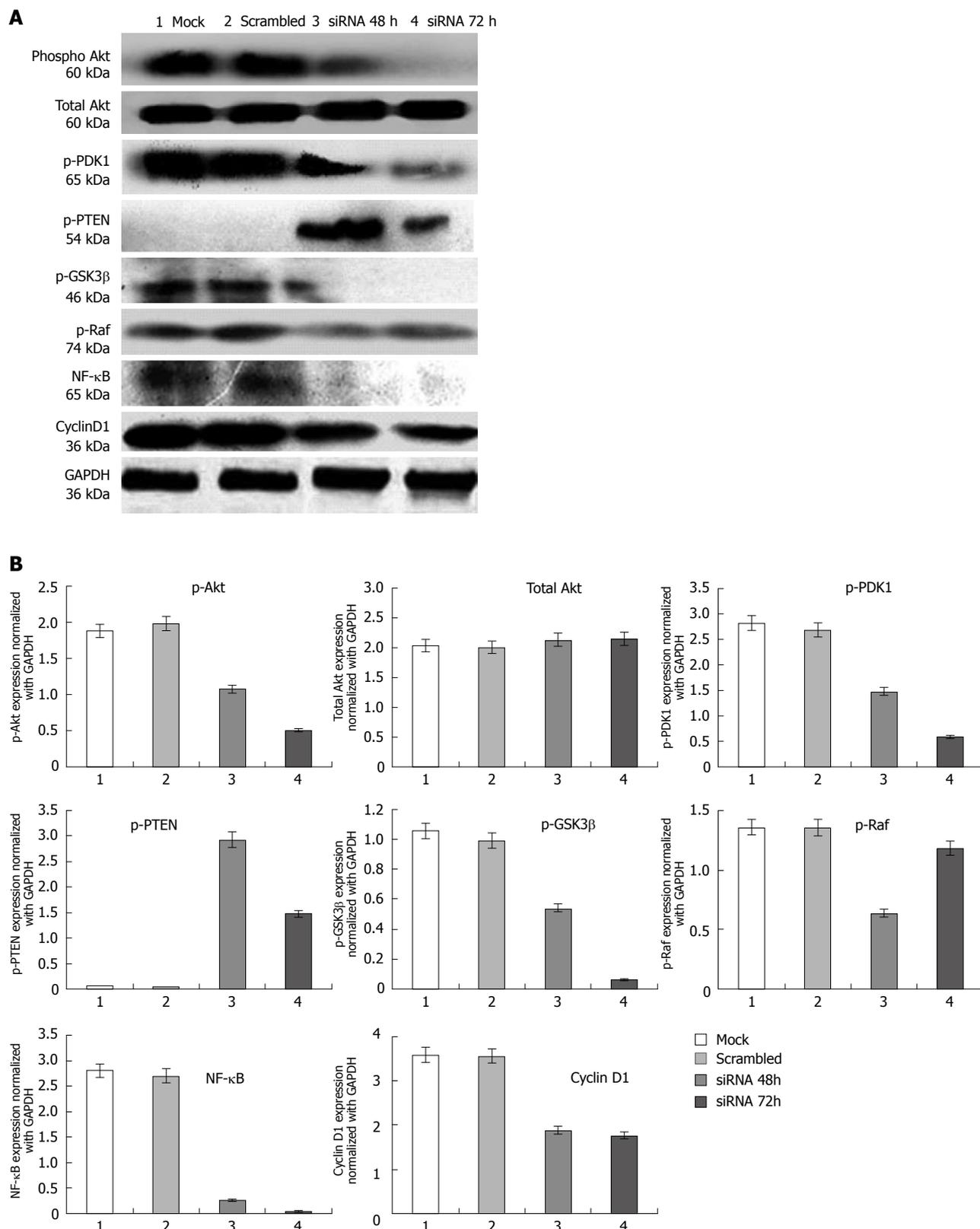


Figure 3 Expression of proteins in esophageal squamous cancer cells TE13 and compared with control. A: Expression analysis of protein kinase B (pAkt), total Akt, protein Glycogen synthase kinase 3β (pGSK3β), pRaf, protein Phosphoinositide-dependent kinase-1 (pPDK1), phosphatase and tensin homolog (pPTEN), nuclear factor-κB (NF-κB) and cyclin D1 proteins compared in esophageal squamous cancer cells TE13. Lane 1: Mock; Lane 2: Scrambled (non-target siRNA); Lane 3: TC21 siRNA transfected for 48 h; Lane 4: TC21 siRNA transfected for 72 h; B: Bar diagram showing relative levels of proteins in comparison with control protein GAPDH. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Table 1 Cell cycle analyses of siRNA-treated TE13 cells using flow cytometry

Experimental parameters	SubG0 (%)	G1 (%)	S (%)	G2/M (%)
Untreated TE13 ¹ cells for 24 h	5.7	37	21	34.8
Cisplatin treatment (8 µg/mL) for 24 h	17.8	30.6	11.8	38.2
Untreated TE13 cells for 48 h	7.9	34.7	10	41
TC21 siRNA ² (20 nmol) transfection for 48 h	14.2	22.8	12.2	42.3
Untreated TE13 cells for 72 h	10.3	36.7	27.7	25.8
TC21 siRNA transfection (20 nmol) for 48 h followed by cisplatin treatment for 24 h (total time of treatment is 72 h)	38.4	23.8	11.9	12.8

¹Esophageal squamous cell carcinoma cells; ²Small interfering RNA.

Knockdown of TC21 results in decreased G1/S population

TC21-knockdown TE13 cells resulted in a marginal increase (14.2%) in the subG0 population (cell death) compared with the mock control cells (7.9%), while the G1/S population decreased from 44% to 35% in the siRNA-treated cells (Figure 5A-2, B and Table 1).

Effect of cisplatin treatment on TE13 cells

The TE13 cells were treated with varying doses of cisplatin (0-10 µg/mL) for 24 h and LD50 was calculated by measuring the cell viability using MTT (Figure 4). LD50 was found to be 9 µg/mL. TE13 were treated with 8 µg/mL cisplatin for 24 h and flow cytometric analysis was performed to determine the cell cycle distribution. Cisplatin-treated TE13 cells showed a marginal increase in cell death (17.8%) compared with untreated cells (5.7%). There was a decrease in the S phase (11.8%) compared with 21% in the untreated control cells (Figure 5A-4, B and Table 1).

Knockdown of TC21 sensitizes TE13 cells to cisplatin treatment

The combined effect of TC21 siRNA and cisplatin treatment on TE13 cells was determined. In TC21 siRNA-transfected TE13 cells, 38.4% cell death was observed after exposure to 8 µg/mL cisplatin compared with 10.3% cell death in the untreated controls; 23.8% cells were found in the G₁ phase compared with 36.7% in the controls. Further, a decrease in S phase fraction (11.9%) was observed compared with the untreated control cells (27.7%) (Figure 5A-6, B and Table 1).

DISCUSSION

This study was focused on the effect of TC21 down-regulation on cell signaling in ESCC and its effect on the cisplatin response by downregulation of TC21 targets, both individually and in combination. In our study, TC21 protein expression correlated with cisplatin sensitivity. Earlier studies have shown that overexpression of TC21 resulted in cisplatin resistance to apoptosis^[29]. Therefore, we investigated whether TC21 downregulation would sensitize TE13 cells to cisplatin. Our results demonstrated that TC21 downregulation resulted in a

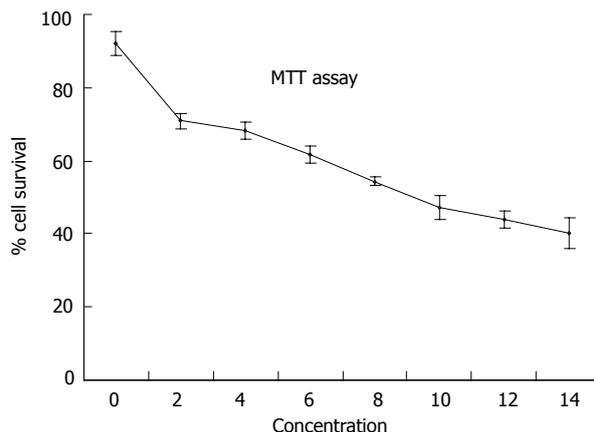


Figure 4 Cell viability assay. TE13 cells were treated with cisplatin in a dose-dependent manner for 24 h. Cell viability was determined using the MTT assay. LD50 was found to be 9 µg/mL. Further treatment with cisplatin was carried out at less than the LD50 dose.

significant reduction in the expression of the Akt pathway components, supporting that Akt pathway proteins serve as important downstream targets of TC21. Akt, an oncogenic protein implicated in human cancer development, is a key component of the PI3-K signaling pathway^[30,31]. Knockdown of TC21 decreased the expression of pAkt/PKB (antibodies specific for Ser 473 and Thr 308 showed similar effects), while the total Akt/PKB levels remained unaffected.

Furthermore, TC21 knockdown decreased the NF-κB levels. The TC21 oncogenic signals are mediated *via* the PI3K/Akt, NF-κB pathway, whereas the role of TC21 in activation of the extracellular signal-regulated kinase/MAPK pathway is less clear^[20].

Significant down-regulation of pGSK3β (Ser 9) in TC21-knockdown cells suggests a role of GSK3β downstream from Akt in TC21-mediated esophageal cancer. TC21 knockdown also suppressed the phosphorylation of the upstream kinase PDK1 (P-Serine 241). The phosphorylation of the p85 subunit of another upstream kinase, PI3K was also suppressed upon TC21 knockdown (data not shown). Suppression of Akt by TC21 siRNA led to the suppression of phosphorylation of GSK3β, the substrate for Akt. The serine/threonine kinase Akt, a key target of PIP3, is activated by TC21^[29], resulting in increased cell proliferation, transformation and survival

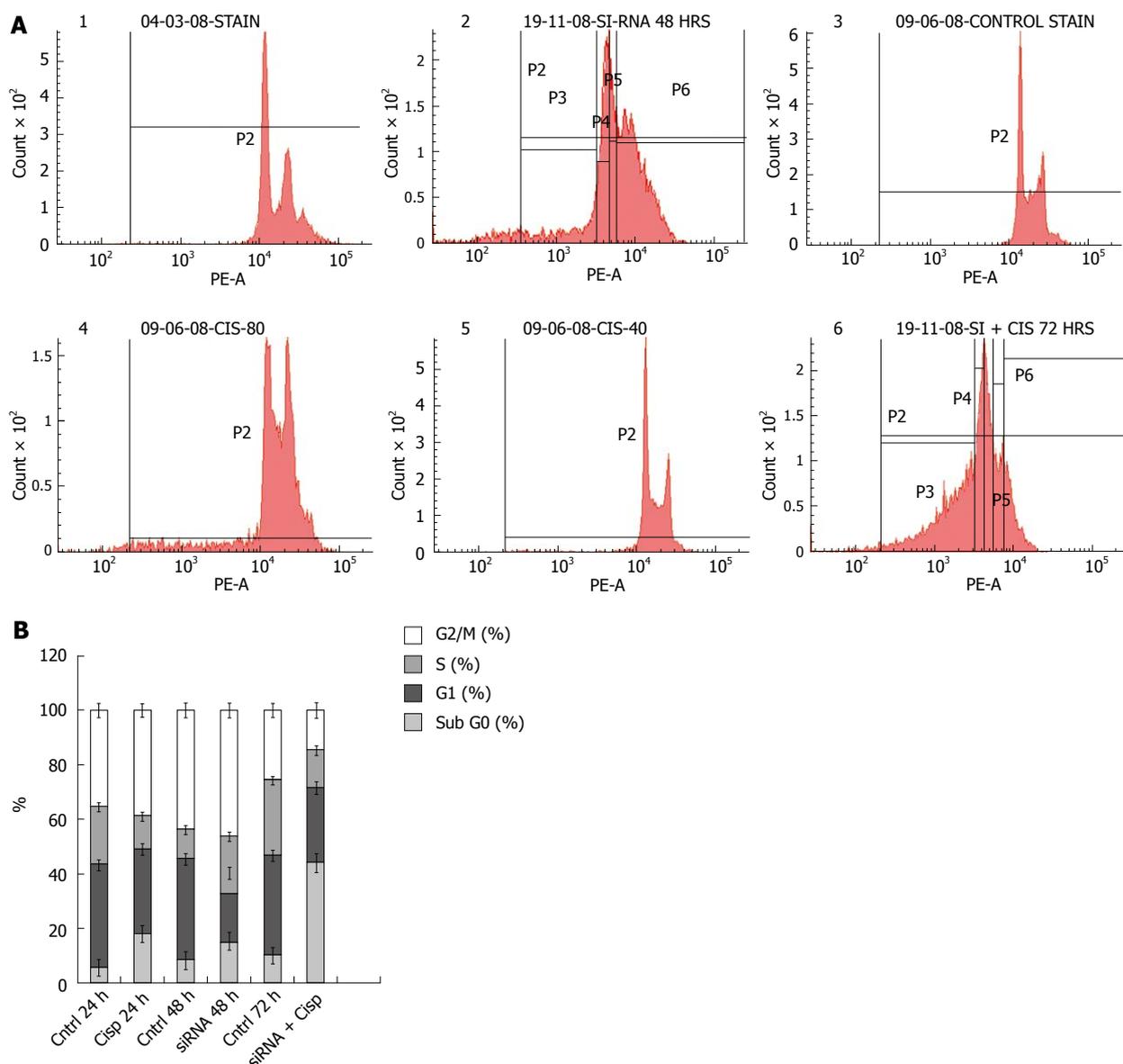


Figure 5 Cell cycle analyses. A: TC21-knockdown cells (20 nmol siRNA for 48 h) were treated with cisplatin (8 μg/mL) for 24 h and cell cycle analysis was performed by labeling cells with DNA binding dye propidium iodide. The panel shows 1: DNA histograms of mock-transfected TE13 cells for 48 h; 2: TE13 cells transfected with TC21 siRNA for 48 h showing increased cell death; 3: TE13 cells without transfection for 24 h; 4: TE13 cells were treated with cisplatin alone for 24 h; 5: TE13 cells without transfection for 72 h; 6: TE13 cells were transfected with TC21siRNA for 48 h for optimal knockdown followed by cisplatin at 8 μg/mL for 24 h; B: Stacked bar graph depicts the cell cycle distribution. Data presented here as mean of percentage events from 3 independent experiments.

through numerous effectors, including Bad, GSK-3β and mTOR^[32]. Notably, a significant increase in pPTEN expression was observed in TC21 siRNA-treated esophageal cancer cells compared with controls. Since pPTEN is a PI3K antagonist and inhibits downstream signaling through Akt, its upregulation in siRNA-treated cells suggests the involvement of PI3K in TC21-mediated esophageal tumorigenesis, suggesting that the PI3K/Akt is a downstream target of TC21.

TC21-knockdown esophageal cancer cells did not affect the level of P-Raf, suggesting that the TC21 pathway is independent of Raf. Our results support the previous report that TC21 is regulated similar to Ras except that it does not interact with full-length Raf 1, B raf, and A raf, suggesting that TC21 uses a Raf-independent

pathway to induce oncogenic transformation^[33]. It also interacts with Ral GDS *in vitro*, which may be responsible for the Raf-independent pathway^[34].

Cyclin D1 is a major regulator of the progression of cells into the proliferative stage of the cell cycle^[35]. Interestingly, TC21 knockdown resulted in reduced expression of cyclin D1, suggesting TC21 may increase cell survival of esophageal cancer by targeting cyclin D1. Thus cyclin D1 may be a target of TC21 signaling through the PI3K/Akt/NF-κB/cyclin D1 pathway. We observed that TC21 gene knockdown by RNAi alone induces an increase in the subG0 population of TE13 cells and a decrease in cyclin D1. Cell cycle progression from G0/G1 to the S phase requires cyclin/cyclin-dependent kinase (CDK) complexes and hyperphosphorylated reti-

noblastoma protein (Rb). In the early G1 phase, the cyclin D1/CDK 4 complex phosphorylates Rb, triggering a cascade of events, including the dissociation of E2F from hyperphosphorylated Rb, the activation of E2F transcription, and progression to the S phase^[36]. Keeping in view the above discussed facts it is possible that a TC21 knockdown-induced decrease in cyclin D1 expression may block transition from G1 to the S phase.

Overexpression of TC21 activated its downstream targets resulting in translocation of NF-κB to the nucleus and stimulated the transcription of anti-apoptotic genes including cyclin D1. Low cellular levels of cyclin D1 have been reported to potentially contribute to increased cisplatin sensitivity in human breast cancer cells CAL-148^[37]. Our results suggest that TC21 may enhance cell survival against cisplatin-induced cell death through activation of the PI3-K/Akt/NF-κB/cyclin D1 signaling pathway in esophageal cancer cells. Knockdown of TC21 sensitized TE-13 cells to cisplatin treatment and resulted in an increase in the subG0 population (cell death), a decrease in G1/S-phase and an increase in G2/M-phase populations. Further studies are needed to reveal new downstream targets of NF-κB responsible for TC21-mediated cell survival.

In summary, cyclin D1 is a downstream target for TC21 and TC21 may be a candidate marker for prediction of cisplatin treatment outcome in esophageal cancer patients. Our study draws attention to the relevance of TC21 in the context of cisplatin pharmacogenetics of esophageal cancer.

COMMENTS

Background

Esophageal cancer is one of the most aggressive malignancies of the gastrointestinal tract, ranking as the sixth most common cancer among males and ninth most common cancer among females globally. Esophageal squamous cell carcinoma (ESCC) is the predominant histological subtype of esophageal cancer in India, characterized by high mortality rate and strong association with dietary habits and lifestyle.

Research frontiers

TC21/R-Ras2 is the only member of the R-Ras subfamily for which overexpression or mutants were detected in human tumor cells, including cells derived from uterine sarcoma, ovarian cancer and mammary tumors. Increased expression of TC21 was found in breast cancer cells.

Innovations and breakthroughs

The effect of TC21 downregulation on cell signaling in esophageal cancer cells was determined by assessing the phosphorylation status of its downstream targets, phosphoinositide 3-kinase, phosphatase and tensin homolog, protein kinase B, nuclear factor-κB (NF-κB), and cyclin D1 using specific antibodies. Cell survival analysis after cisplatin treatment was carried out by a cell viability assay and cell cycle analysis using flow cytometry.

Applications

Cyclin D1 is a downstream target for TC21 and TC21 may be a candidate marker for prediction of cisplatin treatment outcome in esophageal cancer patients. The study draws attention to the relevance of TC21 in the context of cisplatin pharmacogenetics of esophageal cancer.

Peer review

This is a good descriptive study in which authors determine the functional significance of TC21 in ESCC. The results are interesting and suggest that TC21 mediates its effects via PI3K-Akt pathway, NF-κB and cyclin D1 and enhances chemoresistance in esophageal cancer cells.

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Double contrast-enhanced two-dimensional and three-dimensional ultrasonography for evaluation of gastric lesions

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Abstract

AIM: To investigate the value of two-dimensional (2D) and three-dimensional (3D) double contrast-enhanced ultrasonography (DCUS) imaging for evaluation of gastric lesions.

METHODS: 2D and 3D DCUS imaging with both oral and intravenous administrations of contrast agents was used to assess gastroscopically-confirmed gastric lesions in 46 patients with benign and malignant diseases. Initially, liquid-based ultrasound contrast agent (Xinzhang®) was given orally at dose of 500-600 mL for conventional ultrasound examination of the gastric lesions, and then a microbubble-based contrast agent (SonoVue) was injected intravenously at dose of 1.2-2.4 mL in bolus fashion to assess the perfusion pattern of the lesions

using contrast imaging modes. The parameters derived from time-intensity curves including the arrival time (AT), time to peak (TTP), peak intensity (PI) and enhanced intensity (EI) were measured on the 2D DCUS imaging. 3D DCUS of the lesions was acquired to demonstrate the value of this imaging mode.

RESULTS: There were 22 cases with benign lesions including chronic gastritis ($n = 5$), gastric ulcer ($n = 9$), gastric polyps ($n = 3$), gastric stromal tumors ($n = 5$), and 24 cases with malignant lesions including gastric cancer ($n = 20$), gastric cardia carcinoma ($n = 3$) and post-operative recurrent gastric cancer ($n = 1$) in the study. The oral contrast-enhanced ultrasonography (CEUS) imaging of the stomach clearly demonstrated the anatomy of the stomach and morphologic features of gastric lesions. With optimal scanning window and imaging display under oral CEUS, intravenous CEUS clearly showed the perfusion of gastric lesions with various characteristic manifestations. Both 2D and 3D DCUS images clearly demonstrated normal gastric wall as a three-layer structure, from the inside out, hyperechoic mucosa, hypoechoic muscularis and hyperechoic serosa, respectively. There were statistical significant differences of AT (8.68 ± 2.06 vs 10.43 ± 2.75 , $P = 0.017$), PI (34.64 ± 6.63 vs 29.58 ± 8.22 , $P = 0.023$) and EI (29.72 ± 6.69 vs 22.66 ± 7.01 , $P = 0.001$) between malignant lesions and normal gastric wall. However, no differences of AT, PI and EI between benign lesions and normal gastric wall tissue were found. 3D DCUS could intuitively display morphological features and vascularities of the lesions with multiplanar and volume views. 3D DCUS imaging provided comprehensive information complementary to 2D imaging. The crater or wellhead appearances and feeding vessels as well as distorted nourishing vasculature of gastric carcinoma were better seen with 3D imaging than 2D imaging.

CONCLUSION: DCUS imaging can simultaneously

display the anatomic and perfusion features of gastric lesions. 3D DCUS can provide additional information to 2D DCUS for evaluation of gastric lesions.

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Key words: Contrast-enhanced ultrasonography; Gastric lesions; Two-dimensional imaging; Three-dimensional imaging; Contrast media

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INTRODUCTION

The common methods for examination of the upper gastrointestinal (GI) tract are x-ray with oral barium-based contrast agent and endoscopy. Their shortcomings include the fact that they often cannot delineate submucosal mural structures of the GI tract. Limitations to the sonographic assessment of the upper GI tract and adjacent organs include patient body habitus and the presence of gas-filled bowel, which can produce shadowing artifacts^[1,2]. Although ingestion of degassed water has been used to improve sonographic assessments of the GI tract and retroperitoneal structures, water simply displaces gas within the GI tract and can produce inconsistent results. Imaging water-filled stomach usually results in an increase in the wall through transmission, which may cause tissues of otherwise normal echogenicity to appear more echogenic than expected, creating a potential source of diagnostic error. Over the years, researchers attempted to develop oral contrast agents to improve the assessment of the GI tract and adjacent structures by absorbing and displacing bowel gas and provide an acoustic window for sonographic visualization of upper GI tract. One of such oral contrast agent Xinzhang[®] (Huqingyutang Pharmaceuticals Co., Hangzhou, China) has been developed and commercially available in China for ultrasound imaging of upper GI tract in clinical settings^[3-5].

During the last two decades, contrast-enhanced ultrasound imaging (CEUS) with intravascular contrast agents has been investigated and has gradually emerged in clinical settings. The rapid development of contrast agents for sonography is precipitated by the performance limits of grayscale imaging and Doppler techniques. As US Imaging is used to study smaller and deeper structures in the abdomen, the spatial resolution of grayscale imaging and Doppler sensitivity becomes critical to the degree

that it impacts the clinical utility of sonography. Contrast agents promise to improve the sensitivity and specificity of current sonographic diagnoses and have the potential to expand the already broad range of its applications.

Recently, we have explored new technique which combines both oral and intravenous CEUS imaging methods, so called Double contrast-enhanced ultrasound (DCUS), for evaluation of gastric abnormalities. The purpose of this study was to investigate the value of DCUS imaging using both two-dimensional (2D) and three-dimensional (3D) modes for the evaluation of gastric lesions.

MATERIALS AND METHODS

Patients

The study protocol was approved by our hospital ethical committee, and all patients gave informed consent and agreed to participate in the study. During a period from 2007 to 2011, 2D and 3D DCUS imaging with both oral and intravenous administrations of contrast agents was used to assess gastroscopically-detected gastric lesions in 46 patients with 22 benign cases and 24 malignant cases. All final diagnoses are confirmed by endoscopic biopsy or surgical pathological findings. There were 31 males and 15 females, aged from 23 years to 80 years with a mean age of 54.93 ± 12.49 years.

Equipment and contrast agents

The DCUS was performed using full digital ultrasound scanners iU22 (Philips Medical Systems, Bothell, WA) with a C5-2 probe or Sequoia-512 (Siemens Medical Solutions, Mountain View, CA) with a 4C1 probe for 2D imaging. Philips C6-2 volume probe was used for acquiring 3D DCUS imaging. Conventional ultrasound imaging mode was used for oral contrast imaging while contrast imaging modes (Philips Pulse inversion harmonic imaging and Siemens contrast pulse sequencing techniques) were used for intravascular contrast imaging.

The commercially available oral contrast agent Xinzhang[®] (Huqingyutang Pharmaceuticals Co., Hangzhou, China) was supplied as powder which is derived from rice and soya. The 48 g per package was reconstituted by adding 500-600 mL of cooled boiling water and gently agitating by hand to form a homogeneous thin paste.

The intravenous contrast agent SonoVue[®] (Bracco SpA, Milan, Italy) was injected in bolus fashion at doses of 1.2-2.4 mL through brachial vein, followed by 5 mL normal saline flush.

Double oral and intravenous contrast imaging

DCUS examination was performed after patient's fasting for at least 8 h on the day of the study. The stomach of all patients was scanned using real time gray-scale imaging when the patients swallowed the oral agent to expand the cavity of the stomach. Using contrast agent-filled gastric cavity with homogenous moderate echogenicity as an acoustic window, the location, shape and size of any possible lesions and the wall thickness of the stomach were

carefully imaged and recorded under dynamic scanning with patients in the supine and both decubitus positions. The scanning parameters (e.g., the depth, focus, and gain) were adjusted to achieve optimal imaging display as conventional ultrasound examination.

After oral contrast imaging localization of the gastric lesion, vascular CEUS of was performed with a bolus injection of 1.2-2.4 mL of SonoVue *via* a 20-gauge peripheral intravenous catheter under contrast imaging mode with a low mechanical index (0.09-0.21). Initially, each subject underwent 2D imaging to observe the perfusion pattern and measure the time-intensity curve of the lesions and adjacent normal wall of stomach as control. The CEUS parameters of arrival time (AT), time to peak (TTP), infusion time (IT, $IT = TTP - AT$), baseline intensity (BI), peak intensity (PI) and enhanced intensity (EI, $EI = PI - BI$) was obtained and calculated from the time-intensity curve. Next, the regions of interest were selected based on the 2D contrast imaging and 3D images of the region of interest were acquired using a 3D probe with additional contrast agent injection during the arterial phase of enhancement. The 3D imaging volume files was stored digitally with both on-line and off-line imaging process and analysis.

Statistical analysis

SPSS 13.0 (SPSS Inc., Chicago, United States) was used for statistical analysis. The values of measurements were expressed as (mean \pm SD). Two sample *t*-test was used to compare each parameter (AT, TTP, PI, EI and IT) between benign or malignant lesions and normal gastric walls. For all analyses, a *P* value of less than 0.05 was considered statistically significant.

RESULTS

A total of 46 pathologically-proved cases were enrolled in the study. There were 22 cases with benign lesions including chronic gastritis ($n = 5$), gastric ulcer ($n = 9$), gastric polyps ($n = 3$), gastric stromal tumors ($n = 5$), and 24 cases with malignant lesions including gastric cancer ($n = 20$), gastric cardia carcinoma ($n = 3$) and post-operative recurrent gastric cancer ($n = 1$). The 2D DCUS with oral and intravenous contrast enhancement was successfully performed in all 46 patients. While 43 out of 46 patients underwent 3D contrast imaging studies. Both 2D and 3D DCUS images clearly demonstrated normal gastric wall as a three-layer structure, from the inside out, hyperechoic mucosa, hypoechoic muscularis and hyperechoic serosa, respectively (Figure 1). DCUS characteristic findings of gastric lesions were visualized as follows.

Benign lesions

Gastric ulceration lesion: Gastric ulceration lesion appeared as a contrast agent-filled defect on the stomach wall with a spot-like mural hyperechogenic area in 9 cases. There was a lack of localized partial or prominent gastric wall thickening. Intravenous contrast 2D imaging shown

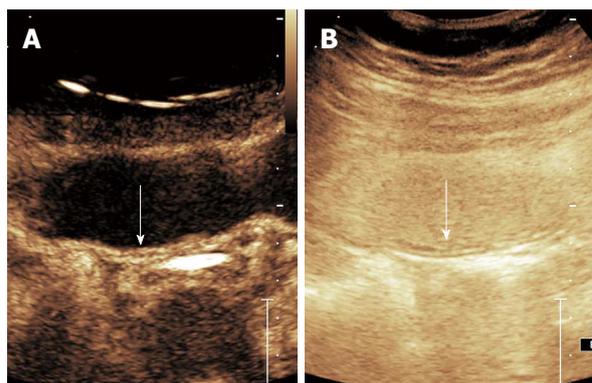


Figure 1 Two-dimensional double contrast-enhanced ultrasound imaging. A: The picture showing intravenous contrast harmonic imaging with the echo-free gastric cavity and three layers of normal gastric wall (arrow); B: The picture showing oral contrast imaging of normal gastric wall (arrow) and cavity filled with echogenic contrast agent.

uniformed enhancement of the gastric wall adjacent to the lesion and 3D DCUS imaging showed the gastric cavity and wall with a focal defect area consistent with an ulcer (Figure 2).

Gastric polyp: Gastric polyp appeared as a hyperechoic beansprout-shaped or a cone-shape mass projecting into the cavity of the stomach in 3 cases (Figure 3). Intravenous contrast 2D imaging shown simultaneous and equal enhancement of both lesions and normal gastric walls.

Gastric stromal tumor: Gastric stromal tumor shown as a hypoechoic or nearly anechoic mass within gastric wall under oral contrast imaging in 5 cases (Figure 4). These lesions had clear demarcation, regular around shape, and homogeneous echotexture. Larger ones protruded into the stomach cavity ($n = 3$) and have inhomogeneous echotexture ($n = 2$). Intravenous contrast 2D imaging demonstrated simultaneous or delayed enhancement of stromal tumors with homogeneous iso- or hypo-enhancement when compared with adjacent normal gastric wall. A ring enhancement appear in hypo-enhancement lesions ($n = 2$).

Gastric inflammatory lesion: Inflammatory thickening of the gastric wall was clearly seen under oral contrast displays in 5 chronic gastritis cases. The focal gastric inflammatory lesion appeared as homogeneous hypoechoic thickening associated with mild elevation of smooth surface of the wall. There was no remarkable change in the layers of the gastric wall. Intravenous contrast 2D imaging of the thickening wall shown uniform, simultaneous and iso- or hyper-enhancement compared to the normal gastric wall.

Malignant lesions

The characteristics of gastric carcinoma were demonstrated with oral contrast imaging in 24 cases. The features of malignant lesions included irregular shape, heterogeneous

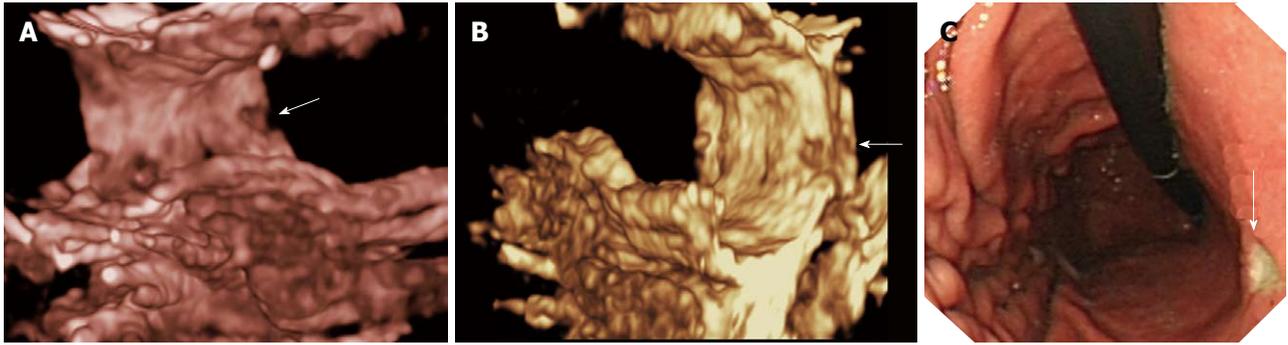


Figure 2 Double contrast-enhanced ultrasound imaging of gastric ulceration. A: Three-dimensional (3D) double contrast-enhanced ultrasound imaging showed the gastric cavity and wall with a focal defect area consistent with an ulcer (arrow); B: Another 3D imaging with different angle showed the ulcerative lesion (arrow) and the folds of gastric wall with pseudo-color which similar to gastroscopic imaging; C: The ulcerative lesion (arrow) is seen on gastroscopic imaging.

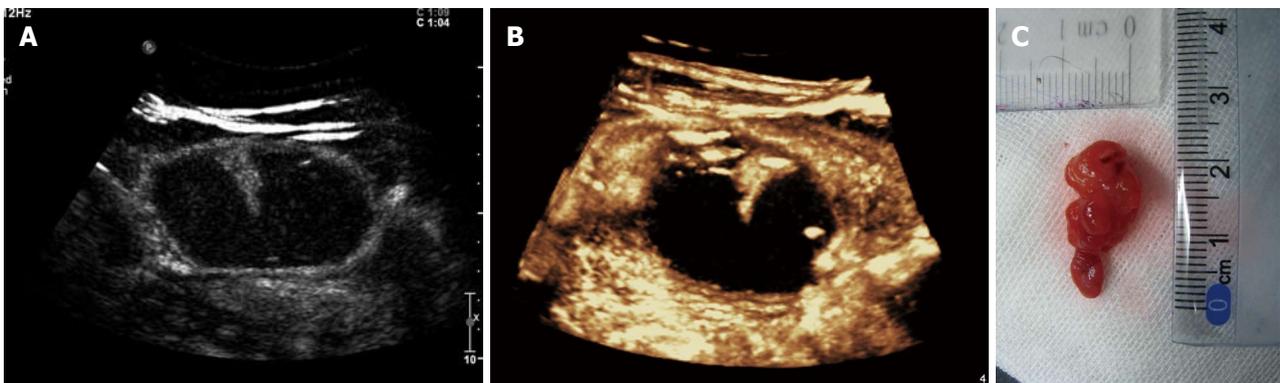


Figure 3 Double contrast-enhanced ultrasound imaging of gastric polyp. A: Two-dimensional double contrast-enhanced ultrasound (DCUS) imaging displayed a polyp with a wide base projecting into the gastric cavity. Contrast enhancement was seen on both polyp and gastric wall; B: Three-dimensional DCUS imaging of the polyp showed in figure A; C: The surgical specimen of the polyp confirmed the DCUS finding.

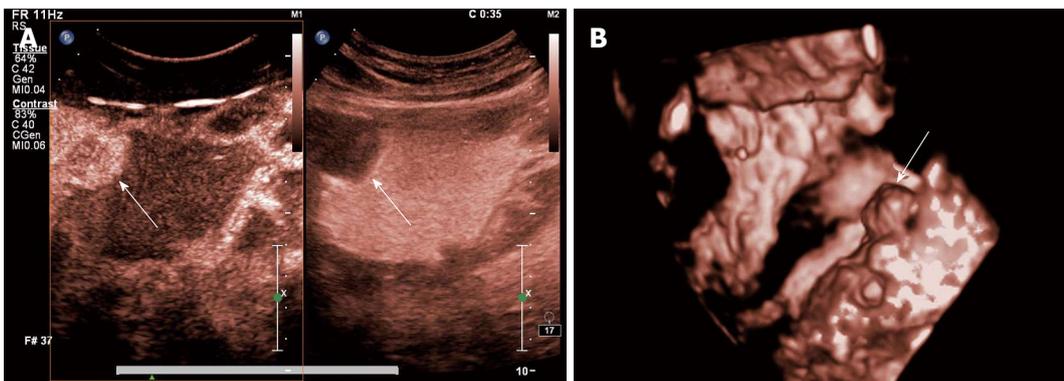


Figure 4 Double contrast-enhanced ultrasound imaging of gastric stromal tumor. A: Two-dimensional double contrast-enhanced ultrasound (DCUS) images displayed a anechoic mass into the gastric cavity in oral contrast ultrasonography (right figure), from which we hardly judged whether it was cystic or solid lesion; but the intravenous contrast imaging (left figure) showed there was contrast agent enhancement in the focus of infection (arrow); B: Three-dimensional DCUS imaging displayed the tumor elevated to the gastric cavity (arrow).

echotexture and disrupted layers of the gastric wall on the 2D oral contrast imaging. The mass-like lesion shown as a solid mass protruding into the cavity while the diffuse lesion appeared as a localized wall thickening and irregular surface of the lesions. The lesions with ulceration in 6 cases shown as filling defects within the lesions (Figure 5). Extensive infiltrative lesions in 9 cases shown diffuse het-

erogeneous thickening of the gastric wall which resulted in gastric cavity narrowing. The passage of oral contrast agent through the narrow gastric cavity can be seen with slow and stiff gastric peristalsis in real-time imaging. In 3 patients with carcinoma of gastric cardia, oral contrast imaging shown hypoechoic wall thickening of the distal esophageal and gastric cardia. Echogenic contrast agents

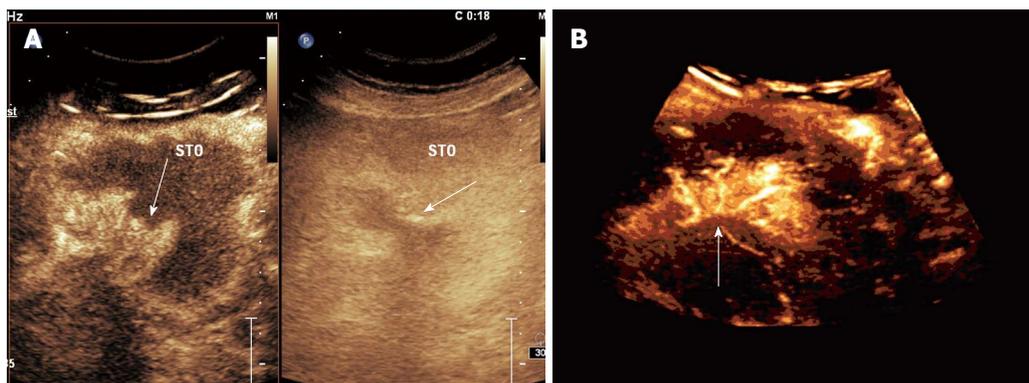


Figure 5 Double contrast-enhanced ultrasound imaging of ulcerative gastric cancer. A: Two-dimensional double contrast-enhanced ultrasound (DCUS) images (conventional imaging on the right and harmonic imaging on the left) showed a contrast-enhanced mass with crater-like ulcerative defect (arrow); B: Three-dimensional DCUS imaging showed distorted nourishing vasculature within the gastric cancer (arrow).

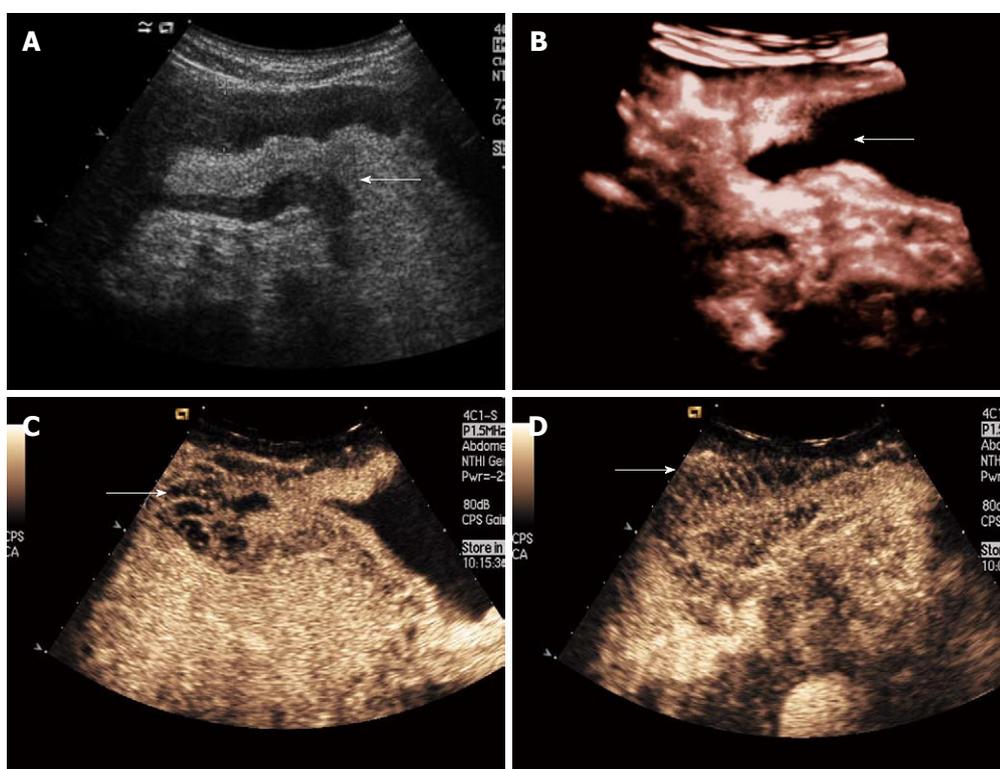


Figure 6 Double contrast-enhanced ultrasound imaging of infiltrative gastric cancer. A: An oral contrast image showed diffusely thickened gastric wall with narrowed gastric cavity (arrow) in a patient with gastric cancer; B: Three-dimensional double contrast-enhanced ultrasound (DCUS) imaging showed thickened gastric wall and narrowed echo-free gastric cavity (arrow); C, D: Two-dimensional DCUS images showed fence-like tumor vessels penetrating through thickened gastric wall in the arterial phase (arrows).

passing through the narrow lumen of gastroesophageal junction was seen during the swallowing of contrast agent. In 6 patients with gastric carcinoma, enlarged lymph nodes adjacent to gastric wall were identified with hypoechogenic and round-shape features.

Combining with oral contrast imaging, intravenous 2D contrast imaging demonstrated variable patterns of enhancement of the lesions. When compared to adjacent normal gastric wall, there were iso-enhancement in 2 lesions, hypo-enhancement in 1 lesions and hyper-enhancement in 21 lesions. The feeding vessels and dis-

torted tumor vasculature was clearly identified with 2D DCUS imaging (Figure 6). All lesions appeared as earlier enhancement in wash-in phase than the normal gastric wall. Under DCUS imaging condition, the enhancement parameters of time-intensity curves in both begin and malignant lesions shown in Table 1.

3D double contrast-enhanced ultrasonography imaging

Reconstructed 3D imaging demonstrated global rendering of DCUS imaging with different prospective of morphology for both normal structures and gastric le-

Table 1 Comparison between benign gastric lesions and normal gastric wall ($n = 22$), malignant gastric lesions and normal gastric wall ($n = 24$) from double contrast-enhanced ultrasound time-intensity curve

	Benign lesions	Normal gastric wall	<i>P</i> value	Malignant lesions	Normal gastric wall	<i>P</i> value
AT (s)	9.43 ± 2.25	9.22 ± 2.37	0.753	8.68 ± 2.06	10.43 ± 2.75	0.017
TTP (s)	16.24 ± 3.67	16.43 ± 3.32	0.862	15.86 ± 3.80	17.86 ± 4.19	0.089
IT (s)	6.85 ± 2.56	7.22 ± 2.57	0.643	7.17 ± 2.45	7.44 ± 3.03	0.344
BI (dB)	5.07 ± 3.49	5.29 ± 4.16	0.846	4.93 ± 3.25	6.92 ± 4.59	0.09
PI (dB)	31.36 ± 8.55	32.96 ± 8.58	0.538	34.64 ± 6.63	29.58 ± 8.22	0.023
EI (dB)	26.28 ± 9.90	27.64 ± 9.59	0.648	29.72 ± 6.69	22.66 ± 7.01	0.001

AT: Arrival time; TTP: Time to peak; IT: Infusion time; BI: Baseline intensity; PI: Peak intensity; EI: Enhanced intensity.

sions. 3D imaging displayed intuitive pictures of lesions and the gastric layers from multiple imaging angles and views, which corresponded well with surgical specimens (Figures 2, 3 and 7).

In cases with thickened wall bulging unevenly or a mass protruding into (or outward) the gastric cavity, 3D DCUS imaging provided comprehensive information to complementary to 2D imaging. The crater or wellhead appearances and feeding vessels as well as distorted nourishing vasculature of gastric carcinoma were better seen with 3D imaging than 2D imaging (Figures 5, 7).

DISCUSSION

Ultrasound imaging is a convenient and noninvasive diagnostic tool for evaluation of abdominal organs. However, its use in diagnosing gastric abnormalities is limited by the interference of the gas in the GI tract. In 1978, Warren first used hydrophilic methyl cellulose oral suspension in ultrasound examination to image retroperitoneal organs such as stomach, duodenum or pancreas^[6]. Since then, researchers have done many studies in oral contrast agent for gastric ultrasound imaging^[7-12]. Early-developed oral contrast agents have short emptying, large required quantity, and an unpalatable taste. The oral contrast agent Xinzhang[®] used in this study is vegetable-based with main components being beans and starch^[13], which is a uniform thin paste with pleasant taste and slow emptying feature without side effects, and thus is easily accepted by patients especially children and the elderly. The thin paste-based agent produces uniform moderate echogenic reflection within well-filled stomach, which clearly shows all normal layers of gastric wall, gastric lesions and surrounding structures under optimal contrast imaging. The gastric lesions revealed by oral contrast ultrasound in this study included mild thickening of the gastric wall, polypoid lesions and other stomach masses. Small lesions such as 0.5 cm diameter ulcer and 1.0 cm diameter stromal tumors can be identified. In addition, oral contrast gastric ultrasound imaging can be carried out using conventional ultrasound systems, which is easily applied in the clinical practice.

Although oral contrast imaging can show normal anatomy of the stomach and the location, shape and size of gastric lesions, its ability to determine internal structures and blood perfusion status of the lesion is limited. For example, gray-scale imaging cannot determine a very

hypoechoic mass (such as gastric stromal tumor) whether it is cystic or solid^[14-16]. Also, color Doppler ultrasound has a poor sensitivity in revealing small blood flow of the gastric wall or lesions. In previous study, intravenous contrast imaging has been used for the evaluation of gastric tumor in canine to determine the blood perfusion status of the tumors^[17-19]. However, without appropriated gray-scale imaging of the stomach, Intravenous contrast imaging cannot achieve useful information of blood perfusion for assessment of tumor vascularity and surrounding structures. Therefore, DCUS imaging is necessary for evaluation of gastric lesions in order to obtain comprehensive information.

Micro-bubble-based SonoVue is a second-generation intravenous ultrasound contrast agent. It has phospholipids as capsule, containing sulfur hexafluoride gas. Its diameter is similar to that of red blood cells, which enables it to reach microvessels of all peripheral organs through intravenous injection. This agent has an average half-life of 12 min *in vivo*. It is removed by lungs through respiration in 15 min and poses no obvious toxic effect to the liver and kidney. In term of the difference from CT contrast agents such as lipiodol, microbubble-based contrast agent does not penetrate vessel wall and leak into interstitial space. Its distribution in the lesion represents the distribution of the microvessels, and the intensity of the lesion enhancement represents the density of those vessels^[20-28]. Therefore, DCUS can be used to evaluate both the morphology and vascularity of gastric lesions.

In this study, we demonstrated that oral contrast imaging can provide excellent acoustic window for evaluation of a variety of gastric lesions by conventional gray-scale imaging. Furthermore, oral contrast imaging serves as important platform for assessment of blood perfusion of the lesions by intravascular contrast imaging. Thus, DCUS is able to demonstrate both morphologic appearances and perfusion status of both normal and abnormal structures, which improves the ability of differential diagnosis. For example, oral contrast imaging revealed 4 gastric stromal tumors as anechoic lesions which was difficult to decide whether they are cystic or solid lesions. However, the use of intravenous contrast imaging demonstrated internal blood perfusion of these tumor lesions, which confirmed they are solid lesions instead of cystic lesions. More important, DCUS can show the relationship of the lesion's vasculature and the gastric wall

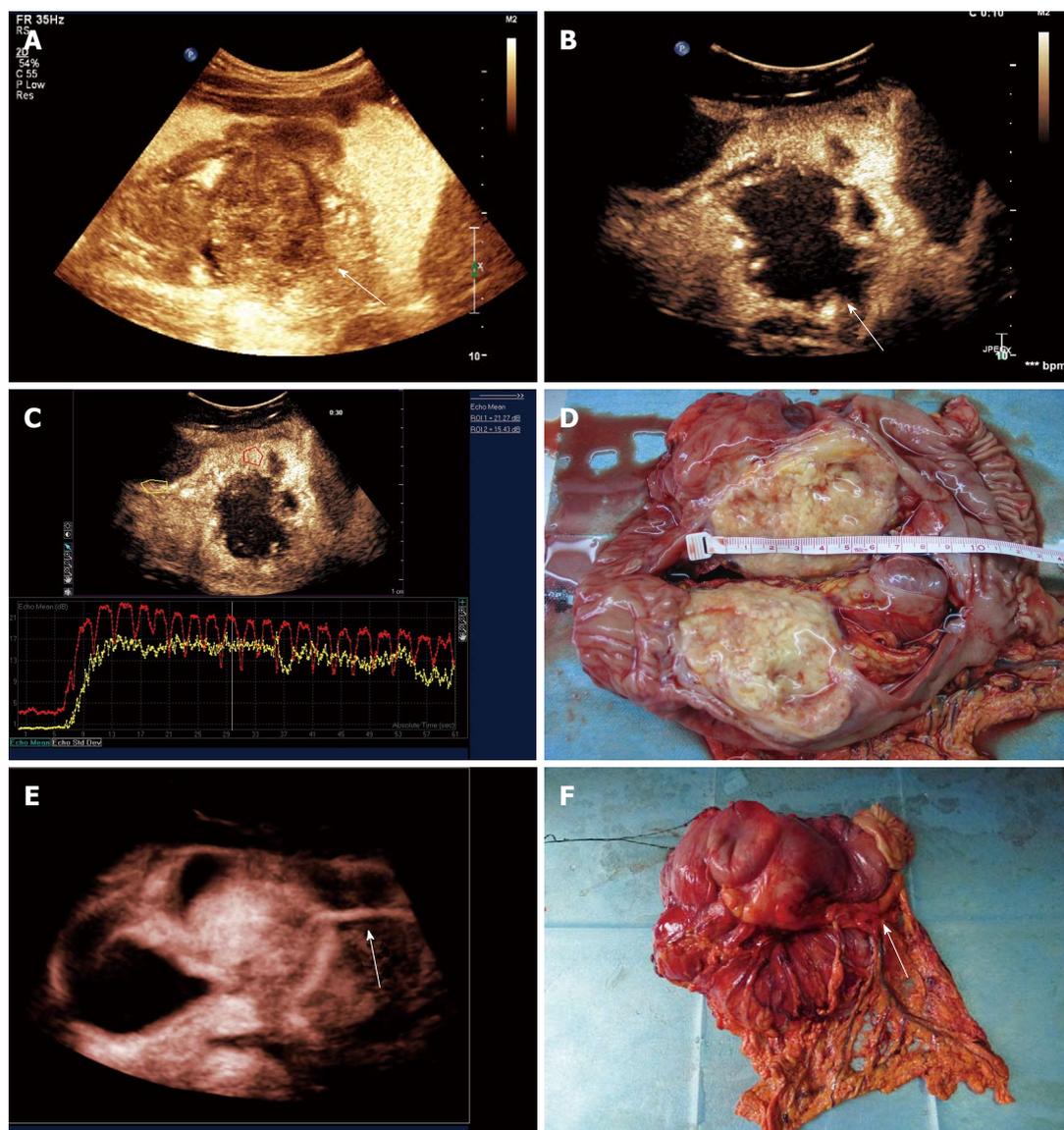


Figure 7 Double contrast-enhanced ultrasound imaging of mass-type gastric cancer. A: Oral contrast imaging revealed a tumor (arrow) extended into the back wall of stomach, which could not be seen by gastroscopy; B: Two-dimensional double contrast-enhanced ultrasound (DCUS) imaging showed the tumor attached to the posterior wall and enhanced in peripheral areas with the center of non-enhancement at early arterial phase; C: A contrast time-intensity curve of the lesion showed that the arrival time was shorter and the peak intensity was higher in the peripheral lesion (red curve) than those in normal gastric wall (yellow curve); D: The surgical specimen demonstrated the tumor with central necrosis which consistent to DCUS findings; E: Three-dimensional DCUS imaging showed the outer-growing lesion with a tumor feeding vessel (arrow); F: The feeding vessel on the gross specimen was seen and coincident with the DCUS imaging.

as well as their contours outlined by contrast imaging, which accentuates pathological features. Indeed, DCUS images can clearly show the pathological features of the lesions, which made sonographic diagnosis to fit with internationally wide adopted Borrmann's classification of advanced gastric cancer, i.e., polypoid lesion, ulcerated lesion, ulcerating infiltrative lesion, and infiltrative lesion.

Comparing malignant lesions with surrounding normal gastric tissue using contrast-enhanced time-intensity curve, values of AT, PI and EI parameters except TTP and IT were statistically significant ($P < 0.05$). Whereas comparing benign lesions with surrounding normal gastric tissue, all parameters including AT, TTP, IT, PI and EI are not statistically significant ($P > 0.05$). Thus, early AT and increased

PI, EI, can be used as potential indexes and indicators for evaluating gastric benign and malignant lesions^[29]. Since EI eliminated baseline intensity factor, it should reflect the actual intensity of the enhancement. Previous study have shown that, in gastric cancer, EI correlates well to the pathological microvessel density ($r = 0.921, P < 0.001$)^[30], which may reflect the density of microvessels with the lesion, and could be a new parameter for evaluating biological behavior and angiogenesis of gastric cancer.

3D DCUS imaging provides comprehensive and observational perspective for gastric wall and lesion morphology. It can show the intuitive appearance of the lesion relevant to the gastric wall which closely correlates to pathological specimen and increases the confidence

of clinical diagnosis. In malignant lesions, 3D imaging shown the dense, tortuous distorted and heavily cluttered vasculatures of the lesions, which is similar to the “tumor vascularity” seen in the liver and other organ malignancies^[31-35]. 3D DCUS imaging can supplement 2D imaging and provide more evidence for the diagnosis of benign and malignant gastric lesions.

It should be pointed out that this is preliminary observation of DCUS imaging applications. The limitations of this study include small number cases with various gastric abnormalities and no blinded comparison with endoscopic examinations. Prospective study design with larger clinical trial is needed for further investigations.

In conclusion, DCUS imaging is able to simultaneously display the sonographic features of various gastric lesions and its vasculatures as well as perfusion patterns. The parameters of AT, PI and EI could serve as potential indicators for differentiating benign and malignant gastric lesions. 3D DCUS could provide additional information to 2D DCUS for evaluation of gastric lesions.

COMMENTS

Background

The common methods for examination of the upper gastrointestinal (GI) tract are x-ray with oral barium-based contrast agent and endoscopy. Their shortcomings include the fact that they often cannot delineate submucosal mural structures of the GI tract. Over the years, oral contrast agents to improve the assessment of the GI tract and adjacent structures by absorbing and displacing bowel gas and provide an acoustic window for sonographic visualization of upper GI tract have been developed and commercially available in China, one of such oral contrast agent was Xinzhang®. Recently, the authors have explored new technique which combines both oral and intravenous contrast-enhanced ultrasonography (CEUS) imaging methods, called Double contrast-enhanced ultrasound, for evaluation both morphologic appearances and perfusion status of gastric abnormalities.

Research frontiers

Two-dimensional (2D) double contrast-enhanced ultrasound (DCUS) could clearly demonstrate normal gastric wall and differentiate normal gastric wall from benign and malignant lesions. Three-dimensional (3D) DCUS could intuitively display morphological features and vascularities of the lesions with multi-planar and volume views.

Innovations and breakthroughs

The oral contrast agent Xinzhang® used in this study is vegetable-based with main components being beans and starch, which is a uniform thin paste with pleasant taste and slow emptying feature without side effects, and thus is easily accepted by patients especially children and the elderly. DCUS is able to demonstrate both morphologic appearances and perfusion status of both normal and abnormal structures, which improves the ability of differential diagnosis. In the study, small lesions such as 0.5 cm diameter ulcer and 1.0 cm diameter stromal tumors can be identified.

Applications

The study results suggest that 2D and 3D double DCUS was safe, highly sensitive and specific and could be applied for evaluation of gastric lesions.

Peer review

This is an interesting and well written study aimed at assessing the role of contrast enhanced 2D and 3D US in the evaluation of gastric lesions. Even if it is a preliminary study and further prospective validation is needed, the manuscript is very clear and very well documented by images.

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A comparison of survival and pathologic features of non-alcoholic steatohepatitis and hepatitis C virus patients with hepatocellular carcinoma

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Abstract

AIM: To compare the clinical outcome and pathologic features of non-alcoholic steatohepatitis (NASH) patients with hepatocellular carcinoma (HCC) and hepatitis C virus (HCV) patients with HCC (another group in which HCC is commonly seen) undergoing liver transplantation.

METHODS: Patients transplanted for HCV and NASH at our institution from January 2000 to April 2011 were analyzed. All explanted liver histology and pre-transplant liver biopsies were examined by two specialist liver histopathologists. Patient demographics, disease free survival, explant liver characteristics and HCC features (tumour number, cumulative tumour size, vascular invasion and differentiation) were compared between HCV

and NASH liver transplant recipients.

RESULTS: A total of 102 patients with NASH and 283 patients with HCV were transplanted. The incidence of HCC in NASH transplant recipients was 16.7% (17/102). The incidence of HCC in HCV transplant recipients was 22.6% (64/283). Patients with NASH-HCC were statistically older than HCV-HCC patients ($P < 0.001$). A significantly higher proportion of HCV-HCC patients had vascular invasion (23.4% vs 6.4%, $P = 0.002$) and poorly differentiated HCC (4.7% vs 0%, $P < 0.001$) compared to the NASH-HCC group. A trend of poorer recurrence free survival at 5 years was seen in HCV-HCC patients compared to NASH-HCC who underwent a Liver transplantation ($P = 0.11$).

CONCLUSION: Patients transplanted for NASH-HCC appear to have less aggressive tumour features compared to those with HCV-HCC, which likely in part accounts for their improved recurrence free survival.

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Key words: Hepatitis C virus; Liver transplant; Hepatocellular carcinoma; Non-alcoholic steatohepatitis; Comparison; Recurrence; Vascular invasion; Poorly differentiated; Survival

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INTRODUCTION

The prevalence of obesity in North American Society continues to rise^[1]. With this increasing rate of obesity there has been a concomitant increase in the prevalence of non-alcoholic fatty liver disease (NAFLD)^[2]. The natural history of NAFLD is quite variable. It includes a spectrum ranging from reversible steatosis to steatohepatitis with hepatic fibrosis (NASH), and ultimately cirrhosis^[3-5]. Up to 30% of adults in North America and Western Europe are known to have excess fat accumulation in the liver^[6]. Of these, nearly 10% have NASH, which represents 2%-3% of all adults. There is speculation that NASH may soon become one of the main causes of End Stage Liver Disease (ESLD) requiring liver transplantation in North America^[7].

Hepatitis C virus (HCV) is one of the most common underlying liver diseases in hepatocellular carcinoma (HCC), accounting for about one-third of the cases of HCC in the United States^[8]. It is well established that patients with NASH can progress to develop HCC with previous reports suggesting that the 5 year prevalence may be as high as 7.6%^[9]. However as increasing numbers of HCC cases arising from NASH are being seen, it is important to clarify the outcomes and recurrence by comparing the clinical and pathological features of HCC due to NASH with those of HCC caused by one the more common underlying liver diseases in HCC, HCV infection, as a benchmark.

Previous studies have suggested that patients with NASH cirrhosis are less likely than those with HCV to get transplanted^[10]. This may be in large part to a higher likelihood of being denied listing for co-morbid conditions. Previous authors have shown that NASH patients with diabetes, hypertension, body mass index (BMI) > 30 years and age > 60 years undergoing liver transplantation have a poor (50%) 1 year mortality^[11]. However in appropriately selected NASH patients post liver transplant survival can fair at least as well as individuals who undergo transplant for other etiologies. The outcome of NASH patients with underlying HCC undergoing a liver transplantation compared to HCV patients with underlying HCC (another group in which HCC is commonly seen) has not been thoroughly investigated. Specifically the tumour characteristics in explanted livers and disease free survival between these groups have not been compared. The goal of the present study was to compare the clinical and pathological parameters as well as disease free survival in the two groups.

MATERIALS AND METHODS

We performed a retrospective review on all patients who underwent liver transplantation (LT) for HCV or NASH cirrhosis from January 2000 to April 2011 at our institution. Patients less than 18 years of age were excluded. Data on these patients were prospectively entered in our transplant database. In order to confirm or refute the

original histological diagnoses, all explanted liver histology and pre-transplant liver biopsies were re-examined by two specialist liver histopathologists who were blinded to the original diagnoses.

The etiology of the original liver disease was diagnosed by set criteria. NASH was determined to be the cause of chronic liver disease in patients with histological evidence of steatohepatitis in pre-transplant liver biopsies or in liver explants (steatosis, portal and/or lobular inflammation, hepatocyte ballooning, pericellular fibrosis and the presence of Mallory bodies)^[12,13], in conjunction with no history of alcohol consumption. HCV-related liver disease was confirmed by explants pathology and the presence of HCV RNA.

All patients with a pre and post-transplant diagnosis of HCC were identified in both the NASH and HCV groups. Listed patients with known HCC all fell within Milan Criteria^[14]. Patients who received pre-transplant radiofrequency ablation were excluded. HCC was confirmed histologically in the explanted liver. All Donation after Cardiac Death (DCD) organs were procured from controlled DCD donors using techniques previously published by our group^[15]. Primary outcomes were patient survival as well as pathologic features of HCC (tumour number, cumulative tumour size, vascular invasion and differentiation). Level of differentiation of HCC tumours was graded using the Modified Edmondson-Steiner grading system^[16]. Additional variables investigated included age at diagnosis, gender and α -feto-protein (AFP) levels. Recurrence free survival was taken at the time point of maximal follow-up.

Statistical analysis

All data are presented as means \pm SD. Differences between groups were analyzed using the unpaired *t* test for continuous variables and by the χ^2 test or continuity correction method for categorical variables. Survival curves for patient and graft survival were generated using the Kaplan-Meier method and compared by the log-rank test. All statistical tests were two-sided and differences were considered significant when $P < 0.05$.

RESULTS

A total of 832 liver transplants were performed at the London Health Sciences Centre during the study period. Of these, 283 (34.0%) recipients were positive for HCV based on the aforementioned criteria. NASH was the indication for liver transplantation in 96 (11.5%) recipients, and 42 (5.1%) recipients were diagnosed with 'cryptogenic' or 'idiopathic' cirrhosis. The remaining 411 (49.4%) recipients had liver failure due to other identifiable causes. Of the 42 cases originally diagnosed as cryptogenic cirrhosis, 6 were re-designated as NASH associated cirrhosis based on current histologic and clinical definitions. Thus the final analysis of HCV and NASH liver transplant recipients was: 283 (34.0%), 102 (12.3%) respectively (Figure 1).

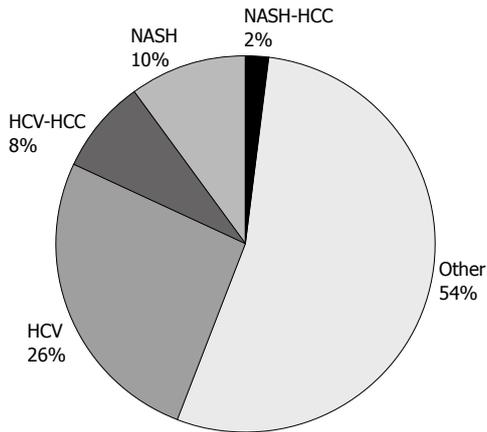


Figure 1 Diagnosis in patients undergoing liver transplantation. HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; NASH: Non-alcoholic steatohepatitis.

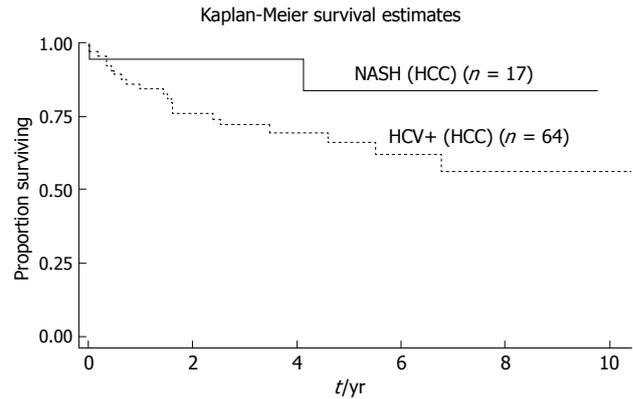


Figure 2 Recurrence free survival in non-alcoholic steatohepatitis-hepatocellular carcinoma vs hepatitis c virus-hepatocellular carcinoma groups. HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; NASH: Non-alcoholic steatohepatitis.

Table 1 Patient and tumour characteristics

	HCV/HCC n = 64	NASH/HCC n = 17	P value
Age at transplant (mean ± SD)	52.6 ± 5.8	58.6 ± 4.2	< 0.001
Gender (% male)	94%	94%	1.000
Donor source (DBD/DCD/LD)	56/8/0	16/0/1	NA
AFP (mean ± SD)	93.1 ± 204.4	20.3 ± 34.0	0.149
Number of tumours (mean ± SD)	1.59 ± 0.81	1.64 ± 0.75	0.819
Cumulative size of tumours (mean ± SD)	3.98 ± 2.4	3.27 ± 2.1	0.270
Vascular invasion	23.40%	6.30%	0.002
Poorly differentiated	4.70%	0%	< 0.001

AFP: α -feto-protein; DBD: Donation after brain death; DCD: Donation after circulatory death; LD: Living donor; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; NASH: Non-alcoholic steatohepatitis; NA: Not available.

The incidence of HCC in NASH recipients was 16.7% (17/102). Importantly, none of the re-categorized NASH patients who were originally designated cryptogenic were found to have HCC. The incidence of HCC in HCV liver transplant recipients at our centre was 22.6% (64/283).

Patients with NASH-HCC were statistically older than HCV-HCC patients (58.6 ± 4.2 years *vs* 52.6 ± 5.8 years, $P < 0.001$). There was no significant difference in gender or preoperative AFP level between the two groups. No patients with NASH-HCC received a DCD liver allograft (Table 1). The diagnosis of HCC was made before liver transplantation using multiple imaging techniques in 65% of NASH patients and 89% of HCV patients. HCC was more likely to be found incidentally in transplanted NASH patients (35%) than in transplanted HCV patients (11%) ($P = 0.015$).

Pathological characteristics of the NASH-HCC tumours were compared with those of HCV-HCC tumours (Table 1). A significantly higher proportion of HCV-HCC patients had vascular invasion (23.4% *vs* 6.4%, $P = 0.002$) as well as poorly differentiated tumours (4.7% *vs* 0%, $P < 0.001$) compared to the NASH-HCC. There was

no significant difference in the mean number of tumours or the mean cumulative size of the tumours between the two groups. In both groups, the tumours satisfied Milan criteria pre-transplantation.

Disease free survival at time of maximal follow-up was not statistically significant between the two groups however there was a clear trend towards lower disease free survival in the HCV-HCC group ($P = 0.11$, Figure 2).

DISCUSSION

The prevalence of NAFLD has continued to increase as the obesity epidemic continues. The rate of progression from NAFLD to the development of NASH or end stage liver disease is unknown. However, the frequency of NASH in patients listed for transplantation in North America has been previously determined to be 2.9%^[17]. This is likely an underestimate as this number was based on data collected from the 1990s, whereas in the last decade the rates of obesity and metabolic syndrome have increased dramatically. A more recent analysis of data from the Scientific Registry of Transplant Recipients (SRTR) reported that the rate had increased to 3.5%^[18]. In our series, 12.3% of patients have NASH as the diagnosis leading to liver failure requiring transplantation. NASH as a primary diagnosis in patients being listed or transplantation has continued to increase at our centre.

The natural history of NAFLD ranging from reversible steatosis to steatohepatitis with hepatic fibrosis (NASH), and ultimately the possibility of developing HCC has been previously described^[19]. In small previously published North American series of patients transplanted for NASH, HCC was found in 22% (2/9) of patients^[20]. In our series of 102 patients with NASH cirrhosis, 16.7% had HCC at the time of transplantation. This is a similar rate to the 22.6% of our HCV cirrhotic patients requiring transplantation, another well known high risk group for developing HCC. The high incidence of HCC in NASH patients undergoing liver transplantation suggests that these patients are at high risk of devel-

oping HCC and should undergo frequent ultrasound surveillance in a similar fashion to that performed in patients with HCV.

It has also been shown that patients with NASH are less likely to be listed for transplantation due to comorbidities, a justifiable practice given suboptimal results in NASH patients possessing these comorbidities^[10]. However in selected NASH patients receiving liver transplantation, others have shown that 5 year disease free survival after transplantation was significantly better than disease free survival in the C and B viral groups, 66%, 29% and 39% respectively^[21]. More recent studies have suggested there is no difference in 1 year and 3 year survival between patients transplanted for NASH and those transplanted for other indications^[22]. The outcome in patients with HCV is clearly affected by possible recurrence of HCV cirrhosis however NASH patients are also at risk for recurrence of NASH cirrhosis with some studies suggesting the incidence of recurrent NASH being as high as 25%^[23-25].

Patients with NASH and HCC post-transplant outcomes have not been previously investigated compared to patients with HCV and HCC (another group in which HCC is commonly seen). In previous studies looking at patients undergoing liver resection for HCC, cumulative survival after resection was comparable among HCV-HCC and NASH-HCC patient groups^[21].

In our study a significantly higher proportion of HCV-HCC patients had vascular invasion as well as poorly differentiated tumours compared to the NASH-HCC group. Vascular invasion is the strongest predictor of recurrence in patients with HCC^[26].

In the present study a trend of poorer recurrence free survival was seen in HCV-HCC patients compared to NASH-HCC who underwent a Liver transplantation. The higher proportion of patients with vascular invasion and poorly differentiated tumours may at least in part account for this difference in recurrence free survival. Based on our results it therefore appears that NASH associated HCC might be a less aggressive form of HCC compared with HCV associated HCC. It must also be entertained that some of the sickest NASH patients may not be listed for transplantation because of significant comorbidities or poor operative candidacy.

Limitations of the present study include its single centre nature as well as the lack of generalizability to non-transplant NASH and HCC populations due to the unique social and biologic factors of patients approved for transplantation.

In summary in those where NASH progresses to cirrhosis, there is a significant proportion that go on to develop HCC suggesting these individuals should undergo aggressive screening protocols directed toward the early detection of HCC, in a similar fashion to patients with HCV-cirrhosis. Patients with NASH-HCC undergoing liver transplantation also appear to be older than HCV-HCC patients undergoing liver transplantation. In appropriately selected patients with NASH and HCC post-transplant outcomes equal if not better than patients with

HCV-HCC (another group in which HCC is commonly seen). This may be related to less vascular invasion and less poorly differentiated pathology.

COMMENTS

Background

There is speculation that Non-alcoholic steatohepatitis (NASH) may soon become one of the main causes of end stage liver disease requiring liver transplantation in North America. The clinical outcome and pathologic features of NASH patients with hepatocellular carcinoma (HCC) undergoing liver transplantation compared with hepatitis c virus (HCV) patients with HCC (another group in which HCC is commonly seen) has not been thoroughly investigated.

Research frontiers

A significantly higher proportion of HCV-HCC patients had vascular invasion (23.4% vs 6.4%) and poorly differentiated HCC (4.7% vs 0%) compared to the NASH-HCC group. A trend of poorer recurrence free survival at 5 years was seen in HCV-HCC patients compared to NASH-HCC who underwent a Liver transplantation.

Innovations and breakthroughs

To knowledge, this represents the first study to compare the tumour characteristics in explanted livers and disease free survival between NASH-HCC and HCV-HCC patients undergoing liver transplantation.

Applications

In appropriately selected patients with NASH and HCC post-transplant outcomes equal if not better than patients with HCV HCC (another group in which HCC is commonly seen). This may be related to less vascular invasion and less poorly differentiated pathology.

Peer review

In this manuscript, the authors investigated the clinical outcome and pathologic features of NASH patients with HCC undergoing liver transplantation compared with HCV patients with HCC. This is a retrospective analysis. The originality of this study was not so high. Nonetheless, the data were properly presented and the manuscript was well prepared. The results of this study may provide useful information to the clinicians.

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Adjusting CA19-9 values to predict malignancy in obstructive jaundice: Influence of bilirubin and C-reactive protein

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Abstract

AIM: To find a possible relationship between inflammation and CA19-9 tumor marker by analyzing data from patients with benign jaundice (BJ) and malignant jaundice (MJ).

METHODS: All patients admitted for obstructive jaundice, in the period 2005-2009, were prospectively enrolled in the study, obtaining a total of 102 patients. On admission, all patients underwent complete standard blood test examinations including C-reactive protein (CRP), bilirubin, CA19-9. Patients were considered eligible for the study when they presented obstructive jaundice confirmed by instrumental examinations and increased serum bilirubin levels (total bilirubin > 2.0 mg/dL). The standard cut-off level for CA19-9 was 32 U/mL, whereas for CRP this was 1.5 mg/L. The CA19-9 level was adjusted by dividing it by the value of serum bilirubin or by the CRP value. The patients were divided

into 2 groups, MJ and BJ, and after the adjustment a comparison between the 2 groups of patients was performed. Sensitivity, specificity and positive predictive values were calculated before and after the adjustment.

RESULTS: Of the 102 patients, 51 were affected by BJ and 51 by MJ. Pathologic CA19-9 levels were found in 71.7% of the patients. In the group of 51 BJ patients there were 29 (56.9%) males and 22 (43.1%) females with a median age of 66 years (range 24-96 years), whereas in the MJ group there were 24 (47%) males and 27 (53%) females, with a mean age of 70 years (range 30-92 years). Pathologic CA19-9 serum level was found in 82.3% of MJ. CRP levels were pathologic in 66.6% of the patients with BJ and in 49% with MJ. Bilirubin and CA19-9 average levels were significantly higher in MJ compared with BJ ($P = 0.000$ and $P = 0.02$), while the CRP level was significantly higher in BJ ($P = 0.000$). Considering a CA19-9 cut-off level of 32 U/mL, 82.3% in the MJ group and 54.9% in the BJ group were positive for CA19-9 ($P = 0.002$). A CA19-9 cut-off of 100 U/mL increases the difference between the two groups: 35.3% in BJ and 68.6% in MJ ($P = 0.0007$). Adjusting the CA19-9 value by dividing it by serum bilirubin level meant that 21.5% in the BJ and 49% in the MJ group remained with a positive CA19-9 value ($P = 0.003$), while adjusting the CA19-9 value by dividing it by serum CRP value meant that 31.4% in the BJ group and 76.5% in the MJ group still had a positive CA19-9 value ($P = 0.000004$). Sensitivity, specificity, positive predictive values of CA19-9 > 32 U/mL were 82.3%, 45% and 59.1%; when the cut-off was CA19-9 > 100 U/mL they were, respectively, 68.6%, 64.7% and 66%. When the CA19-9 value was adjusted by dividing it by the bilirubin or CRP values, these became 49%, 78.4%, 69.4% and 76.5%, 68.6%, 70.9%, respectively.

CONCLUSION: The present study proposes CRP as a new and useful correction factor to improve the diag-

nostic value of the CA19-9 tumor marker in patients with cholestatic jaundice.

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Key words: Tumor marker; CA19-9; C-reactive protein; Bilirubin; Pancreato-biliary malignancy; Biliary stones

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INTRODUCTION

CA19-9 is a tumor marker that increases in pancreatic and biliary malignancy and it has been promoted as a reliable test for the detection of pancreato-biliary malignancy. In pancreatic cancer, CA19-9 has been reported to have 70%-80% sensitivity and 80%-90% specificity in tumor diagnosis, whereas in cholangiocarcinoma without history of sclerosing cholangitis the sensitivity and specificity are, respectively, 77.9% and 76.3%^[1-2]. CA19-9 is unfortunately increased not only in patients with pancreatic or biliary cancers but also in benign biliary diseases which often present with jaundice and is therefore often misleading, reducing significantly the diagnostic accuracy of this marker^[3-5]. The relationship between CA19-9 and jaundice has been analyzed and studied to find possible adjustments to increase the sensitivity, specificity and predictive value of the test in differential diagnosis of hepatobiliary diseases associated with jaundice. Therefore, some authors have suggested to adjust CA19-9 value by dividing it empirically by the serum bilirubin value^[6,7]. Other factors that could influence and alter the values of this tumor marker have not been studied yet. Inflammation contributes to elevating the CA19-9 value and it can be assessed by monitoring the acute-phase proteins: one of these is the C-reactive protein (CRP) which rises in response to infection, injury and neoplasm.

This research was stimulated by our own experience of patients presenting with jaundice of benign etiology who were found to have grossly elevated CA19-9 levels. The purpose of this study was to clarify the clinical interpretation and diagnostic value of an elevated serum CA19-9 level, with special reference to coexistent obstructive jaundice. The present study, first in the literature, analyzes a possible relationship between CA19-9, bilirubin and inflammation, expressed as CRP value, aiming to find a ratio or a better corrective factor to increase predictivity of CA19-9 and reduce the number of misleading false positive results.

MATERIALS AND METHODS

All the patients admitted for obstructive jaundice to the Department of Emergency Surgery, Cannizzaro Hospital, University of Catania, Italy, between September 2005 and September 2009, were considered for the present study. The patients were enrolled prospectively, 51 for each group of benign jaundice (BJ) and malignant jaundice (MJ) patients, obtaining a total of 102 patients. At admission, all patients underwent complete standard blood test examinations, including serum bilirubin levels. Patients were considered eligible for the study when they presented obstructive jaundice confirmed by instrumental examinations and increased bilirubin serum levels (total bilirubin > 2.0 mg/dL). Serum levels of CA19-9 and CRP were also measured at the time of admission. The standard cut-off level of CA19-9 was 32 U/mL, whereas for CRP this was 1.5 mg/L. The definitive diagnosis was obtained by instrumental examinations, surgical exploration and pathology. Instrumental examinations included ultrasonography, computed tomography scan, magnetic resonance imaging, endoscopic ultrasound scan and endoscopic retrograde cholangiopancreatography. The CA19-9 level was adjusted by dividing it by the value of serum bilirubin firstly, and then by the CRP value. The patients were divided into 2 groups: MJ and BJ. After the adjustment a comparison between the serum levels in the 2 groups of patients was obtained by Wilcoxon two sample test. Differences for categorical variables were assessed using the chi-square test or Fisher's exact test when adequate. Spearman correlation between the 3 parameters of CA19-9, bilirubin and CRP in each group of patients was computed. The receiver operating characteristic curve was used to establish the probability that a patient with MJ has a high value of CA19-9. A *P* value less than 0.05 was considered statistically significant.

RESULTS

The present longitudinal study analyzed a total of 102 patients: 53 (52%) men and 49 (48%) women with a median age of 69 years (range 24-96 years). In the group of 51 BJ patients there were 29 (56.9%) males and 22 (43.1%) females with a median age of 66 years (range 24-96 years), whereas in the MJ group there were 24 (47%) males and 27 (53%) females, with a median age of 70 years (range 30-92 years). Causes of BJ included: common bile duct (CBD) stones (66.6%), gallbladder stones with cholangitis (15.7%), biliary pancreatitis (15.7%), papillitis (1.9%). Causes of MJ included: pancreatic cancer (49%), bile duct cancer (19.6%), gallbladder cancer (13.7%), ampullary cancer (9.8%), intrahepatic cholangiocarcinoma (1.9%) and other malignancy (5.8%; 2 patients with metastatic nodes in porta hepatis and one patient with peritoneal carcinomatosis from ovarian cancer). Pathologic CA19-9 serum level was found in 82.3% of MJ, but in two patients the CA19-9 was 2.5 U/mL. CA19-9 did not correlate directly with the grade of biliary obstruction either expressed as bilirubin level in BJ and MJ (Figure 1). CRP

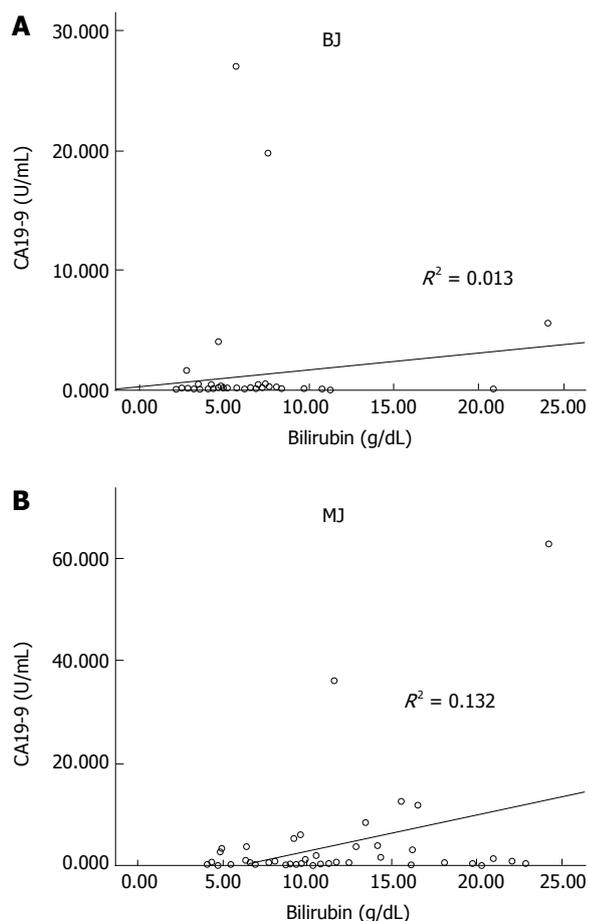


Figure 1 Correlation between total bilirubin and CA19-9 for benign jaundice (exponential line fit, $R^2 = 0.013$) (A) and malignant jaundice (exponential line fit, $R^2 = 0.132$) (B). BJ: Benign jaundice; MJ: Malignant jaundice.

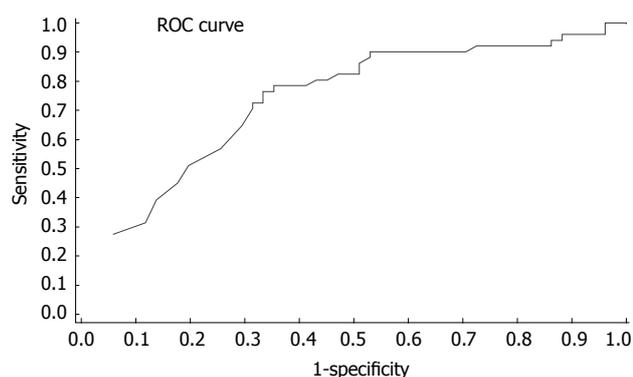


Figure 2 Receiver operating characteristic curve for biomarker CA19-9. ROC: Receiver operating characteristic.

levels were pathologic in 66.6% of the patients with BJ and in 49.0% with MJ. Mean bilirubin and CA19-9 levels were significantly higher in MJ compared to BJ, while the CRP level was significantly higher in BJ (Table 1). Table 2 reports the serum level of bilirubin, CA19-9 and CRP according to the cause of jaundice. There were no relevant differences in bilirubin according to the cause of jaundice. Comparing the CA19-9 value between patients with pancreatic cancer and patients with other causes of MJ and

	Benign	Malignant	P value
No. of patients	51	51	
Bilirubin serum level (median and range) (g/dL)	5.2 (2.12-24.03)	9.7 (4.07-24.25)	< 0.0001
95% CI = 5.0-7.2		95% CI = 9.6-12.5	
CA19-9 serum level (median and range) (U/mL)	36 (2-27019)	405 (2.5-62974)	0.02
95% CI = 0-2.508		95% CI = 699-6282	
> 32 (%)	28/51 (54.9)	42/51 (82.3)	0.003
> 100 (%)	18/51 (35.3)	35/51 (68.6)	0.0007
CRP (median and range) (U/mL)	6.91 (0.1-30)	2.97 (0.1-13.2)	0.0002
95% CI = 4.6-8.3		95% CI = 2.3-4.1	
> 1.5 (%)	34/51 (66.6)	25/51 (49.0)	0.005
> 5 (%)	21/51 (41.2)	7/51 (13.7)	0.004

CRP: C-reactive protein; CI: Confidence interval.

BJ, no difference was reported. CRP was significantly ($P = 0.04$) higher in patients with CBD stones than in pancreatic cancer, and patients with BJ and pancreatitis had the highest value of CRP. In MJ the average level of CRP was significantly higher in gallbladder cancer than in bile duct cancer, but not different from that in pancreatic cancer.

When considering the CA19-9 cut-off level of 32 U/mL, 42 of 51 patients (82.3%) in the malignant group and 28 of 51 (54.9%) in the benign group were positive for CA19-9 (Fisher's exact test: $P = 0.002$). Figure 2 shows that the area under the curve or probability that a patient diagnosed with MJ has a major value of CA19-9 compared to a patient diagnosed with BJ was 0.71. Increasing the cut-off level of CA19-9 to 100 U/mL, the difference between the two groups increases: 35.3% in BJ and 68.6% in MJ (Fisher's exact test: $P = 0.0007$). Changing the cut-off level alters the sensitivity and specificity as shown in Table 3, but by pushing up the cut-off level in spite of an increase of specificity we have obtained a reduction in the sensitivity of the test.

Adjusting the CA19-9 value by dividing it by serum bilirubin level, 11 of 51 patients (21.6%) in the BJ and 26 of 51 patients (51.0%) in the MJ group remained with a positive CA19-9 value (Fisher's exact test: $P = 0.003$), increasing the specificity to 78.4% but reducing the sensitivity to 49%.

The second mode of adjustment of the CA19-9 value was performed by dividing it by serum CRP value. Consequently, 16 of 51 patients (31.4%) in the BJ group and 39 of 51 patients (76.5%) in the MJ group remained with a positive CA19-9 value (Fisher's exact test: $P = 0.000004$). By this adjustment the sensitivity increases to 76.5%, the specificity to 68.6% and the positive predictive value (PPV) to 70.9%.

DISCUSSION

The diagnostic role of CA19-9 as a test for the detection of pancreato-biliary malignancy remains poorly defined, because, as in other diagnostic modalities, the utility of

Table 2 Bilirubin, CA19-9 and C-reactive protein levels according to jaundice cause at time of hospital admission *n* (%)

Cause	No. of cases	Bilirubin (g/dL), median	CA19-9 (U/mL)			CRP (U/mL)				
			Median	<i>P</i> value ¹	> 32	> 100	Median	<i>P</i> value ²	> 1.5	> 5
Malignant	51	9.7	405		42 (8.3)	35 (68.6)	2		25 (49)	7 (13.7)
Pancreatic cancer	25 (49)	11.29	461	-	23 (92)	19 (76)	1.96	0.04	12 (48)	3 (12)
Bile duct cancer	10 (19.6)	9.94	313	0.28	7 (70)	6 (60)	1.26	0.06	1 (10)	0
Gallbladder cancer	7 (13.7)	8.94	3361	0.59	6 (85.7)	5 (71.4)	4.13 ³	0.91	5 (71.4)	2 (28.5)
Ampullary cancer	5 (9.8)	6.49	196	0.42	4 (80)	3 (60)	4.5	0.69	4 (80)	1 (0.2)
Intrahepatic cholangio carcinoma	1 (1.9)	16.13	2600	NA	1	1	5.96	NA	1	1
Others	3 (5.8)	7.65	16	0.53	1	1	4.95	0.84	2 (66.6)	1 (33.3)
Benign	51	5.2	36		28 (54.9)	18 (35.3)	4.5		34 (66.6)	21 (41.2)
CBD stones	34 (66.6)	5.63	42	0.10	19 (55.8)	12 (35.2)	3.47	-	22 (64.7)	11 (32.3)
Gallbladder stones with cholangitis	8 (15.7)	5.26	55	0.54	4 (50)	3 (37.5)	2.31	0.73	5 (62.5)	3 (37.5)
Biliary pancreatitis	8 (15.7)	3.85	61	0.27	4 (50)	3 (37.5)	10.33	0.04	7 (87.5)	7 (87.5)
Chronic papillitis	1 (1.9)	6.6	35	NA	1	0	0.7	NA	0	0

¹*P* = 0.04 *vs* other cancers. ²Wilcoxon test compared to pancreatic cancer; ³Wilcoxon test compared to common bile duct (CBD) stones. CRP: C-reactive protein; NA: Not applicable.

Table 3 Adjustments of the value of CA19-9: Differences in sensitivity, specificity and positive predictive value (%)

	Sensitivity	Specificity	PPV
CA19-9 > 32 U/mL	82.3	45.0	59.1
CA19-9 > 100 U/mL	68.6	64.7	66.0
CA19-9/BIL	49.0	78.4	69.4
CA19-9/CRP	76.5	68.6	70.9

PPV: Positive predictive value; BIL: Bilirubin; CRP: C-reactive protein.

CA19-9 has several confounding limitations. Firstly, false positive elevations in CA19-9 exist in benign conditions such as liver diseases (primary sclerosing cholangitis, primary biliary cirrhosis, chronic hepatitis, acute liver failure), obstructive jaundice, pancreatitis^[3,5,8-11]. Even diseases not related to the hepatobiliary tract such as interstitial pulmonary disease^[12], collagen vascular diseases and, reportedly, heavy tea consumption^[4,13], suggest that CA19-9 may be expressed as a marker of a systemic inflammatory response. Furthermore, CA19-9 has also been shown to be upregulated in other malignant tumors including gastric, ovarian, and colorectal carcinoma^[14]. However, the most common cause of false positive CA19-9 is obstructive jaundice^[10]. Physiologically, biliary epithelial cells secrete mucins carrying the epitope of CA19-9, hence the high level of CA19-9 in serum during the obstructive jaundice, reflecting both inflammatory hypersecretion and leakage of biliary mucins into serum. This process can be reversed by resolution of the jaundice, which is often associated with a fall in CA19-9 greater in benign disease than in malignant^[3], because in malignant disease the synthesis of CA19-9 by proliferating cells contributes to the total level in a manner independent from any associated condition^[5,5]. In this series patient with malignant jaundice had higher mean levels of bilirubin than those with benign jaundice; additionally, the level of CA19-9 was significantly higher in malignant than in benign, especially if we considered a cut off level > 100 U/mL. Unfortunately, in our study,

CA19-9 does not correlate directly with the grade of biliary obstruction either in benign or in malignant jaundice (Figure 1), even if we observed that in patients with benign disease levels of CA19-9 decreased after relief of biliary obstruction (data not shown).

Secondly, the concentration of this tumor marker in the serum may be influenced by the patient's secretor status, because patients who are genotypically negative for the Lewis blood group antigen (a-, b-), approximately 4%-15% of the general population, do not synthesize CA19-9^[15]. As above in the present series the rate of MJ with a CA19-9 value less than 2 U/mL is 3.9%.

Furthermore, not only hyperbilirubinemia can obscure the clinical value of CA19-9, but inflammation can contribute and have a role. CRP, synthesized in hepatocytes, is one of the acute-phase proteins which are components of the innate immune responses that increase after infections, trauma, burns, tissue infarction, inflammatory process and tumors. In general, increased CRP levels in malignant disease could also be caused by an inflammatory response to tumor invasion^[16]. Padillo *et al*^[17], analysing CRP in 24 jaundice patients, found CRP levels significantly higher in patients with cancer. Differently from that study, this series showed the CRP serum levels are higher in benign obstructive jaundice than in malignant. Indeed, Table 2 reports that CRP is significantly (*P* = 0.04) higher in patients with CBD stones than those with pancreatic cancer; in particular, patients with BJ and pancreatitis have the highest value of CRP. Malignancy such as gallbladder cancer and intrahepatic cholangiocarcinoma present values of CRP comparable to BJ, probably due to an intensive inflammatory response to the tumor.

In our series, 54.9% of patients with benign jaundice had positive CA19-9 levels (cut-off 32 U/mL), and 35.5% had CA19-9 value over 100 U/mL; therefore, although the overall increase of the tumor marker in benign jaundice was inferior compared to that observed in malignancies, there was an overlap of values between cancer and non-cancer causes. This resulted in a low

accuracy of CA19-9 to diagnose pancreatic-biliary malignancies in patients with jaundice, especially since this marker is not able to distinguish pancreatic carcinoma from other malignancy or other benign causes of jaundice, as reported in Table 2, differing from what has been shown in other studies^[14,18]. Even when considering a cut-off level of 100 U/mL, the specificity is still 64.7%. As a result of this diagnostic overlap, the American Society of Clinical Oncology does not currently advocate its use for screening, evaluation of resectability or disease follow-up^[19]. For this reason some authors suggested to push up the cut-off level to 300 U/mL in presence of cholangitis and cholestasis to increase CA19-9 specificity, but this was associated with a significant decrease of sensitivity^[20]. To achieve a specificity of 100%, cut-off levels greater than 1000 U/mL should be considered^[21]. We recorded ten BJ patients with CA19-9 level greater than 300 U/mL and five BJ patients with more than 1000 U/mL, and all of them had serum bilirubin level lower than 8 mg/dL. These data can explain why there is no difference in the mean CA19-9 value between each cause of BJ and pancreatic cancer jaundice (Table 2). Indeed, multiple reports of patients with extremely high CA19-9 values in patients with BJ can be found in the literature^[22-24]. Several studies have shown that the association of elevated levels of CA19-9 with the diagnosis of cancer is significantly obscured in the face of obstructive jaundice, and because the bilirubin level correlates with CA19-9, they suggest that this value should be adjusted for hyperbilirubinemia^[10,11]. In our study CA19-9 does not correlate directly with the grade of biliary obstruction, either in BJ or in MJ.

Hence, based on the knowledge that in benign jaundice high levels of CA19-9 are an expression of obstruction and inflammation and CRP levels are higher in this group of patients, the most appropriate adjusting factor could be the CRP and not the bilirubin value. Indeed, 54.9% of patients with BJ had a CA19-9 level greater than 32 U/mL, and using a CA19-9 cut-off of 100 U/mL, the specificity of the test to detect malignancy is increased, with little decrease in the sensitivity. But the majority of benign jaundice patients have a CA19-9 value < 100 U/mL, so by adjusting this value with the CRP it is possible to increase the reliability of the test. As shown in Table 3 using the bilirubin as adjusting factor, even if the specificity reaches 78.4%, the sensitivity falls down to 49%; instead the CRP value better reflects the inflammatory status, obtaining 76.5% sensitivity, 68.6% specificity and 70.9% PPV. Certainly, the CA19-9 value has to be considered as an adjunctive value to the patient's history in the diagnosis of patients with obstructive jaundice, and its value, even after adjustment, should be helpful in planning the type and priority of further investigations with regard to the liver, bile ducts and pancreas.

In conclusion, the present study proposes adjusting to the CA19-9/CRP ratio as a new diagnostic tool in patients with cholestatic jaundice, which has not been reported in similar studies in the literature. This simple

ratio can significantly increase the specificity and the positive predictive value of CA19-9 in the differential diagnosis between malignant and benign jaundice. Other complementary studies of these markers are essential for diagnosis of malignant tumors when jaundice is present.

COMMENTS

Background

CA19-9 is a tumor marker which is increased in both benign and malignant hepatobiliary diseases. Previous studies have shown that CA19-9 has high sensitivity and specificity in pancreatic cancer. However, this tumor marker has been found to be elevated in benign biliary diseases as well. Hence the accuracy of CA19-9 is unreliable. The authors have tried to analyze the relationship between CA19-9, bilirubin and CRP levels to predict the accuracy of CA19-9 in malignant obstructive jaundice.

Research frontiers

After experiencing patients presenting with jaundice of benign etiology and having grossly elevated CA19-9 levels, the authors aimed to clarify the clinical interpretation and diagnostic value of an elevated serum CA19-9 level, with special reference to coexistent obstructive jaundice. CA19-9 is demonstrated to be influenced by bilirubin level, but other factors such as inflammation could influence and alter the values of this tumor marker. The research hotspot is how the inflammation influences the level of CA19-9.

Innovations and breakthroughs

In the past, the accuracy of CA19-9 level was improved by dividing it by bilirubin level, but the high level of CA19-9 in serum during obstructive jaundice reflects also an inflammatory hypersecretion. The inflammation can be assessed by monitoring the acute-phase proteins: one of these is the C-reactive protein (CRP). In this regard, in the present study, the CA19-9 level was adjusted by dividing it by the value of serum bilirubin and by the CRP value to look for a ratio or a better corrective factor to increase predictivity of CA19-9 and reduce the amount of misleading false positives. In fact, this adjustment increases the sensitivity to 76.5%, the specificity to 68.6% and the positive predictive value to 70.9%.

Applications

The study results suggest that considering inflammation as a reliable factor which increases the CA19-9 level in benign disease, abnormal tumor marker values in these patients can be corrected using the CRP in order to reduce the false positive results. In this way, it is possible to concentrate efforts in planning the type and priority of further investigations in the liver, bile ducts and pancreas in patients affected by malignancy.

Terminology

CA19-9: Tumor marker that increases in pancreatic and biliary malignancy, as physiologically biliary epithelial cells secrete mucins carrying the epitope of CA19-9; CRP: Synthesized in hepatocytes, is one of the acute-phase proteins, which are components of the innate immune responses that increase after infections, trauma, burns, tissue infarction, inflammatory process and tumors.

Peer review

This is a good longitudinal study in which authors analyze the relationship between CA19-9, bilirubin and CRP levels to predict the accuracy of CA19-9 in malignant obstructive jaundice. The results are interesting and suggest that the inflammation influences the CA19-9 value and reducing this confounding factor can help to better identify patients with malignant obstructive jaundice.

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Intrahepatic expression of genes related to metabotropic receptors in chronic hepatitis

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Abstract

AIM: To screen for genes related to metabotropic receptors that might be involved in the development of chronic hepatitis.

METHODS: Assessment of 20 genes associated with metabotropic receptors was performed in liver specimens obtained by punch biopsy from 12 patients with autoimmune and chronic hepatitis type B and C. For this purpose, a microarray with low integrity grade and with oligonucleotide DNA probes complementary to target transcripts was used. Evaluation of gene ex-

pression was performed in relation to transcript level, correlation between samples and grouping of clinical parameters used in chronic hepatitis assessment. Clinical markers of chronic hepatitis included alanine and aspartate aminotransferase, γ -glutamyltranspeptidase, alkaline phosphatase and cholinesterase activity, levels of iron ions, total cholesterol, triglycerides, albumin, glucose, hemoglobin, platelets, histological analysis of inflammatory and necrotic status, fibrosis according to METAVIR score, steatosis, as well as anthropometric body mass index, waist/hip index, percentage of adipose tissue and liver size in ultrasound examination. Gender, age, concomitant diseases and drugs were also taken into account. Validation of oligonucleotide microarray gene expression results was done with the use of quantitative real-time polymerase chain reaction (qRT-PCR).

RESULTS: The highest ($0.002 < P < 0.046$) expression among genes encoding main components of metabotropic receptor pathways, such as the α subunit of G-coupled protein, phosphoinositol-dependent protein kinase or arrestin was comparable to that of angiotensinogen synthesized in the liver. Carcinogenesis suppressor genes, such as chemokine ligand 4, transcription factor early growth response protein 1 and lysophosphatidic acid receptor, were characterized by the lowest expression ($0.002 < P < 0.046$), while the factor potentially triggering hepatic cancer, transcription factor *JUN-B*, had a 20-fold higher expression. The correlation between expression of genes of protein kinases PDPK1, phosphoinositide 3-kinase and protein kinase A (Spearman's coefficient range: 0.762-0.769) confirmed a functional link between these enzymes. Gender ($P = 0.0046$) and inflammation severity, measured by alanine aminotransferase activity ($P = 0.035$), were characterized by diverse metabotropic receptor gene expression patterns. The Pearson's coefficient ranging from -0.35 to 0.99 from the results of qRT-PCR and microarray indicated that qRT-PCR had certain

limitations as a validation tool for oligonucleotide microarray studies.

CONCLUSION: A microarray-based analysis of hepatocyte metabotropic G-protein-related gene expression can reveal the molecular basis of chronic hepatitis.

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Key words: Metabotropic receptors; Gene expression; DNA oligonucleotides; Quantitative real-time polymerase chain reaction; Chronic hepatitis

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INTRODUCTION

The natural course of chronic viral hepatitis is affected by progression of fibrosis and the risk of hepatocellular carcinoma development^[1]. Current data indicate that intracellular signaling disturbances have an impact on progression of inflammation and fibrosis, as well as carcinogenesis, in the course of chronic hepatitis.

G-protein-coupled receptors (GPCRs) are a family of cell surface receptors which receive, integrate and enhance the majority of extracellular signals. After stimulation with different signals, GPCRs activate amplifying enzymatic cascades, regulatory proteins and ion channels. This activation regulates cellular responses, including growth, proliferation, and cell survival.

In the present study, using microarray DNA analysis, we attempt to define genes related to metabotropic receptors associated with progression of chronic hepatitis.

DNA technology with genomic profiling and cluster analysis allows determination of the role of genes in the pathogenesis of liver injury^[2]. We assessed the activity of 20 genes encoding metabotropic receptors, some of which have been documented to have probable significance in the progression of chronic hepatitis.

The assessment of expression of genes of the main component of GPCR, such as the G-protein α subunit (*GNA5*), 3-phosphoinositide-dependent protein kinase-1 (*PDPK1*), phosphoinositide 3-kinase (*PIK3CG*), protein kinase A (*AKT1*) and arrestin β (*ARRB2*) was performed. We determined transcription factor *JUN-B*, ETS-domain protein (*ELK4*), early growth response protein 1 (*EGR1*) activated as the result of GPCR stimulation, angiotensinogen (*AGT*), which is a GPCR-ligand,

dual-specificity protein phosphatases 14 (*DUSP14*), which is responsible for dephosphorylation of kinase products, calcitonin receptor proteins (*CALCR*), thyrotropin receptor (*TSHR*), colony stimulating factor-3 (*CSF3*), sphingosine-1-phosphate receptor 1 (*EDG1*), and lysophosphatidic acid receptor (*EDG2*) associated with G-protein. The selection of *EDG2*, *EGR1*, *JUN-B*, chemokine (C-C motif) ligand 4 (*CCLA*), *ELK4* genes was based on their association with cellular proliferation, differentiation and apoptosis^[3-6]. Additionally, the selected group represented genes involved in regulation of inflammatory response and liver fibrosis, which included genes for interleukin 1 β (*IL-1 β*) and its receptors IL1 type I (*IL1R1*), IL1 type II (*IL1R2*), *CALCR*, *EDG1*, *CCLA*, *AGT* and adhesion molecules vascular cell adhesion molecule 1 (*VCAM1*)^[7-14].

MATERIALS AND METHODS

In the group of 12 patients (7 men, 5 women; age 36 ± 10.8 years) with chronic hepatitis type B (2 patients) and C (8 patients) and autoimmune hepatitis (2 patients), according to clinical indications, a liver biopsy was performed by the Menghini technique. From the obtained liver sections, a sample of 2-3 mm in length was frozen at -75°C until the analysis of mRNA of 20 selected genes was performed (gene list Figure 1). During the histopathological investigation of the biopsies, the degree of inflammation and fibrosis was assessed by the METAVIR score and steatosis by the steatosis scoring system.

On the day of liver biopsy, the activity of serum alanine aminotransferase (ALT/GPT Cobas, Roche Diagnostics, Mannheim, Germany), aspartate aminotransferase (ASPART/GOT Cobas), γ -glutamyltransferase (GGT Cobas), alkaline phosphatase (ALP Cobas), cholinesterase (CHE Cobas), the level of iron (Fe Cobas), total cholesterol (CHOD-PAP Cobas), triglycerides (TG Cobas), albumin (ALB plus Cobas), glucose (GLU Cobas), hemoglobin and platelet (Sysmex XE-2100, Sysmex Europe GMBH, Norderstedt, Germany) were assessed. Anthropometric measurements, including body mass index, waist/hip index and the percentage of fatty mass was assessed by skin fold thickness. The size of the liver was measured during ultrasound examination of the abdomen. Patient characteristics included the presence of concomitant illnesses, drug history, as well as the use of such substances as alcohol and cigarettes (Figure 2). In women, the menstrual cycle phase and menopause were taken into account. Biochemical, histological and anthropometric parameters are presented in Figure 2. The gene expression studies were performed using microarray (with low integration level), with DNA oligonucleotides complementary to the investigated transcripts. Total RNA was isolated from liver samples using the TRI Reagent (Sigma-Aldrich, St. Louis, United States), and then purified using the RNeasy MiniElute spin columns with a DNA eliminator (Qiagen, Hilden, Germany). The quantity of isolated RNA was assessed by a spectrophotometer (NanoDrop, Wilmington, United States), and subsequently its degradation level was

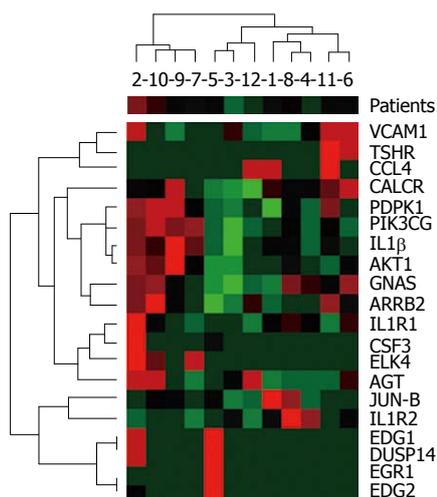


Figure 1 Agglomerative hierarchical clustering methods of genes expression. The dendrogram assigned to the numerical value on the horizontal axis describes the patients' correlation; the dendrogram assigned to individual genes on the vertical axis describes the genes' correlation. Figures on the x-axis denote patient order. *VCAM1*: Vascular cell adhesion molecule 1; *TSHR*: Thyroid stimulating hormone receptor; *CCL4*: Chemokine (C-C motif) ligand 4; *CALCR*: Calcitonin receptor; *PDPK1*: 3-phosphoinositide-dependent protein kinase-1; *PIK3CG*: Phosphoinositide 3-kinase; *IL1β*: Interleukin 1β; *AKT1*: Protein kinase A; *GNAS*: α subunit of G-coupled protein; *ARRB2*: Arrestin β; *IL1R1*: Interleukin 1 receptor, type I; *CSF3*: Colony stimulating factor-3; *ELK4*: ETS-domain protein; *AGT*: Angiotensinogen; *JUN-B*: Transcription factor jun-B; *IL1R2*: Interleukin 1 receptor, type II; *EDG1*: Sphingosine-1-phosphate receptor 1; *DUSP14*: Dual-specificity protein phosphatases 14; *EGR1*: Early growth response protein 1; *EDG2*: Lysophosphatidic acid receptor.

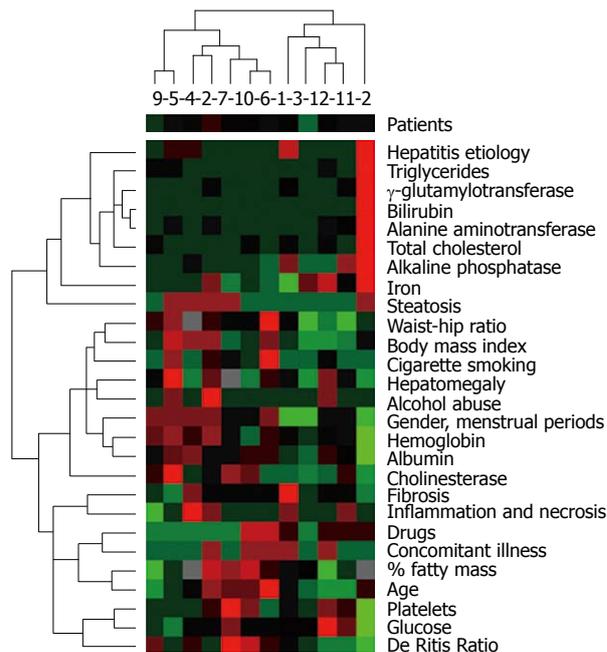


Figure 2 Agglomerative hierarchical clustering methods of clinical features expression. The dendrogram assigned to a numerical value on the horizontal axis describes the patients' correlation; the dendrogram assigned to individual clinical features on the vertical axis describes the clinical features' correlation. Figures on the x-axis denote patient order.

measured by a capillary electrophoresis system (Experion, Bio-Rad, Hercules, United States). For further analysis only samples without evidence of RNA degradation were qualified (RQI > 8.5 Rna Quality Index). Subsequently, based on the obtained RNA, probes were synthesized according to the manufacturer's instructions (SABiosciences, Frederick, United States), and then hybridized to a microarray. We used the Oligo GEArray Human GPCR Signaling Pathway Finder Microarray (OHS-071) supplied by SABiosciences. Detection of the array probes is achieved based on chemiluminescence, using the FujiLAS System (FujiFilm, Tokyo, Japan). The resulting images (the signal density) were quantified using the OligoAnalyser (SABiosciences). The obtained results describing the relative levels of gene expression (with respect to the reference gene) were further examined.

Diversification in gene expression was assessed by agglomerative hierarchical clustering methods. Using the Spearman's rank correlation coefficient for activity of the investigated genes, we searched for pairs of objects and then for clusters with the smallest distance (Figure 1). An analogous classification was carried out using biochemical, histological and anthropometric parameters (Figure 2). Determination of a direct correlation between gene expression and clinical features was done based on agglomerative hierarchical clustering of both the investigated indicators of chronic hepatitis. The genotype-phenotype distinction was analyzed using Fisher's exact test, to de-

termine the difference between clustering of patients achieved on the basis of a dendrogram of clinical signs and a dendrogram of gene expression (Figures 1 and 2). Differentiation of clinical parameters between groups of patients with the biggest difference in gene expression was performed by the Mann-Whitney *U* test and Fisher's exact test. In the present study, these were patients number 2, 10, 9, 7 *vs* the rest (Figure 1). The Wilcoxon signed-rank test was used to assess differential expression between the selected genes, to determine the three groups of genes with the highest, moderate, and lowest activity (Figure 3).

The results of the microarray experiment were verified by means of quantitative real-time polymerase chain reaction (qRT-PCR) for *IL1B*, *VCAM1*, *PIK3CG*, *AGT*, *PDPK1*, *GNAS*, *JUN-B*, *EDG2*, *CCL4*, *EGR1*, *IL1R1*, *IL1R2*, *CALCR*, *AKT1* and *ARRB2*. Total RNA acquired from the tissues of interest was reverse-transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, United States) for the 2-step qRT-PCR assays. The qRT-PCR was performed on a Chromo 4 (Bio-Rad), using the ABI SYBR Green master mix (Applied Biosystems). Primer sets against sequences of genes are indicated by microarray. They are commercially available at OriGene (Rockville, United States), but were additionally checked for specificity with National Center for Biotechnology Information (NCBI) The Basic Local Alignment Search Tool (BLAST). The optimum annealing temperature for each primer set was determined prior to the analysis of experimental samples. Following amplification, dissociation curves were

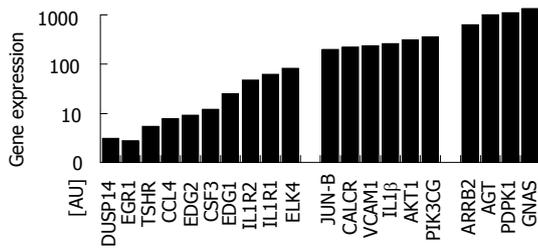


Figure 3 Arithmetic mean of gene activity. There was a statistically significant difference in the Wilcoxon signed-rank test between the genes with the highest expression, *GNAS*, *PDPK1*, *AGT*, and *ARRB2*, and moderate expression, *PIK3CG*, *AKT1*, *IL1 β* , *VCAM1*, *CALCR*, and *JUN-B*, and between the genes with moderate expression and the remaining genes with low expression.

analyzed for each reaction. A sample volume of 20 μ L was used for all assays which contained a 1X final concentration of SYBR green PCR master mix, 100 nmol gene specific primers, and 1 μ L of template. The assays were run using the following protocol: 95 $^{\circ}$ C for 10 min, 95 $^{\circ}$ C for 40 s, gene specific annealing temperature (58-62 $^{\circ}$ C) for 60 s for 40 cycles, followed by a gradual increase in temperature from 55 $^{\circ}$ C to 95 $^{\circ}$ C during the dissociation stage.

Following amplification, the instrument software was used to set the baseline and threshold for each reaction, as well as to determine the reaction efficiency. A cycle threshold (Ct) was assigned at the beginning of the logarithmic phase of PCR amplification and the difference in the Ct values [corrected for reaction efficiency: $Ct = Ct \times \log(\text{efficiency})/\log(2)$] of the housekeeping genes [mean Ct of glucuronidase (GUS) and beta-actin (BAKT)] and the gene of interest were used to determine the relative expression of the gene in each sample. Relative expression levels were then calculated as fold changes to the housekeeping genes, where each PCR cycle represented a 2-fold change.

The data of PCR and microarray experiments were then correlated using Pearson's coefficient.

The study was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association.

RESULTS

Using the agglomerative hierarchical clustering methods we found statistically significant correlations between the *EDG1*, *DUSP14*, *EGR1* and *EDG2* genes and *PDPK1*, *PIK3CG*, *AKT1*, as well as clustering, though not statistically significant, for *GNAS* and *ARRB2* (Figure 1). Spearman's rank correlation coefficients were 0.762 for *PDPK1* and *AKT1*, 0.769 ($P < 0.05$) for *PDPK1* and *PIK3CG*, 0.769 ($P < 0.05$) for *AKT1* and *PIK3CG*, and 0.699 ($P < 0.05$) for *GNAS* and *ARRB2*. In case of *EDG1*, *DUSP14*, *EGR1* and *EDG2*, in 10 out of 12 patients there was no transcriptional activity. Except for the transcriptional factor *JUN-B* and the stage of fibrosis $r = 0.667$ ($P < 0.05$), we did not observe any correlation between the activity of the selected genes and the in-

vestigated clinical factors. Assessing patient distribution based on dendrograms of the investigated genotype and phenotype, a difference on the border of statistical significance was found in Fisher's exact test ($P = 0.08$). The patients with the highest diversity in gene expression (Figure 1) showed a statistically significant difference in gender and ALT activity ($P = 0.0046$ and $P = 0.035$, respectively). Based on expression activity, the genes were divided into a group with high activity, which included *GNAS*, *PDPK1*, *AGT* and *ARRB2* and statistically differed ($0.002 < P < 0.046$) from the genes with moderate and low expression. The group of genes with moderate activity of *AKT1*, *PIK3CG*, *IL1 β* , *VCAM1*, *JUN-B* and *CALCR* also showed a statistically significant difference ($0.002 < P < 0.041$) compared with genes which were classified as belonging to the group with low expression (Figure 3).

For the following genes: *JUN-B*, *EDG2*, *CCL4* and *EGR1*, estimated with the use of microarrays and qRT-PCR, there was a strong positive correlation, with Pearson's coefficient within the range of 0.61-0.99. For other genes, *IL1B*, *VCAM1*, *PIK3CG*, *AGT*, *PDPK1*, and *GNAS*, the correlation was weakly positive, with Pearson's coefficient ranging from 0.07 to 0.43; and for *IL1R1*, *IL1R2*, *CALCR*, *AKT1*, and *ARRB2* the correlation of expression estimated with the two methods was weakly negative (Pearson's coefficient ranging from -0.1 to -0.35).

DISCUSSION

With the exception of the correlation between *JUN-B* and fibrosis stage, we did not reveal any direct relationship between expression of genes related to metabotropic receptors in hepatocytes and anthropometric, histological and biochemical parameters that are commonly used for monitoring progression of chronic hepatitis. In assessing the connection between gene expression and the investigated clinical features, we found an impact of gender and concentration of ALT in the serum on changes in gene expression. The effect of these two factors was detected as the result of the analysis of not any single, but rather all the investigated genes. In the probability test, it was gender which better determined changes in gene activity rather than ALT. In chronic hepatitis C and B, gender is the factor which defines the course and prognosis of the disease^[1]. Changes in gene expression resulting from gender differences can influence the progression of liver disease.

In the present study, among different markers of inflammation and fibrosis in the liver parenchyma, only the ALT level differentiated the gene activity. Among the investigated genes, there were important mediators of inflammation, including IL-1 β , with their receptors, as well as chemokines and adhesion factors. Also protein products of such genes as *EDG1*, *EDG2*, *CALCR* are involved in the induction of the inflammatory response^[9,15]. In contrast to ALT, a small histological difference used to assess the inflammatory process in the

liver, classified in the majority of cases as score 1 or 2 according to METAVIR, were not useful in assessing gene expression.

The reported values of genes expression and their statistical diversity allow distinction between three groups of genes with high, moderate and low activity. Because there is no direct correlation between gene activity and clinical markers of chronic hepatitis, it is difficult to determine if the observed gene expression is induced or constitutional.

In the group of genes with the highest activity there were genes of the main metabotropic receptor proteins, *GNAS*, *PDPK1* and *ARRB2*. Their high transcriptional activity is related to their essential role in the metabotropic receptor system. *GNAS* expression, despite individual differences, showed the highest correlation with *ARRB2* among the investigated genes. Proportional to the level of stimulation of GPCR, *ARRB2* triggers the mechanism of receptor internalization, which is an adaptation to overstimulation^[16]. Also mRNA encoding AGT was characterized by high expression. This observation remains in accordance with current literature and is associated with the liver being the main site of AGT synthesis^[17]. Because AGT is incorporated only indirectly by the renin-angiotensin system in the progression of liver fibrosis^[18,19], its gene activity did not correlate with the histological assessment of this process.

In the group of genes with moderate activity, there were genes which encode products involved in the pathogenesis of liver injury. Disturbances of *PIK3CG* described in chronic hepatitis as caused by NS5 protein of hepatitis C virus and HBx protein of hepatitis B virus destabilize and damage hepatocytes^[20,21]. However, in the present study, expression of *PIK3CG* did not correlate with the markers of liver injury.

As there were no cases with advanced fibrosis among the investigated patients, we did not observe any increased expression of *VCAM1*, which is characteristic only for liver cirrhosis^[22].

The correlation of expression of phosphatidylinositol kinases observed in the study confirms the functional association of the investigated enzymes. A significantly higher expression of *PDPK1* compared with *PIK3CG* is due to the amplification of *PDPK1* during phosphorylation catalyzed by *PIK3CG*. *PDPK1* in turn activates *AKT1* correspondingly; therefore, the 3-fold higher activity of *PDPK1* is noteworthy.

Among the investigated phosphatases that dephosphorylate active kinases, *DUSP14* was measured. However, this phosphatase, which is not functionally associated with the presently studied kinases, showed low expression values and was not correlated with the expression of kinases. Interestingly, a statistically significant correlation was determined between the stage of fibrosis and expression of transcriptional factor *JUN-B*. This factor, in the malfunctioning of the liver, is responsible for reprogramming of hepatocyte genes to the phase of cell proliferation^[23,24]. Its increase is frequently observed

in liver injury and hepatic carcinogenesis^[25].

The higher expression of this factor linked to carcinogenesis correlated with a low activity of the genes *CCLA*, *EGR1*, *EDG2* involved in the induction of apoptosis and suppression of neoplasia^[26].

The results obtained in the present study indicate that qRT-PCR has certain limitations as a validation tool for oligonucleotide microarray studies. Only four genes have shown a similar expression pattern between results obtained with the use of both techniques. A weak positive and negative correlation observed for other genes might result from potential pitfalls inherent in both approaches, and might be a source of errors encountered while employing each method.

However, the range of differences in the correlation coefficient observed in the present study remains within the range described in the literature, from -0.48 to 0.94^[27,28]. The results obtained in this study reflect the debate over which methods produce the most accurate measurements of gene expression.

In conclusion, gender and inflammation activity, as determined by ALT level, were associated with a more diverse pattern of metabotropic receptor gene expression. The highest gene expression was observed for mRNA of the main components of the metabotropic receptor pathway, such as *GNAS*, *PDPK1*, *ARRB2*, and correlated with mRNA of angiotensinogen synthesized in the liver. The correlation of expression of protein kinases *PDPK1*, *PIK3CG* and *AKT1* points to a functional association of these enzymes. The genes suppressing carcinogenesis, *CCLA*, *EGR*, *EDG2*, were characterized by the lowest expression levels among the investigated genes. On the other hand, *JUN-B*, a factor potentially involved in the development of hepatocellular cancer, was characterized by a 20-fold higher level of expression.

COMMENTS

Background

Metabotropic G protein-coupled receptors activate various signaling pathways, which trigger multiple sub-cellular reactions. Microarray-based analysis of expression of hepatocyte genes related to metabotropic receptors can reveal the molecular basis of liver diseases.

Research frontiers

The natural course of chronic viral hepatitis is associated with progression of fibrosis and the risk of hepatocellular carcinoma development. Current data indicate that intracellular signaling disturbances have an impact on progression of inflammation and fibrosis as well as carcinogenesis in the course of chronic hepatitis.

Innovations and breakthroughs

The highest gene expression was in the mRNAs of the main components of the metabotropic receptor pathways, such as the α subunit of G-coupled protein, phosphoinositide-dependent protein kinase (*PDPK1*) and arrestin β and correlated with the mRNA for angiotensinogen synthesized in liver. Carcinogenesis suppressor genes such as chemokine *CCL4*, transcription factor *EGR1* and lysophosphatidic acid receptor were characterized by the lowest expression, while the factor potentially triggering hepatic cancer, *JUN-B*, had 20-fold higher expression. Comparable expression of genes encoding protein kinases *PDPK1*, phosphoinositide 3-kinase and protein kinase A confirms a functional link between these enzymes. Gender and inflammation severity, measured by alanine aminotransferase activity, were characterized by different expression patterns

of genes related to metabotropic receptors.

Applications

Results of the presented work enables better delineation of mechanisms governing the course of chronic hepatitis and form the basis for future investigations.

Terminology

G-protein-coupled receptors are a family of the cell surface receptors, which receive, integrate and enhance most of the extracellular signals implicated in cell growth, proliferation, and survival. Microarray DNA technology with genomic profiling and cluster analysis allows determination of the role of genes in the pathogenesis of liver injury.

Peer review

This is a good descriptive study in which authors screen for genes related to metabotropic receptors family that might be involved in the development of chronic hepatitis. The results are interesting and suggest that a microarray-based analysis of hepatocyte metabotropic G protein-related gene expression can reveal the molecular basis of chronic hepatitis.

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Growth inhibitory effects of *Phyllanthus niruri* extracts in combination with cisplatin on cancer cell lines

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Abstract

AIM: To investigate the cytotoxic effects of spray-dried extracts of *Phyllanthus niruri* in combination with cisplatin on two cancer cell lines.

METHODS: Colorectal carcinoma (HT29) and human hepatocellular carcinoma (HepG2) cells were treated with spray-dried extracts of *Phyllanthus niruri* (SDEPN) either alone or in combination with cisplatin at different concentrations (0.5 mg/mL and 1 mg/mL) for 4 h and 24 h. To verify and quantify cancer cells treated with these products as well as identify the cell cycle stage and cell viability, we stained the cells with propidium iodide and assessed them by flow cytometry. The percentage of cells in different cell cycle phases was quantified and data were expressed as histograms. Significant differences between groups were determined using analysis of variance and Bonferroni's test, as indicated. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS: SDEPN had significantly different cytotoxic effects on HT29 (2.81 ± 0.11 vs 3.51 ± 1.13 , $P > 0.05$) and HepG2 (5.07 ± 0.3 vs 15.9 ± 1.04 , $P < 0.001$) cells when compared to control cells for 4 h. SDEPN also had significantly different cytotoxic effects on HT29 (1.91 ± 0.57 vs 4.53 ± 1.22 , $P > 0.05$) and HepG2 (14.56 ± 1.6 vs 35.67 ± 3.94 , $P < 0.001$) cells when compared to control cells for 24 h. Both cell lines were killed by cisplatin in a dose-dependent manner compared to control cells (HepG2 cells for 4 h: 10.78 ± 1.58 vs 53.89 ± 1.53 , $P < 0.001$; 24 h: 8.9 ± 1.43 vs 62.78 ± 1.87 , $P < 0.001$ and HT29 cells for 4 h: 9.52 ± 0.913 vs 49.86 ± 2.89 , $P < 0.001$; 24 h: 11.78 ± 1.05 vs 53.34 ± 2.65 , $P < 0.001$). In HT29 cells, pretreatment with SDEPN and subsequent treatment with cis-

platin resulted in a greater number of cells being killed (12.78 ± 1.01 vs 93.76 ± 1.6 , $P < 0.001$). HepG2 cells showed significant cell killing with treatment with SDEPN when combined with cisplatin (12.87 ± 2.78 vs 78.8 ± 3.02 , $P < 0.001$).

CONCLUSION: SDEPN is selectively toxic against two cancer cell lines. Moreover, SDEPN in combination with cisplatin induces a synergistic increase in the cell death of both HT29 and HepG2 cells.

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Key words: Cisplatin; Colorectal cancer; Liver cancer; *Phyllanthus niruri*; Cytotoxic effect

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INTRODUCTION

Phyllanthus niruri has many effective traditional uses for a wide variety of diseases. Some of the medicinal uses have been supported in experimental models, suggesting that the plant extracts possess various pharmacological properties. Due to its impressive preclinical therapeutic potential, extracts of species of the genus *Phyllanthus* have been evaluated to treat hypertension, jaundice, diabetes, hypercalciuria, and urolithiasis^[1]. Other studies revealed preclinical pharmacological activity and therapeutic potential of phytochemicals isolated from *Phyllanthus niruri*. The species has demonstrated an antimutagenic and anticarcinogenic action^[2], antitumor^[3], antioxidant^[4], hepatoprotective^[5,6] and antihyperuricemic properties^[7], as well as antihyperlipemic activity^[8,9].

Phytochemicals exhibit different structural characteristics with various pharmacological actions. For example, lignans have excellent hepatoprotective^[10,11] and anti-viral properties^[12], whereas terpenes exhibit anti-microbial activities^[13]. Flavonoids from *Phyllanthus niruri* have been shown to have antioxidant^[14], antileishmanial^[15], and anti-inflammatory properties^[16]. Phytochemical studies have shown that extracts of genus *Phyllanthus* contain a variety of components, including gallic acid^[1,17]. Furthermore, studies have demonstrated cytotoxic activity of gallic acid on the human promyelocytic leukemia HL-60 cell lines^[18,19]. Gallic acid has also been shown to induce apoptotic cell death in HSC-2 and HL-60 cells^[20].

More importantly, no side effects or toxicities have been reported for this herb after many years of research. Although extensive research has been conducted, there is still an abundance of unanswered questions regarding *Phyllanthus niruri*, particularly towards understanding the mechanism of biological activity of phytochemicals from the herb with emphasis on components that have anti-human immunodeficiency virus (HIV)^[21] and anti-hepatitis B^[22] properties. *Phyllanthus niruri* has been found to exhibit a marked inhibitory effect on the hepatitis B virus, as evidenced by its exhaustive utility in cases of chronic jaundice. However, to date, no research has focused on the identification and validation of active pharmacophores of *Phyllanthus niruri* that are responsible for the reported inhibitory effect of its aqueous extract on HIV^[21]. Investigations have examined anti-HIV effects of the alkaloidal extract from *Phyllanthus niruri* on human cell lines. An alkaloidal extract of *Phyllanthus niruri* showed suppressive activity on strains of HIV-1 cultured on the huT-4 cell line^[21].

Directed studies of botanical extracts may lead to the discovery of new agents with improved and intriguing pharmacological properties. This may be achieved by molecular modeling studies that assess the interactions of bioactive phytochemicals from *Phyllanthus niruri* with their respective molecular targets. Moreover, upon improvement of binding affinity to the specified target by virtual chemical modification of existing pharmacophores, new small molecules may be identified and synthesized in the laboratory^[23].

Other species of the genus *Phyllanthus*, such as *Phyllanthus polyphyllus* and *Phyllanthus emblica*, have demonstrated growth inhibitory activity with a certain degree of selectivity towards the two cancer cell lines tested. It has been previously shown that cisplatin can inhibit the growth of colorectal carcinoma (HT29) and human hepatocellular carcinoma (HepG2) cells in a dose-dependent manner^[20,24,25].

In this study, we investigated the antiproliferative or cell-killing activities of spray-dried extracts of *Phyllanthus niruri* on the human colorectal carcinoma HT29 and human hepatocellular HepG2 cell lines. In addition, we assessed the capacity of these extracts to potentiate the action of cis-diamminedichloroplatinum II (cisplatin).

MATERIALS AND METHODS

Preparation of *Phyllanthus niruri* spray dried extracts

A spray-dried extract of *Phyllanthus niruri* (SDEPN) containing 12.33 mg/g of gallic acid^[26] and 94.4 mg/g of total flavonoids^[27] was prepared following the manufacturer's instructions using a Spray Dryer Production Minor (GEA Niro, Denmark).

Cell culture

The human colorectal cancer cell line HT-29 and human hepatocellular carcinoma cell line HepG2 were

purchased from the Culture Collection of the Faculty of Medicine, University of São Paulo. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc., Grand Island, NY, United States) supplemented with 10% (v/v) heat-inactivated fetal bovine serum.

Flow cytometry

To verify and quantify cells treated with SDEPN extract and to identify the cell cycle stage and cell viability, we stained cells with propidium iodide (PI). PI emits different fluorescence intensities depending on the phase of the cell cycle, which in turn are captured by the photomultiplier detectors in a flow cytometer (BD Facs canto II). Cancer cells were plated in 6-well plates at a cell density of 5×10^5 cells/well in 2 mL of medium. Cells were treated with SDEPN alone, cisplatin alone, or SDEPN followed by cisplatin. After 24 h, the cells were treated with a series of SDEPN concentrations (0-75 mmol/L) for 4 h or 24 h. After this incubation, the medium containing SDEPN was replaced with medium containing 2.5 or 12 μ mol/L cisplatin. The cells were harvested by trypsinization when they reached approximately 80% confluence. The cells were placed in 70% ethanol and centrifuged for 5 min at 300 g. They were then resuspended in 200 μ L of a PI solution (20 mL PBS, 20 μ L 0.1% Triton X-100, 200 mg/mL RNase, and 20 μ g/mL PI), placed in FACS tubes, and incubated for 30 min at room temperature protected from light. The labeled cells were captured with a FACScalibur cytometer (BD Bioscience, Franklin Lakes, NJ, United States) and analyzed with the software CELLQuest™ (BD Biosciences). The percentage of cells in different cell cycle phases was quantified and data were expressed as histograms.

Statistical analysis

All experiments were performed at least in triplicate. The significance of differences between groups was calculated by applying analysis of variance and Bonferroni's test, as indicated. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Cytotoxic effects of SDEPN on HT29 and HepG2 cells

The effects of SDEPN on cell killing in two cancer cell lines were determined by flow cytometry. All cell lines were killed in a dose-dependent manner after exposure to the plant extracts (Figure 1). SDEPN had significantly different cytotoxic effects on HT29 (2.81 ± 0.11 vs 3.51 ± 1.13 , $P > 0.05$) and HepG2 (5.07 ± 0.3 vs 15.9 ± 1.04 , $P < 0.001$) cells when compared to control cells for 4 h. SDEPN also had significantly different cytotoxic effects on HT29 (1.91 ± 0.57 vs 4.53 ± 1.22 , $P > 0.05$) and HepG2 (14.56 ± 1.6 vs 35.67 ± 3.94 , $P < 0.001$) cells when compared to control cells for 24 h. These results indicated that the plant extract was selectively toxic against the cancer cells tested. We therefore hypoth-

esized that the combination of these plant extracts with chemotherapeutic drugs may have a synergistic cytotoxic effect on these cells. Primary flow cytometry analyses are shown in Figure 2.

The effect of cisplatin on HT29 and HepG2 cells

Using flow cytometry, we assessed the effects of cisplatin on the proliferation of HepG2 and HT29 cells. Both cell lines were killed in a dose-dependent manner compared to control cells (HepG2 cells for 4 h: 10.78 ± 1.58 vs 53.89 ± 1.53 , $P < 0.001$; 24 h: 8.9 ± 1.43 vs 62.78 ± 1.87 , $P < 0.001$ and HT29 cells for 4 h: 9.52 ± 0.913 vs 49.86 ± 2.89 , $P < 0.001$; 24 h: 11.78 ± 1.05 vs 53.34 ± 2.65 , $P < 0.001$; Figure 1). Primary flow cytometry analyses are shown in Figure 2.

The combination of cisplatin and Phyllanthus niruri extracts has a synergistic cytotoxic effect on HT29 and HepG2 cells

HT29 cells did not undergo cell death when SDEPN was used alone ($P > 0.05$) but HepG2 cells ($P < 0.001$) showed significant cell killing with treatment with SDEPN alone (Figure 1), but also very significantly (12.87 ± 2.78 vs 78.8 ± 3.02 , $P < 0.001$) when combined with cisplatin (Figure 1). However, HT29 cells underwent cell death (12.78 ± 1.01 vs 93.76 ± 1.6 , $P < 0.001$) when treated with SDEPN for 24 h prior to cisplatin treatment (Figure 1). The primary images of flow cytometry analysis can be seen in Figure 2.

In HT29 cells, pretreatment with SDEPN and subsequent treatment with cisplatin resulted in a greater number of cells being killed compared to treatment with cisplatin or SDEPN alone (Figure 1A and B). This increase in the number of HepG2 cells killed in response to combination treatment was observed at the lower concentration of SDEPN (Figure 1C and D). Therefore, pretreatment with SDEPN allowed the cells (HT29) to be sensitized to killing induced by cisplatin. This suggests that this cell type is more able to adapt to stressful conditions.

DISCUSSION

In this study, the growth inhibitory activity of SDEPN extract alone and in combination with cisplatin was investigated in HT29 and HepG2 cells. Our results indicated that SDEPN extract significantly inhibited the growth of both cell lines in a dose-dependent manner when combined with cisplatin.

Cisplatin is a well-known cancer therapeutic agent that causes high toxicity to normal tissues during cancer therapy, inducing cell death through interaction of the platinum complex with DNA molecules which induces crosslinking in DNA^[28,29]. The platinum compound is used to treat various types of human solid tumors^[30]. The major limitation to clinical use of cisplatin is the adverse effects, mainly nephrotoxicity, caused by the compound^[31]. In addition to DNA interactions, cisplatin

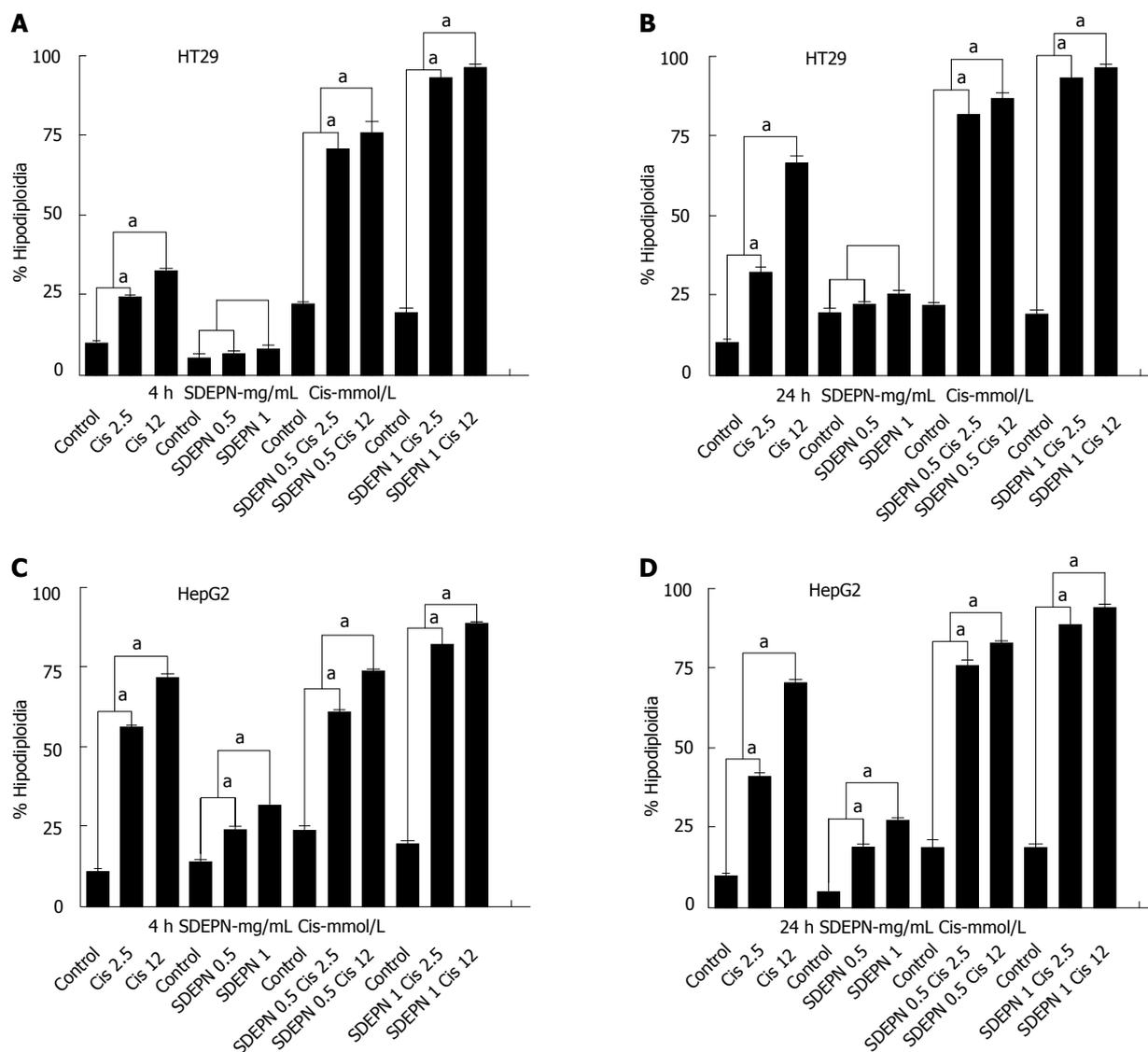


Figure 1 HT29 cells and HepG2 cells were treated for 4 h or 24 h with cisplatin alone, spray-dried extract of *Phyllanthus niruri* alone, or both. A, C: 4 h; B, D: 24 h. ^a $P < 0.05$ vs control group. SDEPN: Spray-dried extract of *Phyllanthus niruri*; Cis: Cisplatin; HT29: Colorectal carcinoma; HepG2: Human hepatocellular carcinoma.

is capable of generating oxidative stress through mitochondrial dysfunction, which results in the increased production of reactive oxygen species (ROS)^[32]. Therefore, our data show that HT29 cells are more resistant to SDEPN- or cisplatin-mediated cell death, and this resistance may be related to their ability to adapt to stressful conditions. In addition, HepG2 cells were more sensitive to both treatments, suggesting that these cells may be less adapted to stress. In this study we found that the combination of SDEPN with cisplatin induced growth inhibition and cell death at different dose levels in HT29 and HepG2 cell lines.

The mechanism of action of some plant extracts is still unclear, since they are not a single active ingredient but rather consist of multiple compounds that could potentially induce the observed effects^[1,23]. Plant-derived polyphenols, including gallic acid^[33,34] and tannins^[35], were reported to be the main components of *Phyllanthus*

niruri extracts^[1]. Therefore, the effects of SDEPN may induce apoptosis and cell death in the cell lines used in this study through multiple pathways due to the multiple components present in the extract. The active ingredients in *Phyllanthus niruri* that exert anticancer effects may include polyphenols, such as gallic acid, flavanoids or tannins, which are abundant in the herb. However, the detailed mechanism responsible for the anticancer effect of SDEPN extract and identification of the actual functional components require further elucidation.

It has been previously demonstrated that the effect of gallic acid on cancer cells, particularly lung cancer cells, involves caspase activation and oxidative processes^[33]. In addition, gallic acid has the capacity to induce apoptosis and increase the efficacy of cisplatin in LL-2 lung cancer cells^[34].

Our study provides corroborative evidence that SDEPN has selective cytotoxic effects against HT29 and HepG2

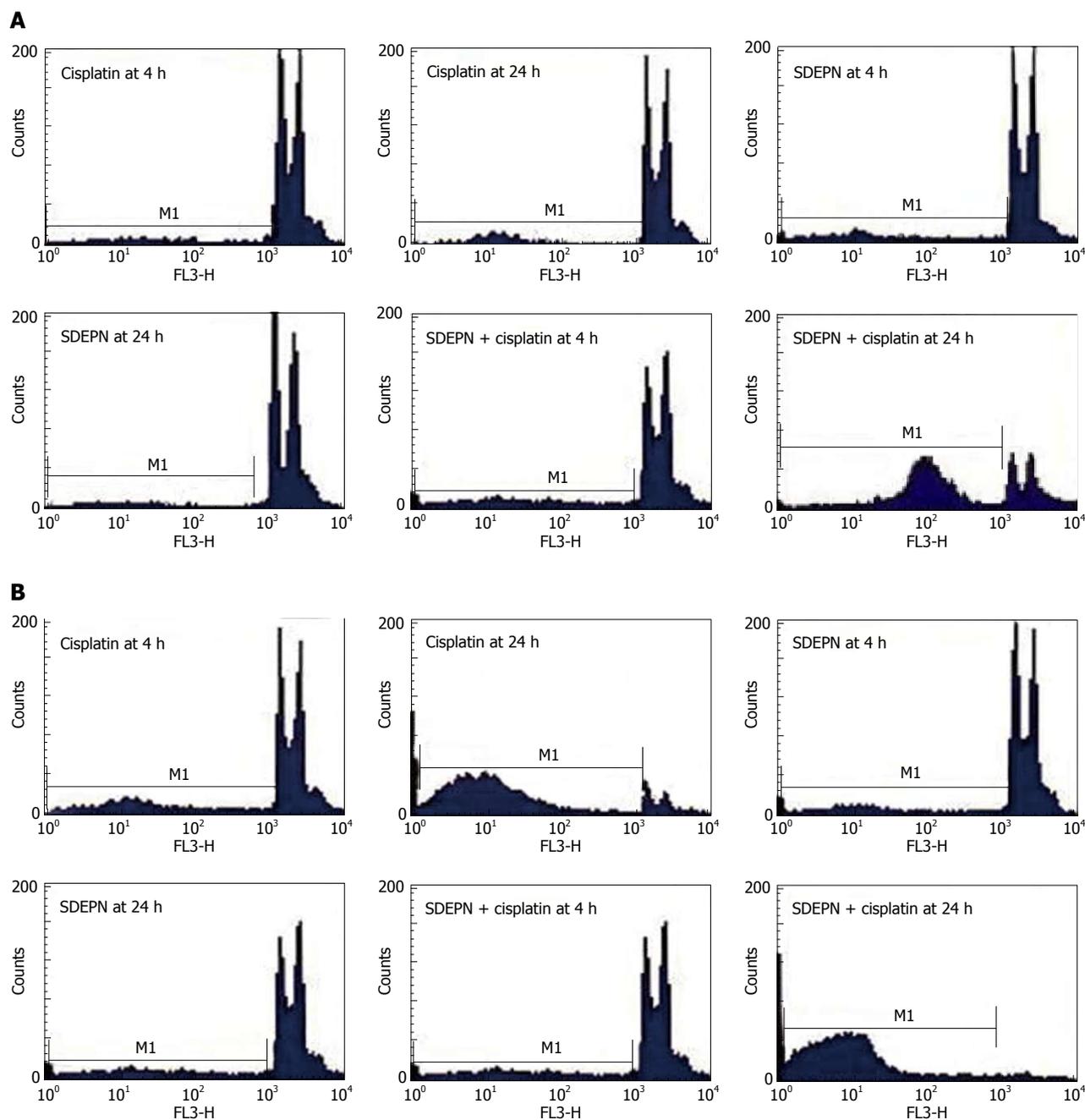


Figure 2 Effects of cisplatin, spray-dried extract of *Phyllanthus niruri*, or both on HT29 cells or HepG2 cells as assessed by flow cytometry. A: HT29 cells; B: HepG2 cells.

cells. Moreover, a synergistic effect was seen when the cells were treated with the extract in combination with cisplatin. This finding supports our hypothesis that combinations of plant extracts and chemotherapeutic agents may allow for a reduction in the dosage of the more toxic chemotherapeutic agent while retaining the therapeutic efficacy and minimizing toxicities. The induction of cell death by SDEPN may increase the sensitivity of HT29 cells to cisplatin-mediated cytotoxicity. Other species of the genus *Phyllanthus* have demonstrated growth inhibitory activity with a certain degree of selectivity towards the two cancer cell lines tested^[20].

The findings of this study support our hypothesis

that combinations of plant extracts and chemotherapeutic agents allow for a reduction in the dosage of the latter (i.e., cisplatin), while maintaining therapeutic efficacy. Moreover, the induction of cell death by SDEPN may be a strategy for increasing the sensitivity of HT29 cells to cisplatin-mediated cell death.

COMMENTS

Background

Phyllanthus niruri is a widespread tropical plant that is commonly found in coastal areas of Mexico, Argentina, and Brazil. In Brazil, some plant extracts have been distributed to patients by the Public Health Programme since 2010

as an adjunctive therapy for various diseases. The extract from aerial sections of *Phyllanthus niruri* was included in this list due to the long history of use in traditional medicine for the treatment of kidney and bladder diseases, intestinal infections, diabetes, and hepatitis B. Furthermore, this study has demonstrated an *in vitro* pro-death potential in cancer cells when *Phyllanthus niruri* is associated with cisplatin. *In vivo* studies are still needed to confirm our findings.

Research frontiers

Currently, a variety of effective phytochemicals have been tested in cancer treatment, which are used alone or in combination with chemotherapeutic agents for treatment. A spray-dried extract from *Phyllanthus niruri* (SDEPN) was found to be selectively toxic against two cancer cell lines and induced an increase in cell death of HT29 and HepG2 cells when used in combination with cisplatin. HT29 cells did not undergo cell death when SDEPN was used alone. The mechanism of this effect is currently unknown, but may involve apoptosis, since a major component of SDEPN extract is gallic acid, which has been shown to induce apoptotic pathways.

Innovations and breakthroughs

In this report, the authors have demonstrated that combinations of SDEPN with cisplatin in HT29 and HepG2 cell lines have a more potent effect than either agent alone.

Applications

Spray-dried extract of SDEPN appears to be cytotoxic against hepatocellular carcinoma and less toxic against colorectal carcinoma. A combination of SDEPN extract with a chemotherapeutic agent may therefore enhance the efficacy of each component against these cancers. Future *in vivo* studies will be performed by our group to study the anticancer activity of SDEPN alone and in combination with cisplatin as well as the toxicity against normal tissues.

Terminology

Dried herbal extracts are widely used as therapeutic products with improved pharmaceutical properties, such as stability and content uniformity. Additionally, dried extracts are successfully used to obtain solid dosage forms that contain a high dose of herbal extracts, such as capsules and tablets. Therefore, spray drying is the most commonly used procedure to obtain dried extracts (spray-dried extracts) in the herbal processing industry.

Peer review

The manuscript is well written and the findings are interesting to some extent. However, some concerns need to be addressed.

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Sensitivity of the suspected blood indicator: An experimental study

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Abstract

AIM: To investigate whether suspected blood indicator (SBI) in capsule endoscopy (CE) is affected by background color and capsule passage velocity.

METHODS: Experimental models of the small intestine constructed from paper in a variety of colors were used to simulate the background colors observed in CE images. The background colors studied included very pale yellow, yellow, very pale magenta, light grayish pink, burnt sienna, and deep and dark brown, and red spots were attached inside them. An endoscopic capsule was manually passed through the models. The rate of detection of the red spots by the SBI was evaluated based on the colors of the models and the capsule passage velocities (0.5 cm/s, 1 cm/s, and 2 cm/s).

RESULTS: The rate of detection of the red spots by

the SBI differed significantly according to the background color of the model ($P < 0.001$). Detection rates were highest for backgrounds of very pale magenta, burnt sienna, and yellow, in that order. They were lowest for backgrounds of dark brown and very pale yellow. The rate of detection of red spots by the SBI tended to decrease at rapid capsule passage velocities (1-2 cm/s) compared to slow velocities (0.5 cm/s) for backgrounds of very pale yellow ($P = 0.042$), yellow ($P = 0.001$), very pale magenta ($P = 0.002$), and burnt sienna ($P = 0.001$). No significant differences in the rate of detection were observed according to velocity for light grayish pink ($P = 0.643$) or dark brown ($P = 0.396$).

CONCLUSION: SBI sensitivity was affected by background color and capsule passage velocity in the models. These findings may facilitate the rapid detection of bleeding lesions by CE.

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Key words: Capsule endoscopy; Suspected blood indicator; Sensitivity; Background color; Passage velocity

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INTRODUCTION

Capsule endoscopy (CE) is a useful method for the diagnosis of small bowel diseases, such as gastrointestinal bleeding of an unknown cause^[1-7]. However, it is relatively

time consuming to examine and interpret the results. To reduce the reading time of this procedure, the RAPID software (Given Imaging Ltd., Duluth, GA, United States) contains a suspected blood identification system, which identifies hemorrhages and suspicious vascular lesions by recognizing red-colored pixels against different colored backgrounds that may be encountered in the small intestine^[8].

Reports on the usefulness of the suspected blood indicator (SBI) generally show a low and variable overall sensitivity for lesions, ranging from 20% to 56.4%^[8-12]. Positive predictive values are also observed to be variable, from 24% to 90.3%. Therefore, SBI generates false-positive and false-negative results, and its clinical usefulness has not been verified.

Active bleeding is the most important factor affecting SBI sensitivity. The sensitivity increases to a range of 58.3% to 93% in cases of active bleeding. Additionally, the sensitivity of SBI may also be affected by other factors. However, not all of the factors that can affect SBI sensitivity have been fully assessed.

The RAPID software includes a tissue color bar function that represents the average color of the region of interest in the intestine and provides information to help determine the anatomical location of a lesion^[13]. When the small intestine is observed *via* CE, on-screen images are primarily composed of the intestinal mucosa and liquid present in the lumen. The background color behind a lesion may vary according to the color of the small intestinal mucosa, which is affected by patient factors, such as hemoglobin and bilirubin levels. Thus, very pale magenta, which is the normal mucous membrane color, can appear very pale yellow in patients with anemia or burnt sienna or deep brown in patients with jaundice. The background color can also be affected by the presence of intestinal fluid. The color and the degree of transparency of the fluid can vary depending on the presence of bile, debris, stool residue, and blood in the intestine. Therefore, a combination of these elements may produce many different colors that can be presented on screen during CE. The various background colors of lesions may influence the detection of SBI in CE images.

The velocity of the passage of a capsule through the small intestine can vary depending on the presence of underlying disease, such as diabetes and disorders of intestinal peristalsis^[14]. Furthermore, the passage velocity can vary in different sections of the small intestine. Capsule movement may be influenced regionally by the gastric emptying time, chronic intestinal motility disorders, small bowel obstruction, intestinal diverticulosis, and other factors^[15]. These differences in capsule passage velocity according to the clinical circumstances may also affect SBI sensitivity. It would be useful to examine the different factors that influence the sensitivity of the SBI for shortening the time required to interpret CE-generated video.

Therefore, we investigated the rate of detection of red spots using SBI according to the background colors of the screen image and the capsule passage velocity in experimental models of the small intestine to determine

the factors that affect SBI sensitivity in diverse clinical situations.

MATERIALS AND METHODS

Experimental small intestine model

To represent a variety of background colors that may be encountered in clinical situations, experimental small intestine models prepared with seven colors of paper were produced. The colors included very pale yellow, yellow, very pale magenta, light grayish pink, burnt sienna, deep brown, and dark brown (Figure 1). Very pale magenta corresponds to the color of a normal intestinal mucous membrane. Lighter colors can be observed in conditions such as anemia, and darker colors are visible in mucosae with concentrated bile. The Hue and Tone 120-color system that was developed by the Nippon Color and Design Institute was used to define the colors and hues used in this study, and all color saturation levels were uniform^[16]. Small bowel models that were 3 cm in diameter and 50 cm in length were constructed, and ten 6-mm red spots were attached inside the models at 5-cm intervals to simulate angiodysplasia.

Investigation of capsule passage

A CE device (M2A capsule; Given Imaging Ltd., Yoqneam, Israel) was fixed to a solid-line cardiac catheter and passed inside the cylinder of each small intestine model at a constant speed (0.5 cm/s). The screen image from the CE instrument as it passed through model was observed using a real-time viewer (Figure 2). To detect differences in the SBI sensitivity according to the background color, 150 red dots were required to achieve a power of 80% ($\alpha = 5\%$) according to a preliminary pilot study. Therefore, through repetition, the CE instrument was guided past 150 red spots on each of the background colors, and the number of red tags on the scroll bar was examined during the application of SBI.

Differences in the SBI sensitivity were examined when the CE was passed through the intestinal models at speeds of 0.5 cm/s, 1 cm/s, and 2 cm/s.

Statistical analysis

For all statistical analysis in this study, SPSS version 12.0 (SPSS Inc., Chicago, IL, United States) was used. Continuous variables were expressed as the mean \pm SD or as the median. For comparative analysis of continuous variables, the Student's *t*-test, the Mann-Whitney *U* test, or the Kruskal-Wallis test were used depending on the normality of the data. For the comparison of nominal variables, Fisher's exact test and a χ^2 test were used. A post-hoc Bonferroni's correction was applied for comparative analysis of the groups. All values with a *P* < 0.05 were considered statistically significant.

RESULTS

Detection according to background color

We compared the number of red tags recognized by the

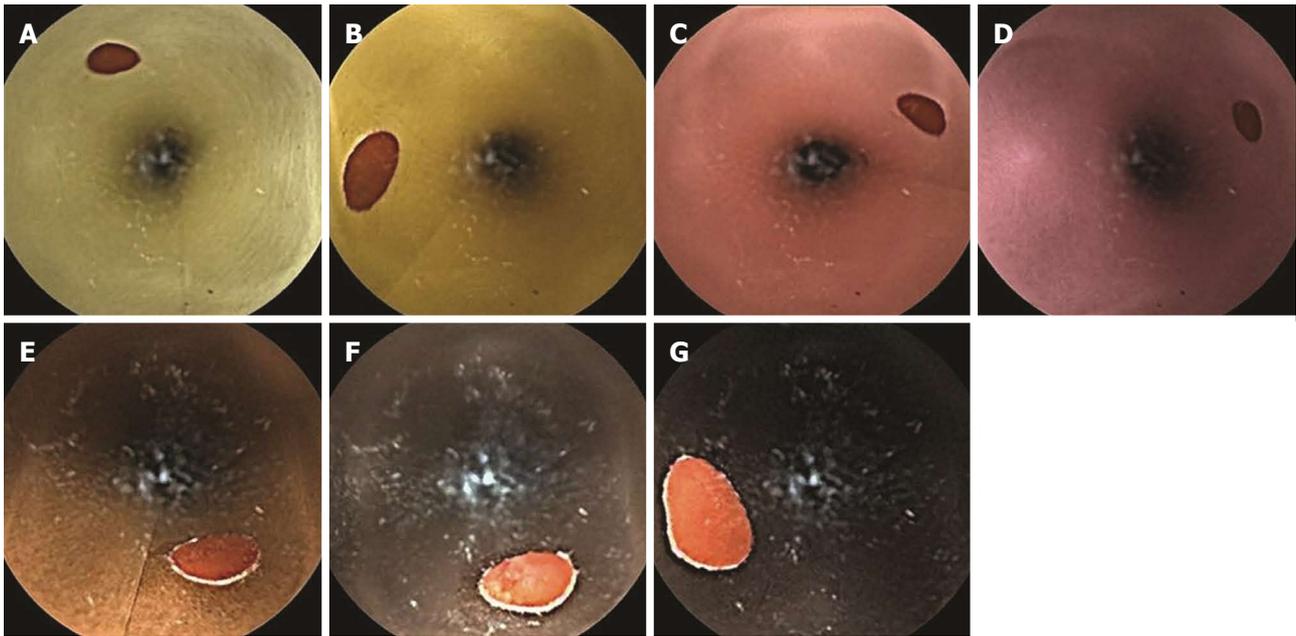


Figure 1 Endoscopic findings on backgrounds of different colors. A: Very pale yellow; B: Yellow; C: Very pale magenta; D: Light grayish pink; E: Burnt sienna; F: Deep brown; G: Dark brown.

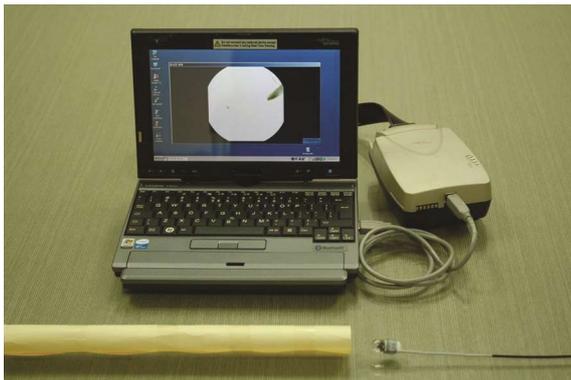


Figure 2 Experimental settings used for this study. A capsule endoscopy (CE) device was manually passed through models of the small bowel. The CE screen was observed using a real-time viewer.

SBI in the small intestine model for each background color. A significant difference was observed in the SBI detection rates on different background colors ($P < 0.001$, Table 1). The detection rates were highest for very pale magenta, burnt sienna, and yellow, in that order, and they were lowest for dark brown. Thus, the sensitivity of the SBI was the highest for a background of very pale magenta, which represented a normal mucosal color with good bowel cleansing, and the sensitivity was the lowest for dark brown, which represented concentrated bile. For a background of very pale yellow, which represented light bile, the detection rate of the red spots was low.

The sensitivity of the SBI for different background colors, analyzed with Bonferroni's correction, showed a significant difference for very pale magenta, for which the detection rate was the highest, compared to that of the other colors. The detection rate with a burnt sienna

Table 1 Detection rates of red spots by suspected blood indicator according to the background color

Color	Detection rate (%)	Rank
Very pale yellow	16/150 (10.7)	6
Yellow	28/150 (18.7)	3
Very pale magenta	64/150 (42.7)	1
Light grayish pink	19/150 (12.7)	4
Burnt sienna	36/150 (24.0)	2
Deep brown	18/150 (12.0)	5
Dark brown	5/150 (3.3)	7
Total	186/1050 (17.7)	$P < 0.001$

background was different from that with a very pale yellow and dark brown background, and the detection rate with a yellow background was different from that when a dark brown background was used (Table 2).

Detection according to capsule passage velocity

Generally, statistically significant differences were observed in the SBI detection rates of the red spots according to the capsule passage velocity in the small intestine models of some colors (Figure 3). Detection of the red spots by SBI tended to decrease at rapid capsule passage velocities (1-2 cm/s) compared to slow velocities (0.5 cm/s) for very pale yellow ($P = 0.042$), yellow ($P = 0.001$), very pale magenta ($P = 0.002$), and burnt sienna ($P = 0.001$) backgrounds. No significant differences were observed according to velocity for light grayish pink ($P = 0.643$) or dark brown ($P = 0.396$). Thus, differences according to velocity were highly pronounced in models constructed from colors that showed high SBI detection rates, and differences in sensitivity were not present for models with colors that had lower detection rates.

Table 2 Differences in detection rate among background colors using Bonferroni's correction

Color	Detection rate (%)	Dark brown	Very pale yellow	Deep brown	Light grayish pink	Yellow	Burnt sienna	Very pale magenta
Dark brown	3.3	1	0.0130	0.0049	0.0029	0.0000	0.0000	0.0000
Very pale yellow	10.7		1	0.7161	0.5901	0.0506	0.0023	0.0000
Deep brown	12.0			1	0.8609	0.1097	0.0069	0.0000
Light grayish pink	12.7				1	0.1535	0.0113	0.0000
Yellow	18.7					1	0.2603	0.0000
Burnt sienna	24.0						1	0.0006
Very pale magenta	42.7							1
Adjusted α	0.002381							

Table 3 Diagnostic accuracy of suspected blood indicator according to the literature (%)

	Liangpunsakul <i>et al.</i> ^[8]		Signorelli <i>et al.</i> ^[12]		D'Halluin <i>et al.</i> ^[10]		Buscaglia <i>et al.</i> ^[9]		Kim <i>et al.</i> ^[11]	
	OL	AB	OL	AB	OL	AB	OL	AB	OL	AB
Sensitivity	25.7	81.2	40.9	60.9	45	83	56.4	58.3	20	93
PPV	90.3	81.3	69.2	53.8	52	23	24	70	44	21

PPV: Positive predictive value; OL: Overall lesion; AB: Active bleeding.

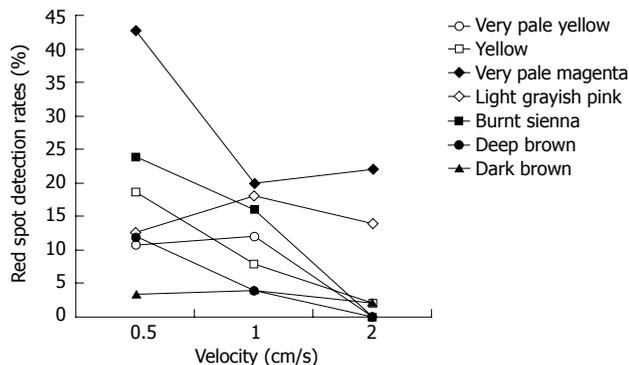


Figure 3 Detection rates of red spots by the suspected blood indicator according to the capsule endoscopy passage velocity.

DISCUSSION

CE was developed to diagnose lesions of the small intestine, and it is a useful method for diagnosing obscure gastrointestinal bleeding, Crohn's disease, polyposis syndrome, and small bowel tumors^[2-4,17-19]. CE has a higher diagnostic rate than radiological diagnosis methods and is less invasive and easier to perform than enteroscopy^[2-4,7,20]. However, CE has the disadvantage of requiring time to interpret multiple images^[21,22]. Usually, 45 min to two hours of viewing are required, although the reading time can be shortened using the multiview function, which allows the simultaneous observation of several images^[22]. To solve these problems, particularly in patients with suspected small intestine bleeding, SBI was developed^[10,23,24].

SBI can display frames that depict red zones during CE. The technique is activated in the first image frame of the duodenum and operates only in frames corresponding to the small intestine. The function is activated by an SBI view button within the software, resulting in the automatic identification of red pixels on the screen as

red hash marks on the scroll bar. Frame with suspected bleeding lesions can be selected in this way. Therefore, SBI helps physicians to review the video quickly, and bleeding lesions can be found easily. SBI provides supplementary information, but it does not replace the analysis of the video image. All frames recognized using the SBI feature should be later examined by a clinician in more detail.

In the first report on the accuracy of SBI in 24 patients by Liangpunsakul *et al.*^[8] in 2003, the sensitivity of the SBI was 25.7% (Table 3). However, when only active bleeding lesions were targeted, the sensitivity increased to 81.2%. In another study by Signorelli *et al.*^[12], the sensitivity was only 40.9% in 100 patients, although it increased to 60.9% in the presence of red blood or actively bleeding lesions. In another study, the overall sensitivity was 45%, with 83% for active bleeding^[10]. In a study by Buscaglia *et al.*^[9], the overall sensitivity was 56.4% and that in patients with active bleeding was 58.3%. According to a Korean report by Kim *et al.*^[11], SBI sensitivity of was as low as 20%; however, for actively bleeding lesions, such as angiodysplasia, a much higher sensitivity was observed (93%). SBI sensitivity showed large differences in these studies, ranging from 20% to 56.4%. The positive predictive value also varied from 24% to 90.3%. Even with active bleeding, sensitivity still differed from 58.3% to 93%. Therefore, SBI is thought to be useful as an adjunct method to screen for bleeding lesions using CE. Still, SBI cannot completely replace the reading of the CE-generated video.

Cases with no significant bleeding, but in which bleeding was still suspected, are problematic and clinically important^[25]. The cause of the variability in SBI sensitivity is likely to be due to the differences in CE images. The presence of bile, debris, stool residue, and blood in the small intestine can vary depending on the patient's condition and bowel preparation. The intestinal fluid may

be yellowish due to bile juices, light grayish pink due to blood, brownish due to stool residue, or dark brownish due to discolored stool. It can also vary according to the concentrations of bile and debris. That is, the intestinal fluid may be very pale yellow or yellow when clear of bile, or it may be burnt sienna or deep brown when thick bile is present. Therefore, the background colors may vary within the same patient depending on the area investigated. Additionally, the color of the intestinal mucosa may be different according to the underlying diseases of patients, such as anemia or jaundice.

The color contrast indicates that the presence of a combination of different colors can influence the detection of individual colors^[26]. The contrast is greatest when a color is combined with its complementary color in the color circle. The color contrast includes contrast related to lightness, hue, saturation, and square. In the color circle, the complementary color of red is blue-green, for which the contrast is greatest. Saturation contrast means that if other vivid colors are located close to the color of interest, the saturation of the color will be reduced. In this study, we investigated whether the ability of SBI to detect red pixels was affected by color contrast, especially in conditions of similar color saturation. We found that the detection rate of red spots was 42.7% for a very pale magenta color, significantly decreasing to 3.3% for dark brown and 10.7% for very pale yellow. A decreased detection rate of 12% was observed when a deep brown background was used. Therefore, significant differences in the SBI detection rates were observed for the different background colors. For colors commonly observed during CE, such as very pale magenta, yellow, and burnt sienna, the SBI sensitivity was similar to that of the clinical data. The sensitivity was decreased for background colors such as dark brown or very pale yellow, which may be infrequently observed in clinical settings. These results suggest that the SBI sensitivity may vary depending on the amount of concentrated bile, food or debris present in the small intestinal lumen and the underlying diseases of patients.

The average time of capsule passage through the intestine is 217 ± 90 min^[14]. However, this can vary from 48 min to more than eight hours depending on the underlying disease, such as diabetes, and on intestinal peristalsis. Significant differences in the sensitivity of SBI were observed at different capsule passage velocities in the experimental intestine models: the faster the capsule moved, the lower the sensitivity.

The results of our experiments suggest that SBI has low detection rates at sites that have colors that are significantly different from those of the normal intestine and in the areas where the capsule passed through relatively quickly. That is, even when the SBI technique fails to detect red pixels, a bleeding spot (i.e., angiodysplasia) may exist in those regions. Physicians must examine the images from these sites to detect false-negatives. The different background colors of the screen image can be easily located by using the tissue color bar in the software.

Therefore, the results of our study may assist physicians in the interpretation of SBI results and reduce the time required for detection of bleeding sites within the small intestine.

This study may be helpful for improving the diagnostic accuracy of SBI. To improve SBI sensitivity, technical improvements should be made. Additionally, the results of this study suggest clinically correctable factors for SBI. Bowel preparation before CE will improve the SBI sensitivity by minimizing the presence of bile and debris in the small intestine. To confirm this hypothesis, further studies investigating the factors that affect SBI sensitivity in different clinical situations are necessary.

The limitation of this study is that experimental models were used. Although the clinical situations of patients vary, if the capsule passes through an average of 6-7 m of small intestine in four hours, the actual average velocity is approximately 0.04 cm/s. The velocities used in this study of 0.5 cm/s, 1 cm/s, and 2 cm/s are not typically experienced in the clinic. Because it is technically difficult to artificially replicate an average capsule passage velocity, we randomly selected a velocity that was easy to replicate. Therefore, our results are difficult to apply directly to a clinical setting. However, it was shown that the sensitivity of SBI can differ depending on the velocity of the capsule.

In summary, the SBI sensitivity was significantly lower for some background colors on CE images, and the sensitivity decreased with faster capsule passage velocities in experimental models of the small intestine. Therefore, physicians should consider these factors when using SBI in the evaluation of CE images.

COMMENTS

Background

It is relatively time consuming to examine and interpret the results of capsule endoscopy (CE). The suspected blood indicator (SBI) is used for rapid screening of gastrointestinal bleeding, and it automatically recognizes frames that include red-colored pixels. However, reports on the usefulness of a SBI generally show low and variable overall sensitivity for lesions.

Research frontiers

SBI generates false-positive and false-negative results, and its clinical usefulness has not been verified. The color of the small intestinal mucosa and luminal fluid, which are the backgrounds on which lesions are detected, and the capsule passage velocity may vary according to bowel preparation and patient factors.

Innovations and breakthroughs

The detection by SBI differed significantly according to the background colors used ($P < 0.001$). The sensitivities on very pale magenta, burnt sienna and yellow backgrounds were significantly higher than on the other colors, such as very pale yellow or dark brown. The sensitivity tended to decrease at rapid capsule velocities in experimental models of the small intestine.

Applications

The results of this study may assist physicians in the interpretation of SBI results and reduce the time required for detecting bleeding sites in the small intestine. Physicians must examine the images that contain sites that could have false negative results more carefully than other sites. Bowel preparation before CE is useful for improving SBI sensitivity by minimizing bile or debris in the small intestine.

Peer review

The paper seeks to assess the automatic diagnostic yield using the suspected

blood identification system from GIVEN Imaging Ltd., designed to reduce the time spent reviewing CE images in patients with intermediate hemorrhage. The authors developed an experimental method to assess the sensitivity of SBI in different conditions.

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Impact of surgical volume on nationwide hospital mortality after pancreaticoduodenectomy

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Abstract

AIM: To evaluate the impact of surgical volume on nationwide hospital mortality after pancreaticoduodenectomy (PD) for periampullary tumors in South Korea.

METHODS: Periampullary cancer patients who underwent PD between 2005 and 2008 were analyzed from the database of the Health Insurance Review and Assessment Service of South Korea. A total of 126 hospitals were divided into 5 categories, each similar in terms of surgical volume for each category. We used hospital mortality as a quality indicator, which was defined as death during the hospital stay for PD, and calculated adjusted mortality through multivariate logistic models using several confounder variables.

RESULTS: A total of eligible 4975 patients were enrolled in this study. Average annual surgical volume of hospitals was markedly varied, ranging from 215 PDs in the very-high-volume hospital to < 10 PDs in the very-low-volume hospitals. Admission route, type of medical

security, and type of operation were significantly different by surgical volume. The overall hospital mortality was 2.1% and the observed hospital mortality by surgical volume showed statistical difference. Surgical volume, age, and type of operation were independent risk factors for hospital death, and adjusted hospital mortality showed a similar difference between hospitals with observed mortality. The result of the Hosmer-Lemeshow test was 5.76 ($P = 0.674$), indicating an acceptable appropriateness of our regression model.

CONCLUSION: The higher-volume hospitals showed lower hospital mortality than the lower-volume hospitals after PD in South Korea, which were clarified through the nationwide database.

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Key words: Hospital mortality; Pancreaticoduodenectomy; South Korea; Databases; Factual; Logistic models; Risk factors

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INTRODUCTION

Nowadays pancreaticoduodenectomy (PD) is considered a common and feasibly-performed abdominal surgery for periampullary tumors, but it is still a high-risk surgical procedure with potential morbidity and mortality rates.

Reducing the morbidity and mortality of this formidable operation is therefore imperative. Although several acceptable results after PD in low-volume hospitals have been reported^[1-4], most studies on volume-outcome correlation in performing PD have purported better outcomes in high-volume hospitals, suggesting centralization or regionalization of PD^[5-13].

Centralization of PD can be affected by the national healthcare system, information on hospital quality, or patient hospital preference. The Korean healthcare system is based on compulsory insurance of the whole population and free selection of medical care and hospital under step-wise referral to tertiary hospital without regional restriction. Although we have no governmental guidelines for distribution of cancer treatment service or information on hospital quality officially provided by the government, PD tends to be centralized in the large and well-equipped hospitals in Seoul, the capital city of South Korea.

Analyzing the present state of centralization of PD and providing information on hospital quality can help facilitate the government to develop nationwide guidelines and help patients to select good-quality hospitals for themselves. Hospital quality can be representatively appraised through PD-related morbidity or mortality. However, perioperative morbidity may vary from institution to institution according to the criteria or definitions of particular complications, making it difficult to obtain reliable nationwide morbidity data. Therefore, more definitive and objective data, such as standardized or risk-adjusted mortality rates, are requisite as an indicator for hospital quality.

With the implication toward public reporting, we performed this study to evaluate the impact of surgical volume on nationwide hospital mortality after PD for periampullary tumors and to validate the utility of surgical volume as a quality indicator of hospitals in South Korea. To date, just a few nationwide studies have been carried out to assess the effect of surgical volume on outcomes after PD. We hope the present study can contribute to nationwide evidence for the volume-outcome correlation in performing PD.

MATERIALS AND METHODS

Data sources and subjects

Data were obtained from the Health Insurance Review and Assessment Service (HIRA), whose database was constructed through the process of medical fee claims. After providing medical treatment, the medical institutions submit treatment details and file medical fee claims through an electronic billing system in the form of diskettes, compact discs or electronic data interchange (EDI). The EDI system, which was developed to review medical fees electronically by converting claim information into an EDI file and automatically reviewing items such as medical and drug fees within the software, occupies 99.7% of all medical claims in South Korea. Each claim contains information on demographic data, diagnoses,

procedures, comorbidity, route of admission, length of stay, discharge status, source of payment, hospital charges, *etc.* Diagnostic data were coded using the International Classification of Diseases, 10th Revision (ICD-10), and procedural data were coded using the health insurance claims code developed by the Ministry of Health and Welfare. From the HIRA database, we obtained anonymous data on patients who underwent PD for periampullary cancers during the period from January 2005 to December 2008. Only the primary cancers were included, and cancers originating at the adjacent organs such as the colon, stomach, or gallbladder were excluded. Benign diseases, including trauma, were also excluded. Additionally, patients with other combined operations which could affect the surgical outcomes such as hepatectomy, gastrectomy, or colectomy were not analyzed.

Categorization of hospitals

A total of 126 hospitals performed at least one PD from 2005 to 2008 in South Korea. Four-year surgical volume of each hospital showed a large gap, ranging from 1 to 861. Therefore, we divided the hospitals into quintiles; very-low, low, medium, high, and very-high categories. For this fractionation, the hospitals were sorted in descending order by total surgical volume, and cut-off points were decided to categorize hospitals into five similarly-sized groups.

Assessment of outcome

For clarification of volume-outcome correlation of PD, we adopted hospital mortality as an outcome indicator, which was defined as death during the hospital stay for PD. Hospital mortality had to be calculated in the form of adjusted mortality, because the hospital and patient characteristics of each category were different. For this adjustment of hospital mortality, we selected several risk factors for death from the HIRA database and the literature; age, sex, admission route as a surrogate for patient's general condition [outpatient department *vs* emergency room (ER)], Charlson comorbidity score^[14] as an index for current comorbid status (≥ 3 *vs* < 3), type of medical security as a surrogate for socioeconomic status (medical aid for the destitute *vs* health insurance for the others), and operation type [classical pancreaticoduodenectomy (CPD) *vs* pylorus-preserving pancreaticoduodenectomy (PPPD)]. However, we were unable to obtain more detailed information on preoperative treatment, tumor node metastasis stage, PD-specific complications, or radicality of PD from the HIRA database, which was a major limitation of the nationwide data. Observed mortality was first obtained according to surgical volume and patient characteristics. Risk-adjusted mortality was then calculated through: (observed hospital deaths/predicted hospital deaths) \times overall mortality rate. The predicted mortality of each category could be produced by summing the probability of death of each patient in that category, and the probability of death was determined by adjustment with significant confounder variables validated through

Table 1 Patient and hospital characteristics by surgical volume

Characteristics	Very-low (<i>n</i> = 92)	Low (<i>n</i> = 20)	Medium (<i>n</i> = 10)	High (<i>n</i> = 3)	Very-high (<i>n</i> = 1)	<i>P</i> value
Age (mean ± SD) (yr)	62.2 ± 10.7	62.1 ± 10.2	62.1 ± 10.5	61.2 ± 9.9	59.9 ± 10.3	0.077
Sex ratio (M:F)	1.5	1.3	1.5	1.6	1.6	0.282
Admission route						< 0.001
Outpatient department (%)	803 (78.6)	774 (77.0)	788 (77.3)	901 (84.4)	593 (68.9)	
Emergency room (%)	218 (21.4)	231 (23.0)	232 (22.7)	167 (15.6)	268 (31.1)	
Charlson comorbidity score						0.193
< 3 (%)	683 (66.9)	651 (64.8)	680 (66.7)	667 (62.5)	554 (64.3)	
≥ 3 (%)	338 (33.1)	354 (35.2)	340 (33.3)	401 (37.5)	307 (35.7)	
Type of medical security						< 0.001
Health insurance (%)	919 (90.0)	925 (92.0)	955 (93.6)	1048 (98.1)	841 (97.7)	
Medical aid (%)	102 (10.0)	80 (8.0)	65 (6.4)	20 (1.9)	20 (2.3)	
Type of operation						< 0.001
CPD (%)	604 (59.2)	420 (41.8)	361 (35.4)	325 (30.4)	301 (35.0)	
PPPD (%)	417 (40.8)	585 (58.2)	659 (64.6)	743 (69.6)	560 (65.0)	
Average annual volume	< 10	10-18	19-35	54-111	215	9.9 ¹

¹Denotes average annual surgical volume of all hospitals. SD: Standard deviation; M: Male; F: Female; CPD: Classical pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy.

multivariate logistic regression.

Statistical analysis

Statistical analysis was carried out using the SAS statistical package version 9.1 (SAS System for Windows, SAS Institute, Cary, NC, United States). Descriptive statistics were used to obtain patient characteristics and hospital mortality in each surgical volume category. Continuous variables were compared with Student's *t* test for two groups and with analysis of variance for multiple groups. Categorical variables were assessed with χ^2 tests. Multivariate logistic regression was used to assess the correlation between surgical volume and hospital mortality, with risk-adjusted mortality as the dependent variable and surgical volume and other risk factors for death as covariates. The result of the Hosmer-Lemeshow test was 5.76 ($P = 0.674$), indicating an acceptable appropriateness of our regression model. Statistical significance was set at P values < 0.05.

RESULTS

Hospital and patient characteristics by surgical volume

Of the patients who underwent PD for periampullary cancers during the period from 2005 to 2008 in South Korea, a total of 4975 patients were eligible for the inclusion criteria and enrolled in this study. Pancreatic cancer (1800, 36.2%) occupied the most common indication for PD and was followed by common bile duct cancer (1433, 28.8%), ampulla of Vater cancer (1280, 25.7%), duodenal cancer (238, 4.8%), and other periampullary cancers (227, 4.5%).

PD patients of each category were arranged to be similar in number; 1021 (20.5%) in the very-low-volume hospitals, 1005 (20.2%) in the low-volume hospitals, 1020 (20.5%) in the medium-volume hospitals, 1068 (21.5%) in the high-volume hospitals and 861 (17.3%) in the very-high-volume hospitals. Only one hospital corresponded

to the very-high-volume hospital, whereas as many as 92 (73.0%) hospitals belonged to the very-low-volume hospitals. Average annual surgical volume of the total 126 participating hospitals was markedly varied; 215 PDs in the very-high-volume hospital and fewer than 10 PDs in the very-low-volume hospitals (Table 1). Even worse, 33 of the very-low-volume hospitals performed less than 1 PD per year.

The mean age of the PD patients was 61.5 years and there were 1.5 times more males than females. Admission *via* ER was significantly more frequent and the percentage of payment by medical aid was significantly lower in the higher-volume hospitals (both $P < 0.001$). However, the Charlson comorbidity score didn't show any association with the surgical volume category. PPPD was performed in 59.6% (2964/4975) and this proportion increased with an increase in surgical volume, ranging from 40.8% in the very-low-volume hospitals to 69.6% in the high-volume hospitals ($P < 0.001$, Table 1).

Observed hospital mortality

The overall hospital mortality rate after PD was 2.1% during the study period. The observed hospital mortality rates were higher in lower-volume hospitals, the medical aid group, and CPD patients ($P < 0.001$, $P = 0.015$, $P < 0.001$, respectively). The mean age of both the mortality and survival group was also significantly different (66.0 *vs* 61.4, $P < 0.001$). Other risk factors didn't affect hospital mortality (Table 2).

Adjusted hospital mortality

All the risk factors with $P < 0.25$ in univariate logistic regression were included for multivariate analysis. These were: age, sex, type of medical security and operation type. Table 3 shows the results of multivariate logistic regression. Hospital mortality had a significant correlation with surgical volume ($P < 0.001$). Although there was no statistical difference in hospital mortality between the

Table 2 Observed hospital mortality by patient characteristics

Characteristics	No. of patients	Mortality (%)	P value
Age			< 0.001
Live (%)	4869 (97.9)	61.4 (10.4) ¹	
Dead (%)	106 (2.1%)	66.0 (8.6) ¹	
Sex			0.193
Male (%)	2 989 (60.1)	1.9	
Female (%)	1 986 (39.9)	2.5	
Admission route			0.291
Outpatient department (%)	3 859 (77.6)	2.3	
Emergency room (%)	1 116 (22.4)	1.7	
Charlson comorbidity score			0.291
< 3 (%)	3 235 (65.0)	2.0	
≥ 3 (%)	1 740 (35.0)	2.4	
Type of medical security			0.015
Health insurance (%)	4 688 (94.2)	2.0	
Medical aid (%)	287 (5.8)	4.9	
Operation type			< 0.001
CPD (%)	2 011 (40.4)	3.0	
PPPD (%)	2 964 (59.6)	1.5	

¹Indicates average age (standard deviation) of survival group and mortality group. No: Number; CPD: Classical pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy.

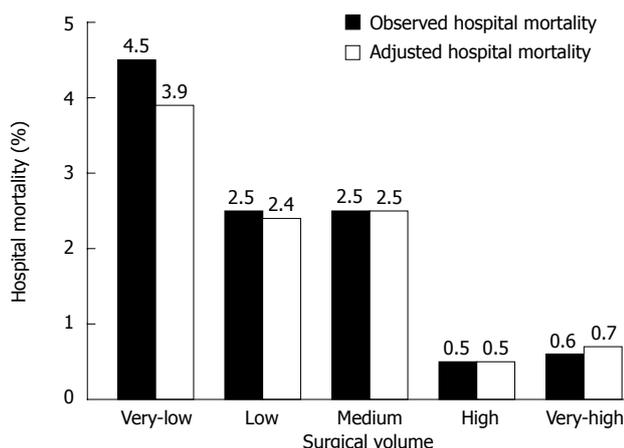


Figure 1 Observed and adjusted hospital mortality according to surgical volume. Observed hospital mortality rates showed a significant decreasing trend as surgical volume increased ($P < 0.001$). Adjusted hospital mortality rates were then calculated through multivariate logistic regression with hospital mortality rates as a dependent variable and age and operation type as significant confounder variables.

very-low-volume and medium-volume hospitals, or just a small statistical difference between the very-low-volume and low-volume hospitals, adjusted odds ratios (ORs) for hospital death of the high-volume and very-high-volume hospitals were significant lower than those of the very-low-volume hospitals (both $P < 0.001$). Age and operation type were the only significant confounder variables ($P < 0.001$ and $P = 0.025$, respectively) and were used for adjustment to produce the predicted mortality. Risk-adjusted hospital mortality rates according to surgical volume are depicted in Figure 1, ranging from 0.5% to 3.9%.

DISCUSSION

We used the nationwide database to validate the associa-

Table 3 Logistic regression for hospital mortality

Characteristics	Odds ratio (95% CI)	P value
Surgical volume		
Very-low	1.00	
Low	0.59 (0.36-0.98)	0.042
Medium	0.61 (0.37-1.01)	0.056
High	0.13 (0.05-0.32)	< 0.001
Very-high	0.16 (0.06-0.41)	< 0.001
Age ¹	1.04 (1.02-1.06)	< 0.001
Sex		0.329
Male	1.00	
Female	1.22 (0.82-1.80)	
Type of medical security		0.120
Health insurance	1.00	
Medical aid	1.60 (0.89-2.88)	
Operation type		0.025
CPD	1.00	
PPPD	0.64 (0.43-0.96)	

¹Per year increase in age. CI: Confidence Interval; CPD: Classical pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy.

tion of surgical volume and hospital mortality. Nationwide data has the power to yield reliable and objective results by itself, mainly due to a large number of subjects and the least influence of selection bias. Of the nationwide reports, however, there have been just a few studies on only PD^[5,8,10,11,13,15,16], whereas the majority included all types of pancreatic resections, including PD^[17-21]. It is known that PD is different from left-sided pancreatectomy, at least in postoperative morbidity and mortality. Nationwide hospital mortality results on PD would be reliable evidence and a base to support governmental or administrative guidelines for the establishment of policy on medical service, to select high-quality hospitals for patients, and to compare quality of care providers within and beyond South Korea.

The inverse relationship between surgical volume and hospital mortality in performing PD has been clarified in South Korea through this study. The risk-adjusted hospital mortality rates of the high-volume and very-high-volume hospitals were very low (0.5% and 0.7%) compared to those of the very-low-volume, low-volume and medium-volume hospitals (3.9%, 2.4% and 2.5%, respectively). This difference in the adjusted hospital mortality rates was found to be similar to that in the observed hospital mortality rates (0.5% and 0.6% *vs* 4.5%, 2.5% and 2.5%). In other words, the ORs for hospital mortality in the low-volume and medium-volume hospitals *vs* the very-low-volume hospitals were around 0.6 (a 40% decrease in the probability of hospital mortality), whereas the ORs in the high-volume and very-high-volume hospitals *vs* the very-low-volume hospitals were as low as 0.13 and 0.16 (a decrease of more than 80%).

The overall hospital mortality rate after PD in South Korea between 2005 and 2008 was 2.1%. This value was much lower than mortality rates from other statewide^[12,22,23] or nationwide^[5,8,10,11,13,15] databases, but a slightly higher than those from high-volume single insti-

tutions^[7,24-27]. Hospital mortality after PD is affected by many independent variables. Significant risk factors for hospital mortality, other than surgical volume as mentioned above, were age and operation type in this study. Type of medical security showed statistical significance in univariate analysis but not in multivariate analysis. Significant confounder variables for hospital mortality in PD were similar within the literature. Age, gender, body mass index, and urgent admission were advocated in other studies^[8,11,28].

Regionalization and centralization in severe medical illnesses and high-risk surgical procedures are worldwide trends which may occur naturally by patients' free selection, or intentionally by governmental policy across the world. About 40% of PDs were undertaken in the high-volume and very-high-volume hospitals, or the big 4 hospitals in South Korea. Again, more than 50% of PDs were performed only in 14 (11.1%) out of 126 hospitals. With these data, South Korea can be said to show a typical example of centralization for PD. Despite trends toward regionalization of care^[5,9,12,13,29], not a few PDs were safely performed in community hospitals by surgeons with varying degrees of experience^[28], as evidenced by a comparably low mortality rate and a high one-year survival rate^[1-4]. About 20% of patients still received PD in as many as 92 very-low-volume hospitals (73.0%) performing fewer than 10 PDs per year in South Korea. There could be several community hospitals with comparably low mortality rates, because the overall mortality rate of the very-low-volume hospitals in South Korea was not so high.

Hospital quality can be assessed with diverse outcome indicators. These are divided into short-term and long-term outcomes, with short-term outcomes including mortality, morbidity, hospital cost, and postoperative hospital stay. Survival outcome represents the long-term outcome. Considering that PD is a very complicated surgical procedure, postoperative morbidity results are very useful in comparing short-term outcomes between hospitals. However, our nationwide study didn't include morbidity results due to difficulty in data collection through the medical fee claims system of the HIRA. Additional drawbacks of this study were as follows; no long-term outcome, total hospital stay not postoperative hospital stay, no pathology-related data, or no surgeon volume. Of these drawbacks, surgeon volume is worthy of note. Surgeon volume^[19,22,28,30-32] or experience^[30], could be a more exact and detailed indicator of hospital outcome after PD than total hospital surgical volume. The HIRA database does not yet have the surgeon identifier which was used in the study by Eppsteiner *et al.*^[33]; therefore we couldn't analyze the correlation of surgeon volume and hospital outcome. In one study^[30], an experienced surgeon was defined as one performing 50 or more PDs, and experienced surgeons had comparable outcomes irrespective of annual volume. Learning curves also projected that less experienced surgeons would achieve morbidity and mortality rates equivalent to those of experienced surgeons when they reached 20 and 60 PDs, respectively. In other studies,

a high-volume surgeon was defined as having an average of 10 or more PDs per year^[28], or 5 or more PDs per year^[33]. Like stratification of hospitals by surgical volume, defining experienced or high-volume surgeons is difficult and varies according to the medical situation or surgeon training system of each country.

Quality indicators other than surgical volume have been introduced. Some researchers focused on the importance of surgery residency training programs, reporting a greater impact on outcomes after PD than hospital volume or surgeon frequency^[34]. Similarly, Joseph *et al.*^[35] put emphasis on hospital clinical resources, such as the Leapfrog Safe Practice Score, HealthGrades 5-star rating, or interventional radiology services, as well as surgical volume for lower operative mortality after PD. A pathologic indicator was also proposed by reporting that patients undergoing PD at low-volume centers were more likely to have margin-positive resections^[36].

Categorization of hospitals was carried out by two methods in the previous reports; by cut-off points of hospital similar in size in each category like our study and by cut-off points of surgical volume that were arbitrarily determined. These cutoff points of surgical volume were varying according to the medical situation and total surgical volume of states or countries. For example, Birkmeyer *et al.*^[10] defined > 5/year for high-volume hospitals in United States between 1992 and 1995, and Topal *et al.*^[5] did > 10/year for high-volume and > 20/year for very-high-volume hospitals in Belgium between 2000 and 2004, while Balzano *et al.*^[11] used a cut-off point of 14-51/year for high-volume hospitals and 89-104/year for very-high-volume hospitals in Italy in 2003. For this stratification of hospitals by surgical volume, the size of each category was uneven according to the cut-off points. In our study, the high-volume hospitals corresponded to 54-111/year and the very-high-volume hospital did 215/year between 2005 and 2008, as a result of dividing hospitals into 5 similar-sized categories. In addition, quintile division^[5,17] was rarely used in the previous studies, with the majority being performed in three or four stratifications. Accordingly it is not easy to reach an international consent on established stratification of hospitals by surgical volume.

In conclusion, the nationwide database clarified the impact of surgical volume on hospital mortality after PD in South Korea. The higher-volume hospitals had a better mortality outcome than the lower-volume hospitals. PD performance showed centralization in South Korea and the overall hospital mortality rate was comparable among countries. Further nationwide studies with surgeon volume, morbidity data, and long-term survival results after PD are warranted for more detailed information, and for a domestic and international comparison.

COMMENTS

Background

Pancreaticoduodenectomy (PD), performed for various diseases around the

duodenal ampulla, is one of the high-risk surgical procedures which tend to be centralized in high-volume hospitals across the world. Previous studies have reported that high-volume hospitals show better surgical outcomes than low-volume hospitals. Up to now, however, there have been no reports on surgical outcomes after PD, or whether surgical volume is a good quality indicator of care providers in South Korea.

Research frontiers

The correlation of surgical volume and hospital outcome after PD is well known between individual institutions or in a limited area. For research into clarifying this relationship, comprehensive results from nationwide databases are important for patients and government, as well as medical personnel.

Innovations and breakthroughs

Although there have been many studies on the relationship between surgical volume and hospital outcome after all types of pancreatic surgery from nationwide databases or after only PD from databases of institutions or states, nationwide results on only PD, which is still an operation with high morbidity and mortality rates, are very rare. Moreover, this is the first study of its type performed in South Korea and having a recent study period of four years.

Applications

With the information on the relationship between surgical volume and nationwide hospital mortality after PD in South Korea, reference guidelines for establishing medical policy and selecting good-quality hospitals could be supported.

Peer review

This is a frontier study on the relationship between surgical volume and hospital outcome after one type of major surgery in South Korea. This is a well-analyzed and clear manuscript that describes the impact of hospital volume on mortality following pancreaticoduodenectomy.

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Diabetes but not insulin is associated with higher colon cancer mortality

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Abstract

AIM: To evaluate whether diabetic patients had a higher risk of colon cancer mortality and its associated risk factors.

METHODS: The sex-specific crude and age-standardized (to the 2000 World Health Organization population) mortality rates of colon cancer in the Taiwanese general population were first calculated from 1995 to 2006. The trends were evaluated by linear regression. A total of 113 347 diabetic men and 131 573 diabetic women aged ≥ 25 years at recruitment from 1995 to 1998 were followed up until the end of 2006. Age/sex-specific colon cancer mortality rate ratios were calculated comparing the mortality rates of the diabetic patients with the average mortality rates of the general population within 12 years (1995-2006). A sub-cohort of diabetic patients (42 260 men and 49 405 women) was interviewed using a baseline questionnaire and Cox's regression was used to evaluate the risk factors for colon cancer mortality in these diabetic patients.

RESULTS: The crude and age-standardized trends of colon cancer mortality from 1995 to 2006 increased significantly for both sexes in the general population. A total of 641 diabetic men and 573 diabetic women died of colon cancer, with a mortality rate of 74.4 and 54.3 per 100 000 person-years, respectively. Mortality rate ratios [95% confidence intervals (CIs)] showed a significantly higher risk of mortality from colon cancer for the diabetic patients compared to the general population, with the magnitude increasing with decreasing age: 1.65 (1.40-1.95), 2.01 (1.78-2.27), 2.75 (2.36-3.21) and 5.69 (4.65-6.96) for ≥ 75 , 65-74, 55-64 and 25-54 years old, respectively, for men; and 1.46 (1.24-1.72), 2.09 (1.84-2.38), 2.67 (2.27-3.14) and 3.05 (2.29-4.06), respectively, for women. Among the sub-cohort of diabetic patients who had been interviewed with the baseline questionnaire, including information on age, sex, diabetes duration, diabetes type, body mass index, smoking, insulin use and area of residence, age and smoking were significantly predictive for colon cancer mortality, with respective adjusted hazard ratios (HRs) (95% CIs) of 1.077 (1.066-1.088) and 1.384 (1.068-1.792). Diabetes duration became a significant factor when those who died of colon cancer within 5 years of diabetes diagnosis were excluded to minimize the possible contamination of diabetes caused by incipient colon cancer, with an adjusted hazard ratio of 1.021 (1.007-1.034). Sex, diabetes type, insulin use, body mass index and area of residence were not significant predictors for colon cancer mortality in the diabetic patients. Although insulin use was categorized into subgroups of duration of use (non-users and users < 5 years, 5-9 years and ≥ 10 years), none of the HRs for colon cancer mortality was significant with regards to different durations of insulin use.

CONCLUSION: Colon cancer mortality is increasing in Taiwan. A higher risk is observed in diabetic patients. Smoking, but not insulin use, is a modifiable risk factor.

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Key words: Colon cancer; Diabetes mellitus; Mortality; Secular trend

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INTRODUCTION

Colorectal cancer is a major cause of death in developed countries^[1]. In Taiwan, it is the third most common cause of cancer-related death^[2], and the trend of its age-adjusted mortality showed an increase from 1971 to 1996^[3]. A meta-analysis concluded there was a 30% higher risk in diabetic patients^[4]. However, most studies were done in western countries, and the only one involving Asians in the meta-analysis was conducted in Korea, which showed a 28% higher risk of mortality in diabetic men, but not women^[5]. On the other hand, some studies showed an association in women^[6,7].

Most previous studies did not distinguish between type 1 and type 2 diabetes. A recent prospective study in the United States identified patients with type 2 diabetes and nondiabetic subjects aged 50-74 years in 1992-1993 and followed biannually by questionnaires from 1997 to 2007^[8]. Diabetes was significantly associated with colorectal cancer in men who were either insulin users or non-users; but diabetes and insulin use were not associated with a higher risk among women^[8].

Whether insulin use is associated with colon cancer mortality has rarely been studied. Furthermore, no previous studies have examined prospectively the confounding effects of diabetes duration and age; both being highly associated with insulin use. Therefore, this study evaluated: (1) the trends of colon cancer mortality in the Taiwanese general population; (2) the age/sex-specific mortality rate ratio between diabetic patients and the general population; and (3) the risk factors for colon cancer mortality in diabetic patients, including age, sex, diabetes duration, diabetes type, body mass index, smoking, insulin use/duration of insulin use and area of residence.

MATERIALS AND METHODS

Colon cancer mortality in the general population

The study was approved and supported by the Department of Health, Executive Yuan, Taiwan. In Taiwan, every resident has a unique identification number and events like birth, death, marriage or migration should be registered. If a person dies, a death certificate should be

reported to the household registration offices within 30 d as required by law. The death certificate database includes the identification number, date of birth, sex, and date and cause of death. The causes of death coded in the ninth revision of the International Classification of Diseases are used. Colon cancer has a code of 153.

The age/sex-specific population numbers are reported annually by the government. The sex-specific trends of crude and age-standardized (to the 2000 World Health Organization population) mortality rates for colon cancer in the general population were first calculated from 1995 to 2006 for all ages. Linear regression was used to evaluate whether the trends changed with regard to calendar years, where the mortality rate was the dependent variable and the calendar year the independent variable.

Colon cancer is rare in young individuals, therefore, we analyzed the data for those aged ≥ 25 years in the following groups: 25-54 years, 55-64 years, 65-74 years and ≥ 75 years old. Age/sex-specific average mortality rates during 1995-2006 were calculated by dividing the average numbers of colon cancer deaths by the average mid-year population of the specific age and sex within the period.

Colon cancer mortality in diabetic patients

Figure 1 shows a flow chart for the follow-up of diabetic patients. In March 1995, a compulsory and universal National Health Insurance (NHI) program was implemented and covered $> 96\%$ of the population. From 1995 to 1998 a cohort of 256 036 diabetic patients ("the original cohort") using the NHI was established (detailed elsewhere)^[9,10].

All patients were followed until 2006. The date and cause of death were obtained from the death certificate database. Mortality rates were computed using a person-years denominator: the duration from enrollment until the end of 2006 for those who were alive or to the date of death. The patients were categorized into age subgroups by their age at enrollment. Age/sex-specific mortality rates and mortality rate ratios were calculated. The mortality rate ratio was calculated using the average mortality rate of that subgroup within the 12 years in the general population as a reference. To reduce the aging effect on age subgroup categorization, analyses for the original cohort were also performed by splitting the follow-up duration into two periods: (1) from enrollment to the end of 2000: age categorized at enrollment and mortality followed from enrollment to 2000; and (2) from 2001 to the end of 2006: those who died before the end of 2000 were excluded, age calculated at 2001 and mortality followed from 2001-2006.

For sub-cohort analyses, we calculated the mortality rates and mortality rate ratios in the patients who had been interviewed with a baseline questionnaire (detailed elsewhere)^[11-13]. The number interviewed was 93 484 and among them 91 665 patients (42 260 men and 49 405 women) were aged ≥ 25 years ("sub-cohort diabetic patients"). To evaluate whether an association was found in

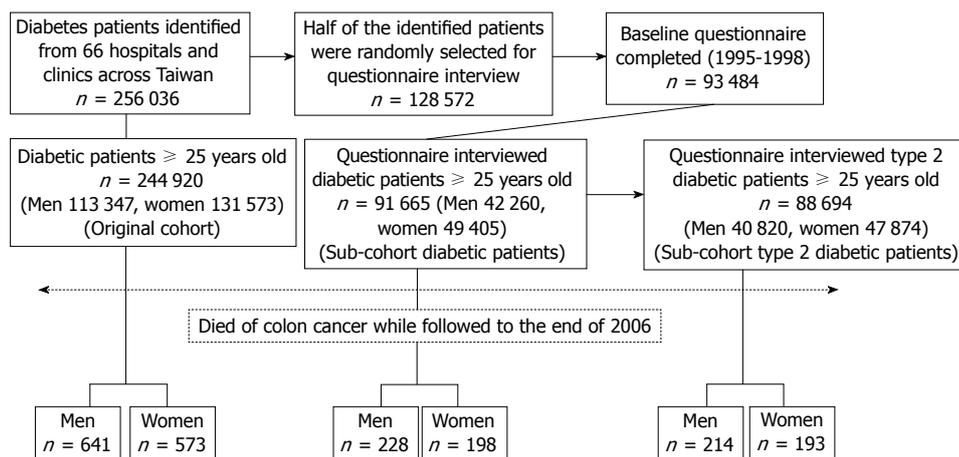


Figure 1 Flow chart showing the procedures in the calculation of colon cancer mortality in the diabetic cohorts.

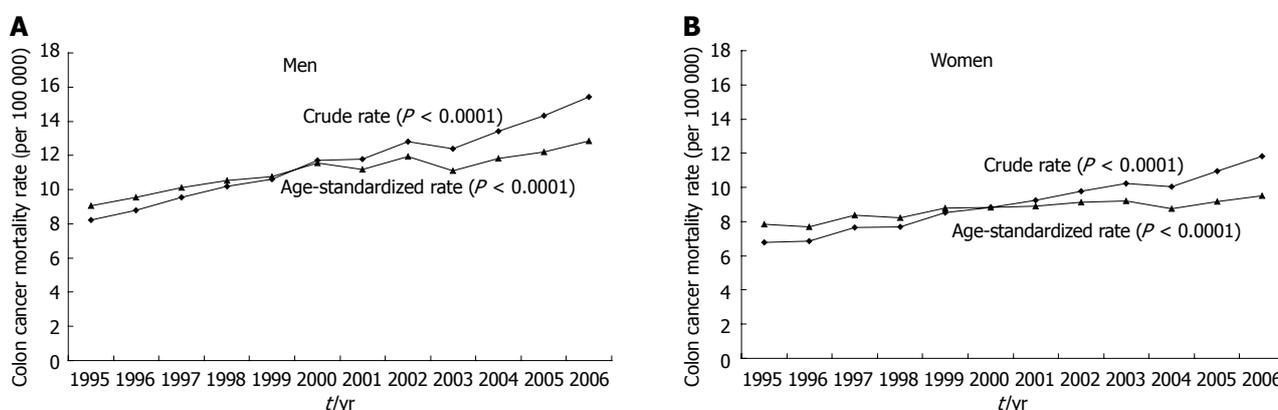


Figure 2 Secular trends of mortality from colon cancer from 1995 to 2006 in the general population of Taiwan in men (A) and women (B), respectively. The 2000 World Health Organization population was used as reference for age standardization.

patients with type 2 diabetes, mortality rates and mortality rate ratios were calculated after excluding patients with type 1 diabetes based on diabetic ketoacidosis at diabetes onset, or the need for insulin injection within 1 year after diabetes diagnosis. There were 40 820 diabetic men and 47 874 diabetic women after this exclusion (“sub-cohort type 2 diabetic patients”). Only 1440 men and 1531 women were excluded for type 1 diabetes, and among them only 14 men and five women died of colon cancer, therefore, we did not analyze the association with type 1 diabetes. To minimize the possibility that diabetes might be caused by incipient colon cancer, analyses were also done by dividing the patients into subgroups with a diabetes duration at enrollment < 10 years and ≥ 10 years.

Risk factors for colon cancer mortality in diabetic patients

The baseline characteristics of the sub-cohort diabetic patients who had been interviewed (Figure 1) were compared between men and women by either the *t* test for continuous variables or the χ^2 test for categorical variables. Cox proportional hazards models were then used to identify the risk factors for colon cancer mortality. Colon cancer mortality was the dependent variable in the models and the independent variables included age,

sex (men *vs* women), diabetes duration, diabetes type (type 2 *vs* type 1), body mass index, smoking (yes *vs* no), insulin use (yes *vs* no) and area of residence (urban *vs* rural). The area of residence was defined as urban for the Metropolitan Taipei area including Taipei City and Taipei County (New Taipei City) and other administratively named cities across Taiwan; and as rural for administratively named counties and offshore islands. To evaluate whether the duration of insulin use could be associated with colon cancer mortality, Cox models were also created by comparing insulin use at < 5 years, 5-9 years and ≥ 10 years to insulin non-users, before adjustment, at adjustment for age, sex, diabetes type, diabetes duration, body mass index, smoking and area of residence one at a time, and at adjustment for all these factors simultaneously (full model). The analyses were done before and after excluding patients who died of colon cancer within 5 years of diabetes onset, to minimize the possibility that diabetes might be caused by incipient colon cancer or might occur after the diagnosis of colon cancer.

RESULTS

The trends of crude and age-standardized colon cancer mortality showed a significant increase in both sexes in

Table 1 Age/sex-specific mortality rates (per 100 000 person-years) for colon cancer in diabetic patients and their mortality rate ratios compared to the average mortality rates in the general population of Taiwan

Age (yr)	Men					Women				
	<i>n</i>	<i>N</i>	PY	MR	MRR (95% CI)	<i>n</i>	<i>N</i>	PY	MR	MRR (95% CI)
Original diabetic cohort										
Followed from enrollment to the end of 2006										
25-54	76	42107	363948.2	20.88	5.69 (4.65, 6.96) ^a	43	43749	399556.9	10.76	3.05 (2.29, 4.06) ^a
55-64	158	28867	225706.1	70.00	2.75 (2.36, 3.21) ^a	146	37317	314033.6	46.49	2.67 (2.27, 3.14) ^a
65-74	265	30704	211811.1	125.11	2.01 (1.78, 2.27) ^a	238	35194	258155.6	92.19	2.09 (1.84, 2.38) ^a
≥ 75	142	11669	59761.6	237.61	1.65 (1.40, 1.95) ^a	146	15313	83905.5	174.01	1.46 (1.24, 1.72) ^a
Followed from enrollment to the end of 2000										
25-54	23	42107	143238.6	16.06	4.38 (3.00, 6.37) ^a	16	43749	153584.7	10.42	2.95 (1.84, 4.71) ^a
55-64	52	28867	96078	54.12	2.12 (1.62, 2.78) ^a	55	37317	129321.2	42.53	2.44 (1.88, 3.17) ^a
65-74	112	30704	94488.8	118.53	1.90 (1.58, 2.29) ^a	91	35194	113687.6	80.04	1.81 (1.48, 2.23) ^a
≥ 75	70	11669	30353.6	230.61	1.60 (1.27, 2.03) ^a	75	15313	42092.9	178.18	1.49 (1.19, 1.87) ^a
Followed from 2001 to the end of 2006										
25-54	30	32626	186021.3	16.13	4.39 (3.16, 6.11) ^a	22	34400	201514.2	10.92	3.09 (2.07, 4.60) ^a
55-64	68	22743	122446.4	55.53	2.18 (1.72, 2.76) ^a	52	29880	167205.9	31.10	1.79 (1.36, 2.34) ^a
65-74	172	26548	133463.8	128.87	2.07 (1.78, 2.40) ^a	131	33591	176023.3	74.42	1.69 (1.42, 2.00) ^a
≥ 75	114	13653	59608.3	191.25	1.33 (1.11, 1.60) ^a	131	17808	79994.6	163.76	1.37 (1.16, 1.63) ^a
Sub-cohort diabetic patients										
Diabetes of any duration at enrollment										
25-54	25	13837	100793.2	24.80	6.76 (4.81, 9.50) ^a	16	12768	95960.8	16.67	4.72 (3.02, 7.37) ^a
55-64	62	12454	84551.8	73.33	2.88 (2.26, 3.66) ^a	53	16717	118278.5	44.81	2.57 (1.98, 3.35) ^a
65-74	99	12395	76349.6	129.67	2.08 (1.71, 2.53) ^a	86	15029	96058.4	89.53	2.03 (1.64, 2.50) ^a
≥ 75	42	3574	17710.4	237.15	1.65 (1.22, 2.23) ^a	43	4891	25384.8	169.39	1.42 (1.05, 1.91) ^a
Diabetes duration < 10 yr at enrollment										
25-54	15	11631	86016.8	17.44	4.75 (3.00, 7.53) ^a	12	10389	78983.7	15.19	4.30 (2.56, 7.23) ^a
55-64	42	8892	61949.1	67.80	2.66 (1.99, 3.57) ^a	37	11636	84435.4	43.82	2.52 (1.84, 3.45) ^a
65-74	62	7887	49825.0	124.44	2.00 (1.56, 2.55) ^a	47	9246	61226.8	76.76	1.74 (1.31, 2.31) ^a
≥ 75	29	2138	10946.7	264.92	1.84 (1.29, 2.64) ^a	23	2814	15302.6	150.30	1.26 (0.84, 1.90)
Diabetes duration ≥ 10 yr at enrollment										
25-54	10	2206	14773	67.69	18.44 (11.81, 28.80) ^a	4	2379	16988.5	23.55	6.66 (2.85, 15.56) ^a
55-64	20	3562	22608.3	88.46	3.47 (2.30, 5.25) ^a	16	5081	33862.5	47.25	2.71 (1.69, 4.35) ^a
65-74	37	4508	26541.7	139.40	2.24 (1.63, 3.07) ^a	39	5783	34845.7	111.92	2.54 (1.87, 3.44) ^a
≥ 75	13	1436	6785.4	191.59	1.33 (0.77, 2.29)	20	2077	10103.5	197.95	1.66 (1.07, 2.56) ^a
Sub-cohort type 2 diabetic patients										
Diabetes of any duration at enrollment										
25-54	23	13239	96635.9	23.80	6.49 (4.54, 9.26) ^a	16	12260	92076.3	17.38	4.92 (3.16, 7.66) ^a
55-64	60	12082	82183.2	73.01	2.87 (2.24, 3.66) ^a	51	16288	115494.5	44.16	2.54 (1.94, 3.32) ^a
65-74	92	12051	74413.3	123.63	1.98 (1.62, 2.43) ^a	83	14570	93223.1	89.03	2.02 (1.63, 2.50) ^a
≥ 75	39	3448	17171.3	227.12	1.58 (1.16, 2.16) ^a	43	4756	24716.7	173.97	1.46 (1.08, 1.97) ^a
Diabetes duration < 10 yr at enrollment										
25-54	14	11248	83333.9	16.80	4.58 (2.84, 7.38) ^a	12	10082	76622.8	15.66	4.43 (2.64, 7.44) ^a
55-64	40	8730	60901.6	65.68	2.58 (1.91, 3.48) ^a	36	11453	83223.4	43.26	2.48 (1.81, 3.42) ^a
65-74	59	7742	48999.2	120.41	1.93 (1.50, 2.49) ^a	46	9068	60169.4	76.45	1.73 (1.30, 2.31) ^a
≥ 75	27	2088	10708.6	252.13	1.75 (1.21, 2.55) ^a	23	2760	15063.9	152.68	1.28 (0.85, 1.93)
Diabetes duration ≥ 10 yr at enrollment										
25-54	9	1991	13298.7	67.68	18.44 (11.53, 29.50) ^a	4	2178	15465.0	25.86	7.32 (3.17, 16.88) ^a
55-64	20	3352	21284.3	93.97	3.69 (2.45, 5.56) ^a	15	4835	32290.5	46.45	2.67 (1.64, 4.35) ^a
65-74	33	4309	25428.1	129.78	2.08 (1.49, 2.91) ^a	37	5502	33067.7	111.89	2.53 (1.85, 3.47) ^a
≥ 75	12	1360	6484.3	185.06	1.29 (0.73, 2.26)	20	1996	9674.1	206.74	1.73 (1.12, 2.67) ^a

^a*P* < 0.05 vs general population. PY: Person-years; MR: Mortality rate; MRR: Mortality rate ratio; CI: Confidence interval; *n*: Case number of colon cancer; *N*: Case number observed.

the general population during the period from 1995 to 2006 in Taiwan (Figure 2). The average mortality rates for colon cancer during the period in the general population aged 25-54 years, 55-64 years, 65-74 years and ≥ 75 years were 5.1, 24.75, 76.6 and 160.92 per 100 000 for men, respectively, and 4.51, 18.01, 46.08 and 130.90 for women.

A total of 113 347 diabetic men and 131 573 diabetic women in the original cohort were followed (Figure 1). Among them, 641 men and 573 women died of colon cancer, with mortality rates of 74.4 and 54.3 per 100 000

person-years, respectively. The age/sex-specific mortality rates in the diabetic patients and their mortality rate ratios compared to the general population are shown in Table 1. Except for those aged ≥ 75 years and with a diabetes duration of ≥ 10 years at enrollment in men, and with a diabetes duration of < 10 years at enrollment in women (Table 1), the mortality rate ratios were all significant, and especially remarkable in those aged 25-54 years. Diabetes was unlikely to be caused by colon cancer, because diabetes diagnosed ≥ 10 years before colon

Table 2 Baseline characteristics of the sub-cohort of diabetic men and women who had been interviewed with a baseline questionnaire

Variable	Men	Women	P value
n	42 260	49 405	
Age, yr	59.8 (11.7)	61.5 (10.8)	< 0.0001
Diabetes duration, yr	7.0 (6.6)	7.5 (6.6)	< 0.0001
Diabetes type, % type 1	1440 (3.4)	1531 (3.1)	0.0085
Body mass index, kg/m ²	24.4 (3.4)	24.7 (3.8)	< 0.0001
Smoking, % yes	26 522 (62.8)	1703 (3.5)	< 0.0001
Use of insulin, % yes	3717 (8.8)	5059 (10.2)	< 0.0001
Area of residence, % urban	20 231 (47.9)	22 264 (45.1)	< 0.0001
Colon cancer mortality	228 (0.5)	198 (0.4)	0.0021

Data are expressed as mean (SD) or n (%).

cancer mortality can hardly be a consequence of the carcinogenic process. The aging effect during follow-up on age subgroup categorization was also minimal because the results were similar when the long duration was split into two shorter periods in the original cohort analyses.

All baseline characteristics of the sub-cohort diabetic patients who had been interviewed with a baseline questionnaire differed significantly between the diabetic men and women (Table 2). The unadjusted and mutually-adjusted HRs for different age groups are shown in Table 3. In the adjusted models, age and smoking (especially in those aged < 65 years) were significant. When diabetic patients who died of colon cancer within 5 years of diabetes diagnosis were excluded, diabetes duration was significant (especially in those aged \geq 65 years). Sex, diabetes type, insulin use, body mass index and area of residence were not significant after adjustment.

Table 4 shows the HRs for different durations of insulin use compared to non-users in unadjusted and adjusted models. Before exclusion of patients with a duration of < 5 years between the onset of diabetes and colon cancer mortality, none of the HRs was significant. In models after exclusion, insulin use \geq 10 years might be associated with a higher risk. However, in the models after respective adjustment for age, diabetes type or diabetes duration, and in the full model, insulin use of any duration was not predictive, suggesting that the association with insulin use might be due to the effects of some confounders.

DISCUSSION

Contrary to the decreasing trend since the mid-1980s in the United States^[14], colon cancer mortality in Taiwan is increasing (Figure 2), and has been since 1971 if the observation of Chen *et al*^[3] is considered simultaneously. Although the etiology of the increasing trend remains to be explored, it may be due to the westernization of the Taiwanese lifestyle in recent decades, with increased fat intake, and the high prevalence of metabolic syndrome and diabetes. The higher risk among diabetic patients of both sexes (Table 1) was also contrary to Korean^[5] and

United States^[8] studies showing an association in men but not in women, and to others showing an association only in women^[7].

It is interesting that the increased mortality rate ratio was more remarkable at the youngest age of 25-54 years (Table 1). This has public health importance because the incidence of diabetes is increasing dramatically in the younger generation^[9]. One explanation is that age *per se* is a strong risk factor, and therefore, the impact of diabetes might not be as obvious in the elderly, resulting in a remarkably higher incidence rate ratio and a higher mortality rate ratio in the younger age group. Other explanations include that diabetes has a different impact on colon cancer mortality in different age groups, and that younger diabetic patients with colon cancer would have a poorer prognosis than non-diabetic patients. Mucin production in colorectal cancer has an inverse effect on survival among Taiwanese patients^[15]. Taiwanese patients with colon cancer and aged < 40 years also have significantly poorer 5-year survival^[16]; age is inversely associated with tumor stage at diagnosis, tumor differentiation and mucin production^[17]. In addition, the healthy survivor effect might also lead to a reduced mortality rate ratio in the elderly.

Metabolic syndrome is associated with a 35% higher risk of colon cancer in Taiwan^[18]. Similarly, a cluster of three components of the metabolic syndrome (hypertension, body mass index \geq 25 kg/m² and high-density lipoprotein cholesterol < 40 mg/dL) was associated with a 58% higher risk in a Finnish study^[19]. In Taiwan, metabolic syndrome is present in 76.2% of diabetic patients^[20], in contrast to 15% in the general population^[21]. Therefore, the higher prevalence rates of hypertension, obesity and dyslipidemia in diabetic patients might explain partly their higher risk of colon cancer.

Contrary to other studies showing an association between body mass index and distal colon adenoma^[22] or colorectal cancer^[19], body mass index was not predictive for colon cancer mortality in the present study (Table 3). One possibility is that the risk factors for incidence and mortality or for different ethnicities might be different. It is also possible that if diabetes *per se* incurred a markedly higher risk, the impact of other risk factors might be overshadowed. Therefore, risk factors in diabetic patients might not be the same as those observed in non-diabetic subjects.

The association between colorectal neoplasm and insulin use in patients with type 2 diabetes has been controversial^[8,23,24]. Although two retrospective studies suggested a positive link, a recent prospective study concluded a lack of association^[8]. A study conducted in Korea suggested a threefold higher risk of colorectal adenoma associated with insulin therapy^[23]. However, this study used a retrospective case-control design and evaluated adenoma rather than cancer. Another retrospective cohort study using the General Practice Research Database from the United Kingdom showed a significantly twofold higher risk of colorectal cancer associated with

Table 3 Cox proportional hazards models showing hazard ratios and 95% confidence intervals for colon cancer mortality in diabetic patients by age before and after exclusion of patients with a duration of < 5 years between onset of diabetes and colon cancer mortality

Variables	Interpretation	Hazard ratio (95% CI)							
		Age \geq 25 yr				Age 25-64 yr		Age \geq 65 yr	
		Unadjusted	P value	Mutually adjusted	P value	Mutually adjusted	P value	Mutually-adjusted	P value
Before exclusion									
Age	Every 1-yr increment	1.077 (1.066-1.088)	< 0.0001	1.077 (1.066-1.088)	< 0.0001	1.090 (1.062-1.119)	< 0.0001	1.072 (1.049-1.096)	< 0.0001
Sex	Men vs women	1.395 (1.150-1.691)	0.0007	1.256 (0.976-1.618)	0.0769	1.181 (0.758-1.841)	0.4622	1.288 (0.946-1.753)	0.1081
Diabetes duration	Every 1-yr increment	1.032 (1.019-1.045)	< 0.0001	1.006 (0.992-1.020)	0.3845	0.993 (0.964-1.023)	0.6652	1.009 (0.994-1.025)	0.2329
Diabetes type	Type 2 vs type 1	0.682 (0.430-1.081)	0.1036	0.750 (0.432-1.301)	0.3057	0.914 (0.345-2.425)	0.8574	0.680 (0.348-1.328)	0.2588
Body mass index	Every 1-kg/m ² increment	0.956 (0.929-0.984)	0.0021	0.976 (0.948-1.005)	0.1062	0.966 (0.920-1.014)	0.1616	0.981 (0.946-1.018)	0.3080
Smoking	Yes vs no	1.484 (1.219-1.808)	< 0.0001	1.384 (1.068-1.792)	0.0140	1.746 (1.121-2.720)	0.0136	1.215 (0.881-1.676)	0.2351
Insulin use	Yes vs no	1.344 (0.992-1.821)	0.0564	1.235 (0.843-1.809)	0.2782	1.318 (0.686-2.532)	0.4076	1.215 (0.759-1.946)	0.4168
Area of residence	Urban vs rural	0.901 (0.743-1.094)	0.2928	0.852 (0.702-1.034)	0.1046	0.734 (0.530-1.016)	0.0621	0.931 (0.731-1.187)	0.5660
After exclusion									
Age	Every 1-yr increment	1.082 (1.070-1.094)	< 0.0001	1.080 (1.068-1.092)	< 0.0001	1.096 (1.064-1.128)	< 0.0001	1.070 (1.046-1.095)	< 0.0001
Sex	Men vs women	1.454 (1.186-1.784)	0.0003	1.281 (0.980-1.676)	0.0704	1.355 (0.836-2.195)	0.2172	1.249 (0.903-1.728)	0.1787
Diabetes duration	Every 1-yr increment	1.046 (1.034-1.059)	< 0.0001	1.021 (1.007-1.034)	0.0022	1.025 (0.996-1.054)	0.0883	1.020 (1.004-1.035)	0.0120
Diabetes type	Type 2 vs type 1	0.640 (0.398-1.028)	0.0647	0.710 (0.402-1.254)	0.2386	0.637 (0.229-1.771)	0.3878	0.737 (0.371-1.465)	0.3837
Body mass index	Every 1-kg/m ² increment	0.960 (0.932-0.989)	0.0078	0.984 (0.955-1.015)	0.3034	0.983 (0.933-1.035)	0.5160	0.984 (0.948-1.022)	0.4153
Smoking	Yes vs no	1.555 (1.263-1.914)	< 0.0001	1.445 (1.099-1.899)	0.0083	1.814 (1.130-2.913)	0.0137	1.268 (0.905-1.778)	0.1672
Insulin use	Yes vs no	1.431 (1.044-1.961)	0.0258	1.159 (0.781-1.722)	0.4639	0.927 (0.440-1.954)	0.8419	1.274 (0.798-2.035)	0.3106
Area of residence	Urban vs rural	0.909 (0.741-1.116)	0.3639	0.848 (0.691-1.041)	0.1158	0.815 (0.573-1.158)	0.2536	0.876 (0.680-1.128)	0.3040

CI: Confidence interval.

insulin use^[24]. On the other hand, a prospective cohort study conducted in the United States did not suggest an association between colorectal cancer and insulin use in either men or women^[8]. The finding of the present study was in line with the United States study, suggesting a lack of association between insulin use and colon cancer mortality (Tables 3 and 4). Insulin use is essential in patients with type 1 diabetes and is always seen in older patients with type 2 diabetes who might have prolonged duration of diabetes. Therefore, it is worth mentioning that adjustments should be made simultaneously for age, diabetes duration and diabetes type in the analyses evaluating the risk of cancer associated with insulin use. The present study is probably the longest follow-up study showing that insulin use was not predictive for colon cancer mortality after adjustment for confounders, including all of these factors (Tables 3 and 4).

Consistent with some prior studies^[25,26], smoking was significantly predictive, especially in those aged < 65 years (Table 3). A recent Swedish retrospective cohort study evaluating the use of snuff and colorectal and anal cancer found no significant association^[27]. We were not able to evaluate the effect of snuff use because of a lack

of information. Although we could not evaluate the impact of socioeconomic status, this study did not show an association with area of residence (Table 3).

Incidence and mortality are two different entities, and probably linked to different factors. If diabetic patients with colon cancer had a poorer prognosis, the mortality rate ratio would not properly reflect the incidence rate ratio. A recently published prospective study (Cancer Prevention Study-II Nutrition Cohort) conducted among 2278 patients with colorectal cancer suggested that patients with type 2 diabetes had a higher risk of mortality than those without diabetes; especially a higher risk of death from cardiovascular disease^[28]. A study from Taiwan also showed that diabetic patients with colon cancer had an overall 21% higher mortality than nondiabetic patients^[29]. However, this was only observed in stage II cancer. It was believed that the 21% higher case-fatality rate could not explain the several-fold higher mortality rate ratios in the diabetic patients (Table 1).

The strengths of this study included a prospective follow-up of a large cohort of diabetic patients over a long duration; the completeness of the ascertainment of vital status by matching with the death certificate

Table 4 Cox proportional hazards models for mortality from colon cancer by duration of insulin use (reference group: diabetic patients not using insulin) before and after exclusion of patients with a duration of < 5 years between onset of diabetes and colon cancer mortality

Variables adjusted	Duration of insulin use								
	< 5 yr			5-9 yr			≥ 10 yr		
	HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value
Before exclusion									
Unadjusted	1.128	0.734-1.735	0.5824	1.245	0.664-2.333	0.4947	1.539	0.918-2.579	0.1018
Age	1.388	0.902-2.136	0.1355	1.487	0.793-2.787	0.2163	1.441	0.860-2.416	0.1654
Sex	1.146	0.745-1.763	0.5339	1.267	0.676-2.375	0.4605	1.562	0.932-2.618	0.0907
Diabetes type	1.101	0.682-1.777	0.6931	0.817	0.362-1.843	0.6259	1.321	0.589-2.964	0.4991
Diabetes duration	1.009	0.655-1.556	0.9663	1.028	0.546-1.935	0.9330	1.018	0.590-1.756	0.9491
Body mass index	1.089	0.707-1.675	0.6995	1.210	0.645-2.268	0.5530	1.505	0.898-2.523	0.1208
Smoking	1.143	0.743-1.759	0.5416	1.263	0.674-2.367	0.4666	1.549	0.924-2.596	0.0966
Area of residence	1.122	0.729-1.726	0.6005	1.237	0.660-2.318	0.5074	1.538	0.918-2.578	0.1023
Full model ¹	1.217	0.764-1.937	0.4082	0.943	0.442-2.010	0.8788	1.003	0.454-2.212	0.9948
After exclusion									
Unadjusted	1.108	0.698-1.761	0.6630	1.416	0.755-2.656	0.2782	1.746	1.041-2.931	0.0348
Age	1.384	0.871-2.199	0.1696	1.704	0.908-3.197	0.0971	1.633	0.973-2.740	0.0635
Sex	1.129	0.710-1.793	0.6085	1.445	0.771-2.711	0.2510	1.776	1.058-2.981	0.0297
Diabetes type	1.089	0.651-1.821	0.7447	1.010	0.446-2.287	0.9807	1.700	0.770-3.757	0.1893
Diabetes duration	0.943	0.592-1.501	0.8045	1.079	0.573-2.034	0.8133	0.968	0.561-1.669	0.9057
Body mass index	1.072	0.674-1.703	0.7702	1.375	0.733-2.581	0.3215	1.710	1.019-2.870	0.0424
Smoking	1.126	0.708-1.788	0.6164	1.440	0.768-2.701	0.2557	1.760	1.049-2.954	0.0323
Area of residence	1.103	0.694-1.752	0.6786	1.409	0.751-2.643	0.2855	1.746	1.040-2.929	0.0349
Full model ¹	1.133	0.691-1.857	0.6214	1.038	0.491-2.195	0.9221	1.003	0.462-2.178	0.9949

¹Adjusted for age, sex, diabetes type, diabetes duration, body mass index, smoking and area of residence. HR: Hazard ratio; CI: Confidence interval.

database; and the consistency observed in both sexes, and in different age groups, enrollment periods and sub-cohorts of diabetic patients.

There were limitations to the study. First, diabetic patients might have visited their physicians more frequently, resulting in a higher probability of detecting cancer. However, this might only suggest a higher rate of detection of early colon cancer with a better prognosis, which might have attenuated the magnitude of the mortality rate ratios. Second, the use of cause of death on the death certificate as the only source of colon cancer diagnosis might have underestimated the mortality related to colon cancer, because some patients with colon cancer might have died without having colon cancer listed as the cause of death. Therefore the impact of this possible effect awaits further investigation. Third, multiple drug therapy in diabetic patients might have complicated the situation. For example, statin, aspirin and nonsteroidal anti-inflammatory drugs are possibly preventive for colorectal cancer^[30,31]. A higher proportion of diabetic patients might have been using these medications for the prevention of cardiovascular diseases. Different oral antidiabetic agents may have different effects on cancer development. For example, metformin has been shown to be preventive for cancer^[32], but the use of sulfonylureas may be associated with a higher risk of cancer^[33]. We could not evaluate the effects of these medications because such information was not collected. Fourth, this study did not consider confounders identified in Taiwan, including less exercise, less vegetable and fruit consumption, increased meat intake, and alcohol intake^[25]. Furthermore, we were not able to adjust for some other

confounders, as discussed below. For example, hyperhomocysteinemia has been shown to be a risk factor for type 2 diabetes and is also associated with abnormal DNA methylation, which has the potential to inactivate tumor suppressor genes leading to colorectal cancer^[31]. Family history and inflammatory bowel disease are also significant risk factors for colorectal cancer^[34-36]. None of these potential confounders were measured and could not be considered for adjustment.

In summary, we have demonstrated an increasing trend in colon cancer mortality in the Taiwanese general population from 1995 to 2006. The risk is increased in diabetic patients, with the magnitude of the mortality rate ratio becoming larger with decreasing age. Smoking is a risk factor, but insulin use is not. Given that the population is aging and the incidence of type 2 diabetes is increasing, the impact of the link between diabetes and colon cancer on the mortality of the population warrants public health attention.

COMMENTS

Background

Diabetic patients may have a higher risk of colon cancer, but whether insulin use in the diabetic patients can be a risk factor is controversial. Studies related to these issues are rarely conducted in Asian populations.

Research frontiers

A meta-analysis suggested a 30% higher risk of colorectal cancer in diabetic patients. However, most studies were done in western countries. In Korea, a 28% higher risk of mortality was observed in diabetic men, but not women. Whether insulin use is associated with colorectal cancer has rarely been studied. A recent prospective United States study showed that diabetes was significantly associated with colorectal cancer in men who were either insulin users or

non-users, but diabetes and insulin use were not associated with a higher risk among women.

Innovations and breakthroughs

The author demonstrated an increasing trend in colon cancer mortality in the Taiwanese general population from 1995 to 2006. The risk is increased in diabetic patients, with the magnitude of the mortality rate ratio becoming larger with decreasing age. In patients with diabetes, smoking is a risk factor for colon cancer mortality, but insulin use is not. The strengths of this study included a prospective follow-up of a large cohort of diabetic patients over a long duration of 12 years; the completeness of the ascertainment of vital status by matching with the national death certificate database; the consistency observed in both sexes, and in different age groups, enrollment periods and sub-cohorts of diabetic patients; and consideration of the confounding effects of diabetes duration and age - both being highly associated with insulin use.

Applications

Given that the population is aging and the incidence of diabetes is increasing, the impact of the link between diabetes and colon cancer on the mortality of the population warrants public health attention. Insulin is commonly used in diabetic patients, therefore, clarification of a lack of insulin effect on colon cancer development relieves concern about the use of insulin.

Terminology

Secular trend of mortality: a systematic change in mortality rates over a period of (calendar) time; Mortality rate ratio: ratio of two mortality rates.

Peer review

This was a well-performed study that may be published when some corrections have been done.

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Role of body mass index in colon cancer patients in Taiwan

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Abstract

AIM: To determine the effect of body mass index (BMI) on the characteristics and overall outcome of colon cancer in Taiwan.

METHODS: From January 1995 to July 2003, 2138 patients with colon cancer were enrolled in this study. BMI categories (in kg/m²) were established according to the classification of the Department of Health of Taiwan. Postoperative morbidities and mortality, and survival analysis including overall survival (OS), disease-free survival (DFS), and cancer-specific survival (CSS) were compared across the BMI categories.

RESULTS: There were 164 (7.7%) underweight (BMI < 18.5 kg/m²), 1109 (51.9%) normal-weight (BMI = 18.5-23.9 kg/m²), 550 (25.7%) overweight (BMI = 24.0-26.9 kg/m²), and 315 (14.7%) obese (BMI ≥

27 kg/m²) patients. Being female, apparently anemic, hypoalbuminemic, and having body weight loss was more likely among underweight patients than among the other patients ($P < 0.001$). Underweight patients had higher mortality rate ($P = 0.007$) and lower OS ($P < 0.001$) and DFS ($P = 0.002$) than the other patients. OS and DFS did not differ significantly between normal-weight, overweight, and obese patients, while CSS did not differ significantly with the BMI category.

CONCLUSION: In Taiwan, BMI does not significantly affect colon-CSS. Underweight patients had a higher rate of surgical mortality and a worse OS and DFS than the other patients. Obesity does not predict a worse survival.

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Key words: Body mass index; Colon cancer; Survival; Morbidity; Outcome

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INTRODUCTION

The prevalence of obesity and the incidence of colon cancer continue to increase in Taiwan as in other developed countries. The findings of many studies support the idea that obesity is a risk factor for the development of colon cancer^[1-3]. However, the few studies that have investigated the influence of body mass index (BMI) on the outcomes of colon cancer patients have produced

inconsistent findings^[4-7]. These previous reports were mostly from Western countries and based on clinical trial data. We investigated herein the role of BMI in general colon cancer patients in Taiwan; the population of this study may represent both Chinese people and Asians in general, in terms of the clinical characteristics, postoperative morbidities and mortalities, and long-term outcome defined by overall survival (OS), disease-free survival (DFS), and cancer-specific survival (CSS).

MATERIALS AND METHODS

Study population

From January 1995 to July 2003, 2138 patients from a total population of 2765 patients with histologically confirmed adenocarcinoma of the colon were enrolled in this study; 14 nonmetastatic patients without available BMI data and 613 patients with metastatic disease were excluded. The included patients had regular follow-up visits with a healthcare professional until December 2008 (i.e., a postoperative follow-up period of at least 5 years) or until death.

The demographic and clinicopathologic data evaluated for each patient included age, gender, body height and weight, tumor stage (as defined according to the American Joint Committee on Cancer TNM staging system, sixth edition, New York: Springer-Verlag, 2002), tumor location, degree of tumor differentiation, timing of surgery (i.e., elective or emergent), medical illness, and preoperative laboratory data.

BMI was calculated as the weight in kilograms divided by the height in meters squared. The following BMI categories were established according to the classification of the Department of Health (DOH) of Taiwan:^[8] underweight (BMI < 18.5 kg/m²), normal weight (BMI = 18.5-23.9 kg/m²), overweight (BMI = 24.0-26.9 kg/m²), and obese (BMI ≥ 27 kg/m²). For long-term outcome, BMI was also categorized according to the World Health Organization (WHO) classification^[9] as follows: underweight (BMI < 18.5 kg/m²), normal weight (BMI = 18.5-24.9 kg/m²), overweight (BMI = 25-29.9 kg/m²), and obese (BMI ≥ 30 kg/m²) to enable comparison with the Taiwanese classification, since The Expert Consultation on BMI in Asian populations recommended that the current WHO BMI cut-off points be retained as the international classification^[10].

Local recurrence or distant metastasis was confirmed histologically or radiographically, while postoperative mortality was defined as death within 30 d of the primary surgery. The patients were divided into three age groups: younger than 40 years (younger group), 40-75 years (middle age group), and older than 75 years (elderly group). The carcinoembryonic antigen (CEA) level was considered to be abnormal at > 5 ng/mL. Tumor location was categorized as right (from the cecum to the transverse colon) or left (from the splenic flexure to the sigmoid colon). Hypoalbuminemia was defined as a serum albumin level of < 35 g/L and apparent anemia was defined as a hemoglobin level of < 10 g/dL.

Table 1 Patient and tumor characteristics stratified according to body mass index category

	Percentage of patients within each BMI category				P value
	Under-weight (n = 164)	Normal weight (n = 1109)	Over-weight (n = 550)	Obese (n = 315)	
Age at diagnosis (yr)					< 0.001
≤ 40	12.8	8.7	5.8	4.8	
41-75	57.3	75.5	81.6	83.2	
> 75	29.9	15.9	12.5	12.1	
Gender (%)					
Female	65.9	47.2	44.7	48.3	< 0.001
Tumor location					
Right	47.0	41.6	35.5	29.5	< 0.001
Tumor grade					
High-to-moderate differentiation	82.9	83.7	85.6	87.9	0.239
Tumor stage					< 0.001
I	9.1	9.2	15.3	19.4	
II	48.2	50.9	44.7	45.1	
III	42.7	39.9	40.0	35.6	
Emergent operation	3.7	3.4	2.5	2.2	0.584
Body weight loss					< 0.001
CEA > 5 ng/mL	44.2	34.6	36.3	36.0	0.150
Albumin < 35 g/L	42.3	21.0	13.5	11.6	< 0.001
Hemoglobin < 10 g/dL	40.9	32.9	23.2	21.3	< 0.001
Comorbidities					
None	57.3	51.7	50.1	41.6	< 0.001
Hypertension	11.0	19.7	26.2	34.9	< 0.001
Heart disease	8.5	6.8	7.6	12.7	0.008
Previous stroke	3.0	3.4	4.5	4.8	0.527
Asthma	3.7	3.3	3.6	2.9	0.936
Diabetes mellitus	4.9	10.6	12.4	20.6	< 0.001
Peptic ulcer disease	14.0	10.8	9.1	8.9	0.229
Chronic hepatitis	1.2	4.3	3.3	4.1	0.225
Liver cirrhosis	1.8	1.4	1.1	0.6	0.650
Renal insufficiency	5.2	8.9	6.8	9.4	0.202
Others	16.6	13.8	12.0	15.1	0.555

Except where stated otherwise, data are the percentage of the particular body mass index (BMI) category population. CEA: Carcinoembryonic antigen.

Statistical analysis

Survival curves were constructed using the Kaplan-Meier method and then compared using the log-rank test. OS was calculated as the number of years from primary surgery to the date of death. DFS was calculated as the number of years from primary surgery to either the first disease recurrence or death. CSS was calculated as the number of years from primary surgery to the first of disease recurrence. The two arms were compared by Pearson χ^2 test and independent-samples *t* tests to

Table 2 Univariate analysis of survival of colon cancer patients stratified according to body mass index category (Taiwan *vs* World Health Organization)

	Underweight (<i>n</i> = 164)	Normal weight (<i>n</i> = 1109)	Overweight (<i>n</i> = 550)	Obese (<i>n</i> = 315)
BMI category in Taiwan				
Overall survival				
5-yr survival rate	60.0	73.6	78.2	75.4
HR (95% CI)	1.67 (1.31-2.13)	Reference	0.83 (0.69-0.99)	0.93 (0.74-1.16)
<i>P</i> value	< 0.001		0.046	0.500
Disease-free survival				
5-yr survival rate	57.4	70.8	73.4	71.0
HR (95% CI)	1.60 (1.26-2.04)	Reference	0.86 (0.72-1.04)	0.97 (0.79-1.20)
<i>P</i> value	< 0.001		0.103	0.807
Cancer-specific survival				
5-yr survival rate	71.6	78.5	78.9	77.3
HR (95% CI)	1.31 (0.94-1.82)	Reference	0.95 (0.77-1.19)	1.01 (0.78-1.32)
<i>P</i> value	0.107		0.657	0.932
BMI categories of WHO				
	Underweight (<i>n</i> = 164)	Normal-weight (<i>n</i> = 1331)	Overweight (<i>n</i> = 553)	Obese (<i>n</i> = 90)
Overall survival				
5-yr survival rate	60.0	74.0	78.2	75.0
HR (95% CI)	1.70 (1.34-2.15)	Reference	0.85 (0.71-1.02)	0.99 (0.66-1.39)
<i>P</i> value	< 0.001		0.072	0.833
Disease-free survival				
5-yr survival rate	57.4	71.0	73.2	70.4
HR (95% CI)	1.62 (1.28-2.06)	Reference	0.89 (0.74-1.05)	1.05 (0.73-1.49)
<i>P</i> value	< 0.001		0.169	0.808
Cancer-specific survival				
5-yr survival rate	71.6	78.2	79.5	75.0
HR (95% CI)	1.30 (0.94-1.80)	Reference	0.92 (0.74-1.13)	1.10 (0.72-1.70)
<i>P</i> value	0.113		0.418	0.661

Except where stated otherwise, data are the percentage of the particular body mass index (BMI) category population. WHO: World Health Organization; HR: Hazard ratio; CI: Confidence interval.

detect any differences in proportions and means. A Cox regression model was used for multivariate analysis. All *P* values were two-tailed, and they were considered to be statistically significant at < 0.05 .

RESULTS

Disease and patient characteristics

The study population comprised 2138 patients with colon cancer, of whom 1109 were males and 1029 were females. BMI in this study ranged from 12.2 kg/m² to 49.0 kg/m², with a mean of 23.4 kg/m². Of the 2138 patients, 164 (7.7%) were underweight, 1109 (51.9%) were normal weight, 550 (25.7%) were overweight, and 315 (14.7%) were obese.

The characteristics of the patients and tumors are presented stratified according to BMI category in Table 1. The age of the entire study population was 61.4 ± 13.9 years (mean ± SD); those of the underweight, normal-weight, overweight, and obese patients were 63.5 ± 17.3 years, 61.8 ± 14.0 years, 61.9 ± 12.4 years, and 61.7 ± 12.3 years, respectively. The mean age did not differ significantly with the BMI category (*P* = 0.828). However, the distribution of age groups differed significantly with the BMI category, with the proportions of younger and elderly patients being higher in underweight-patients group than in the other groups (*P* < 0.001). The gender distribution also differed significantly between the underweight patients and the other groups, with underweight

patients being more likely to be female. The tumor location also differed significantly, with the proportion of right colon cancer decreasing as the BMI category increased (*P* < 0.001).

With respect to the distribution of tumor stage among the BMI categories, the number of stage I tumors was lower in the underweight and normal-weight patients than in the overweight and obese patients. The obese patients had the lowest number of stage III tumors.

The proportion of emergent operations did not differ significantly with the BMI category: 3.7% of underweight, 3.4% of normal-weight, 2.5% of overweight, and 2.2% of obese patients (*P* = 0.584).

Underweight patients were the most likely to exhibit apparent anemia (hemoglobin < 10 g/dL) and have hypoalbuminemia and body weight loss (*P* < 0.001). The proportion of patients with body weight loss, hypoalbuminemia, and apparent anemia decreased as the BMI category increased. The percentage of patients with an abnormal preoperative CEA level did not differ significantly with the BMI category.

Finally, with regard to associated medical illnesses, the percentage of patients with hypertension or diabetes mellitus increased with the BMI category. Obese patients were the most likely to have heart disease. The other associated comorbidities including previous stroke, asthma, peptic ulcer disease, chronic hepatitis, renal insufficiency, and others (e.g., gall stones and thyroid disease) did not differ significantly with the BMI category.

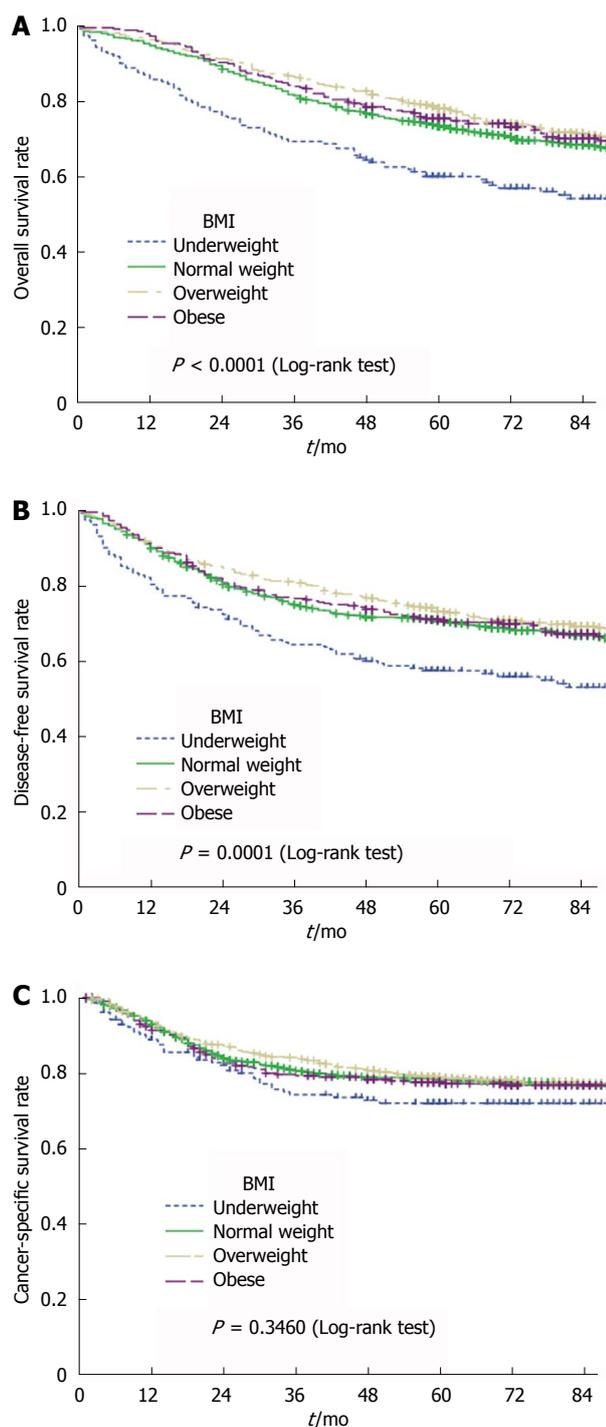


Figure 1 Overall survival curves (A), disease-free survival curves (B) and cancer-specific survival curves (C) relative to body mass index category using the Kaplan-Meier method, and comparison using the log-rank test. BMI: Body mass index.

Short-term outcome

The postoperative morbidity, anastomotic leakage, and mortality rates did not differ significantly between the underweight, normal-weight, overweight, and obese patients: 12.8%, 12.9%, 13.3% and 15.6%, respectively ($P = 0.667$); 1.2%, 1.4%, 1.6%, and 1.9%, respectively ($P = 0.880$); and 3.7%, 1.3%, 1.1%, and 0%, respectively ($P = 0.007$).

Long-term outcome

Data from all patients who received surgery were used to analyze the long-term outcome. Univariate analysis was performed using the Kaplan-Meier method, along with the log-rank test and Cox proportional-hazards model. The results of our statistical analysis are presented in Table 2, and the survival curves are shown in Figure 1A (OS), Figure 1B (DFS), and Figure 1C (CCS).

Univariate analysis revealed that OS ($P < 0.001$) and DFS ($P = 0.001$) were lowest in the underweight patients. The OS was marginally higher ($P = 0.046$) in overweight patients than in normal-weight patients, but the DFS ($P = 0.103$) did not differ significantly between the two groups. No significant difference in OS ($P = 0.500$) or DFS ($P = 0.807$) was observed between normal-weight and obese patients.

CSS did not differ significantly with the BMI category ($P = 0.346$). The results were similar when these patients were categorized according to the WHO BMI classifications.

Adjuvant chemotherapy was offered to 58.0%, 70.1%, 73.9%, and 70.8% of underweight, normal-weight, overweight, and obese patients with stage III tumors, respectively ($P = 0.096$). In total, 70.2% of patients with stage III tumors received adjuvant chemotherapy.

Since the compositions of patients and tumors differed with the BMI category, multivariate analysis was used to determine the effect of BMI along with other confounding factors on the long-term outcome. The variables of the Cox regression model included BMI, TNM stage, age group, gender, comorbidities (patients were divided into three groups: without, with one or two kinds, and with more than two kinds of comorbidity), CEA (normal *vs* abnormal), hemoglobin (< 10 g/L *vs* ≥ 10 g/L), albumin (normal *vs* hypoalbuminemia), timing of surgery (elective *vs* emergent), postoperative morbidity (with *vs* without), tumor location (right *vs* left), histologic type (adenocarcinoma *vs* mucinous *vs* signet-ring type), and histologic grade (high *vs* moderate *vs* low differentiation). The hazard ratio (HR) of each BMI category was compared with that of the normal-weight patients. The results of multivariate analysis for OS, DFS, and CSS are listed in Table 3.

As documented in Table 4, underweight patients had a significantly worse OS [HR, 1.58; 95% confidence interval (CI), 1.23-2.05; $P < 0.001$] and DFS (HR, 1.49; 95% CI, 1.16-1.93; $P = 0.002$), but their CSS did not differ significantly (HR, 1.33; 95% CI, 0.94-1.87; $P = 0.107$) when compared with the normal-weight patients. OS, DFS, or CSS did not differ significantly between the normal-weight, overweight, and obese patients.

To determine whether the prognostic effect of BMI was related to patient gender, a multivariate analysis was applied to separated data from male and female patients. The results of this statistical analysis yielded the same findings between the genders: CSS did not differ significantly with the BMI category for all patients or for male

Table 3 Multivariate analysis of colon cancer survival (overall, disease-free, and cancer-specific) by Cox regression model

Variable	Overall survival		Disease-free survival		Cancer-specific survival	
	P value	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)
BMI group	0.000		0.002		0.369	
Underweight <i>vs</i> normal	0.000	1.58 (1.23-2.05)	0.002	1.49 (1.16-1.93)	0.107	1.33 (0.94-1.87)
Overweight <i>vs</i> normal	0.060	0.83 (0.68-1.01)	0.123	0.86 (0.71-1.04)	0.751	0.96 (0.76-1.22)
Obese <i>vs</i> normal	0.578	0.94 (0.74-1.18)	0.972	1.00 (0.79-1.25)	0.677	1.06 (0.80-1.41)
TNM stage	0.000		0.000		0.000	
II <i>vs</i> I	0.721	1.06 (0.77-1.45)	0.981	1.00 (0.74-1.35)	0.025	1.78 (1.07-2.95)
III <i>vs</i> I	0.000	1.90 (1.39-2.60)	0.000	1.80 (1.34-2.42)	0.000	3.94 (2.39-6.48)
Age group (yr)	0.000		0.000		0.876	
41-75 <i>vs</i> ≤ 40	0.054	1.45 (0.99-2.11)	0.052	1.44 (0.99-2.09)	0.672	1.09 (0.74-1.58)
> 75 <i>vs</i> ≤ 40	0.000	3.14 (2.11-4.67)	0.000	2.88 (1.94-4.25)	0.607	1.12 (0.72-1.74)
Gender (male <i>vs</i> female)	0.329	1.08 (0.92-1.27)	0.220	1.10 (0.94-1.29)	0.734	1.03 (0.85-1.26)
Medical illness (comorbidity)	0.003		0.003		0.769	
One or two kinds <i>vs</i> none	0.358	1.09 (0.91-1.32)	0.442	1.08 (0.89-1.29)	0.927	0.99 (0.79-1.24)
> two kinds <i>vs</i> none	0.001	1.40 (1.16-1.71)	0.001	1.39 (1.15-1.68)	0.478	0.91 (0.70-1.18)
CEA (> 5 <i>vs</i> ≤ 5 ng/mL)	0.000	1.86 (1.58-2.18)	0.000	1.93 (1.65-2.25)	0.000	2.15 (1.77-2.61)
Hemoglobin (< 10 <i>vs</i> ≥ 10 g/dL)	0.913	1.01 (0.84-1.22)	0.760	1.03 (0.86-1.23)	0.835	1.03 (0.81-1.29)
Albumin (normal <i>vs</i> hypoalbuminemia)	0.000	0.65 (0.54-0.79)	0.000	0.68 (0.57-0.83)	0.297	0.87 (0.67-1.13)
Operation timing (emergent <i>vs</i> elective)	0.562	1.21 (0.64-2.26)	0.416	1.28 (0.70-2.34)	0.687	1.18 (0.53-2.66)
Morbidity (yes <i>vs</i> no)	0.000	1.55 (1.26-1.91)	0.000	1.50 (1.23-1.84)	0.279	1.17 (0.88-1.56)
Tumor location (left <i>vs</i> right)	0.785	1.03 (0.86-1.22)	0.666	1.04 (0.88-1.23)	0.583	1.06 (0.86-1.32)
Histologic type	0.079		0.144		0.055	
Signet ring cell <i>vs</i> adenocarcinoma	0.039	2.19 (1.04-4.63)	0.065	2.03 (0.96-4.30)	0.017	2.68 (1.19-6.01)
Mucinous <i>vs</i> adenocarcinoma	0.323	1.14 (0.88-1.49)	0.465	1.10 (0.85-1.43)	0.690	1.07 (0.77-1.48)
Histologic grade (differentiation)	0.282		0.213		0.173	
Moderate <i>vs</i> high	0.363	1.11 (0.89-1.37)	0.143	1.17 (0.95-1.45)	0.061	1.32 (0.99-1.76)
Low <i>vs</i> high	0.114	1.34 (0.93-1.94)	0.121	1.33 (0.93-1.92)	0.403	1.24 (0.75-2.04)

HR: Hazard ratio; CI: Confidence interval; BMI: Body mass index; TNM: Tumor, nodes, metastasis; CEA: Carcinoembryonic antigen.

or female patients.

DISCUSSION

BMI is a simple and easy-to-determine index used to classify individuals as being underweight, overweight, or obese. The classification of BMI was established to evaluate health risks such as type 2 diabetes and cardiovascular disease^[9,10]. The associations between BMI, percentage of body fat, and distribution of body fat differ across populations, which has resulted in different cut-off points for classifications being used in different countries. Furthermore, the cut-off points for certain risks vary between different Asian populations^[10]. The Expert Consultation on BMI in Asian populations recommended that the current WHO BMI cut-off points be retained as the international classification^[10]. In the present study, BMI classifications were based on the definition of the DOH, Taiwan. In addition, the WHO BMI classifications were used to evaluate the long-term outcome of colon cancer survivors and to compare with the Taiwan BMI classification system. In the present study, the prevalence rates of diabetes and hypertension increased significantly with the BMI category; this finding is highly consistent with the intended meaning of the BMI classification system. However, the risks of other comorbidities such as asthma, hepatitis, peptic ulcer disease, and renal insufficiency were not correlated with the BMI category.

Obesity is a growing problem in Taiwan and is known to increase the risk of developing colon cancer^[1-3]. It has also been reported that obese patients have a higher risk of surgical complications^[11-13]. Previous studies have produced controversial results regarding the relationship between obesity and anastomotic leakage^[14,15]. Sorensen *et al*^[14] reported that obesity is not a risk factor for anastomotic leakage in colonic resection, while Biondo *et al*^[15] reported that obesity is an independent risk factor, but is only associated with emergent procedures of the left colon. Miransky *et al*^[16] reported that obesity and a contaminated surgical procedure independently predicted surgical-site infection in colorectal procedures. Riou *et al*^[17] reported that obesity was a significant independent risk factor for wound dehiscence. Obesity has also been reported to be a risk factor for the postoperative occurrence of pulmonary complications^[18,19]. The postoperative morbidity rate and anastomotic leakage rate did not differ significantly with the BMI category in our study. However, one of the limitations of this study is that we did not examine whether the occurrence rate of the other type of complications differed with the BMI category.

In the present study, the risk of postoperative mortality was highest in underweight patients. This finding is similar to that of Hickman *et al*^[20]. Although obese patients have higher rates of comorbidities with cardiovascular disease and diabetes, the postoperative morbidity and mortality rates were comparable with the normal-weight patients. Conversely, the underweight patients had

Table 4 Results of multivariate analysis of colon cancer survival stratified according to body mass index category

	Underweight	Normal weight	Overweight	Obese
All patients (<i>n</i> = 2138)				
Overall survival				
HR (95% CI) ¹	1.58 (1.23-2.05)	Reference	0.83 (0.68-1.01)	0.94 (0.74-1.18)
<i>P</i> value ²	< 0.001		0.060	0.578
Disease-free survival				
HR (95% CI) ¹	1.49 (1.16-1.93)	Reference	0.86 (0.71-1.04)	1.00 (0.79-1.25)
<i>P</i> value ²	0.002		0.123	0.972
Cancer-specific survival				
HR (95% CI) ¹	1.33 (0.94-1.87)	Reference	0.96 (0.76-1.22)	1.06 (0.80-1.41)
<i>P</i> value ²	0.107		0.751	0.677
Males (<i>n</i> = 1109)				
Overall survival				
HR (95% CI) ¹	1.55 (1.03-2.35)	Reference	0.77 (0.58-1.01)	0.91 (0.66-1.25)
<i>P</i> value ²	0.036		0.056	0.565
Disease-free survival				
HR (95% CI) ¹	1.60 (1.04-2.44)	Reference	0.84 (0.65-1.09)	1.01 (0.75-1.37)
<i>P</i> value ²	0.031		0.192	0.937
Cancer-specific survival				
HR (95% CI) ¹	1.46 (0.84-2.52)	Reference	0.96 (0.69-1.32)	1.21 (0.83-1.77)
<i>P</i> value ²	0.179		0.789	0.328
Females (<i>n</i> = 1029)				
Overall survival				
HR (95% CI) ¹	1.55 (1.11-2.16)	Reference	0.95 (0.71-1.27)	0.99 (0.69-1.41)
<i>P</i> value ²	0.011		0.724	0.945
Disease-free survival				
HR (95% CI) ¹	1.41 (1.01-1.97)	Reference	0.91 (0.68-1.22)	1.01 (0.71-1.43)
<i>P</i> value ²	0.042		0.544	0.957
Cancer-specific survival				
HR (95% CI) ¹	1.16 (0.75-1.82)	Reference	0.96 (0.69-1.36)	0.93 (0.60-1.43)
<i>P</i> value ²	0.508		0.839	0.727

¹HR was calculated using the Cox regression model; the variables in the multivariate analysis included TNM stage, age, gender (when analyzing all patients), comorbidities, CEA, hemoglobin, albumin, operative timing, postoperative morbidity, tumor location, histologic type, and histologic grade; ²Each category compared to normal-weight patients. HR: Hazard ratio; CI: Confidence interval; BMI: Body mass index; TNM: Tumor, nodes, metastasis; CEA: Carcinoembryonic antigen.

a lower rate of comorbidities but a higher rate of postoperative mortality than did the other patients. However, a higher proportion of underweight patients in this study were hypoalbuminemic and anemic. The observed higher postoperative mortality rate may be at least partially attributed to the associated disease conditions.

Whether obese colon cancer patients have a worse long-term outcome than other patients remains a matter of controversy. Sinicrope *et al*⁷¹ reported that underweight patients had a significantly worse OS ($P = 0.0258$), and that BMI ≥ 35 kg/m² patients exhibited a trend toward a worse DFS ($P = 0.0725$) and OS ($P = 0.0805$) compared with normal-weight patients, but there was no significant difference. When they analyzed the data according to patient gender, males with BMI ≥ 35 kg/m² exhibited a reduced OS, and females with obesity (BMI = 30-34 kg/m²) had a reduced OS when compared with their normal-weight counterparts. BMI category was significantly associated with both DFS and OS in multivariate analysis in their study. Meyerhardt *et al*⁶¹ reported that neither BMI nor weight change was significantly associated with colon cancer patient survival indicators,

including the OS, DFS, and recurrence-free survival, even for underweight patients. Dignam *et al*⁵¹ reported that OS and DFS were significantly worse for underweight patients (BMI < 18.5 kg/m²) and very obese patients (BMI ≥ 35 kg/m²) than for normal-weight patients. Very obese patients had a greater risk of cancer recurrence or secondary primary tumors. In the present study, we found that BMI by itself was not a significant factor of CSS in colon cancer, but OS and DFS did tend to be worse for underweight patients than for the other patients. We found no differential effect of gender on either BMI or obesity. Compared with other patients, underweight patients had a worse OS but a similar CSS. This implies that many underweight patients died from noncancer events. It would have been reasonable to conclude that underlying comorbidities caused the higher mortality risk among the underweight patients, but in the present study this group actually had the smallest number of comorbidities. Further research should be conducted to establish the mechanisms responsible for the observed higher mortality risk in underweight patients. Furthermore, previous studies have shown that highly

obese patients (BMI ≥ 35 kg/m²) or males may have a worse long-term outcome than normal-weight patients, but the obese cohort of the present study was not large enough to allow analysis of the difference.

In this study, tumor location was found to be correlated with BMI, such that the proportion of patients with right colon cancer increased as the BMI category decreased. Whether patients with a lower BMI tend to have right-side colon cancer is not well known or studied. However, since the lumen is larger for the right than the left colon, symptoms related to the tumor such as small-caliber or bloody stool need more time to be sensed by patients with right colon cancer, resulting in a longer period of nutrition depletion and body weight loss. Minoo *et al.*^[21] reported that proximally located tumors are significantly larger than those found in the distal colon. We have shown previously that the prevalence of malnutrition (hypoalbuminemia) is higher for right colon tumors than for left colon tumors^[22]. Moreover, body weight loss is more common in right colon cancer than in left colon cancer (48.8% and 33.5%, respectively; $P < 0.001$). BMI is reduced in patients with body weight loss, and more patients with right colon tumors have body weight loss resulting in a lower BMI, which may partly explain the greater number of left-side tumors in the groups with a higher BMI.

The findings of this study suggest that a low BMI is a marker of weight loss, blood loss, nutrition depletion, and more-advanced disease, all of which are associated with a worse DFS and OS^[22-24]. This could be the reason why the low-BMI group had a lower DFS and OS.

This study was subject to some limitations. It lacked data regarding changes in body weight before and after surgery, measurement of central obesity, physical activity, and diet changes after surgery, and involved a smaller sample than did previous studies. However, the study's cohort came from a single medical institution with a standard collection of patients' data, and so there was no selection bias as might be expected in a clinical trial. The results of this study pertain to patients from Taiwan and hence may not be generalizable to other populations.

For the population of Taiwan, which represents both Chinese people and Asians in general, BMI does not appear to be a significant factor of colon-CSS, but underweight patients appear to have a higher postoperative mortality and worse OS, and are less likely to experience DFS than those in the other BMI categories. The obese patients had a higher wound complication rate, but exhibited a similar survival rate when compared to the normal-weight patients.

COMMENTS

Background

The prevalence of obesity and the incidence of colon cancer continue to increase in Taiwan as in other developed countries. Studies that have investigated the influence of body mass index (BMI) on the outcomes of colon cancer patients have produced inconsistent findings. These previous reports were mostly from Western countries, and lacked data regarding Asian people. The authors

therefore investigated the role of BMI in general colon cancer patients of Taiwan, in terms of the clinical characteristics and short- and long-term outcomes.

Research frontiers

BMI is a simple index that is used to classify individuals as being underweight, overweight, or obese. The cut-off points of BMI for classifications vary in different countries. The World Health Organization BMI classifications were used to evaluate the long-term outcome of colon cancer survivors and were compared with those determined using the Taiwan BMI classification system.

Innovations and breakthroughs

The findings of the present study demonstrate that a low BMI could be a marker of weight loss, blood loss, nutrition depletion, and more-advanced disease. For the population of Taiwan, which represents both Chinese people and other Asians in general, BMI does not appear to significantly affect the colon-cancer-specific survival, although underweight patients do appear to have a higher postoperative mortality and worse overall survival, and are less likely to experience disease-free survival than patients in the other BMI categories.

Applications

BMI is a simple index that can be used to evaluate the likelihood of colon cancer patients developing weight loss, blood loss, nutrition depletion, and more-advanced disease.

Terminology

BMI was calculated as the weight in kilograms divided by the height in meters squared. The classification of BMI was established to evaluate health risks such as type 2 diabetes and cardiovascular disease.

Peer review

This study investigated the effect of body mass index on the characteristics and overall outcome of colon cancer in Taiwan. This manuscript addresses a highly relevant subject and could be important for the cancer field.

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Oxymatrine liposome attenuates hepatic fibrosis *via* targeting hepatic stellate cells

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Abstract

AIM: To investigate the potential mechanism of Arg-Gly-Asp (RGD) peptide-labeled liposome loading oxymatrine (OM) therapy in CCl₄-induced hepatic fibrosis in rats.

METHODS: We constructed a rat model of CCl₄-induced hepatic fibrosis and treated the rats with different formulations of OM. To evaluate the antifibrotic effect of OM, we detected levels of alkaline phosphatase, hepatic histopathology (hematoxylin and eosin stain and Masson staining) and fibrosis-related gene expression of matrix metalloproteinase (MMP)-2, tissue inhibitor of metalloproteinase (TIMP)-1 as well as type I procollagen *via* quantitative real-time polymerase chain reaction. To detect cell viability and apoptosis of hepatic stellate cells (HSCs), we performed 3-(4,5)-dimethylthiazoliazolo(2-yl)-2,5-diphenyltetrazolium bromide assay and flow cytometry. To reinforce the

combination of oxymatrine with HSCs, we constructed fluorescein-isothiocyanate-conjugated Arg-Gly-Asp peptide-labeled liposomes loading OM, and its targeting of HSCs was examined by fluorescent microscopy.

RESULTS: OM attenuated CCl₄-induced hepatic fibrosis, as defined by reducing serum alkaline phosphatase (344.47 ± 27.52 U/L *vs* 550.69 ± 43.78 U/L, $P < 0.05$), attenuating liver injury and improving collagen deposits ($2.36\% \pm 0.09\%$ *vs* $7.70\% \pm 0.60\%$, $P < 0.05$) and downregulating fibrosis-related gene expression, that is, MMP-2, TIMP-1 and type I procollagen ($P < 0.05$). OM inhibited cell viability and induced apoptosis of HSCs *in vitro*. RGD promoted OM targeting of HSCs and enhanced the therapeutic effect of OM in terms of serum alkaline phosphatase (272.51 ± 19.55 U/L *vs* 344.47 ± 27.52 U/L, $P < 0.05$), liver injury, collagen deposits ($0.26\% \pm 0.09\%$ *vs* $2.36\% \pm 0.09\%$, $P < 0.05$) and downregulating fibrosis-related gene expression, that is, MMP-2, TIMP-1 and type I procollagen ($P < 0.05$). Moreover, *in vitro* assay demonstrated that RGD enhanced the effect of OM on HSC viability and apoptosis.

CONCLUSION: OM attenuated hepatic fibrosis by inhibiting viability and inducing apoptosis of HSCs. The RGD-labeled formulation enhanced the targeting efficiency for HSCs and the therapeutic effect.

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Key words: Oxymatrine; Arg-Gly-Asp peptide; Hepatic stellate cell; Hepatic fibrosis; Target therapy

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INTRODUCTION

Hepatic fibrosis is characterized by excessive deposition of extracellular matrix (ECM) components in the interstitial space of the liver^[1,2]. The fibrogenesis is triggered by a variety of events that lead to chronic injury, including viral infection, drug or alcoholic toxicity, autoimmune disorders and metabolic diseases. As a consequence of liver damage, nonparenchymal cells are activated by a concert of mediators released from injured hepatocytes. A population of nonparenchymal cells, the hepatic stellate cells (HSCs), have been identified and recognized for their contributing role in the fibrotic process after transformation towards myofibroblasts^[3]. Thus, HSCs represent an attractive target for the development of antifibrotic strategies incorporating a selective targeting approach for hepatic fibrosis^[4]. Recently, several therapeutic strategies have been developed by means of targeting hepatic fibrosis, including inhibition of collagen synthesis^[5], interruption of matrix deposition^[6], stimulation of matrix degradation, modulation of HSC activation^[7], or induction of HSC death^[8]. Despite advance in understanding hepatic fibrogenesis, therapeutic repertoire for hepatic fibrosis treatment is still limited.

Oxymatrine (OM), an alkaloid extracted from the medicinal plant *Sophora alopecuroides* L, has received increasing attention for its multiple pharmacological functions. OM has been demonstrated to exert an inhibitory effect on the replication of hepatitis B^[9] and C^[10] viruses *in vitro*. Preclinical and clinical studies have shown that OM effectively inhibited infection with hepatitis B virus^[11]. In addition to antiviral effects, OM has been reported to have a beneficial effect on progression of CCl₄-induced hepatic fibrosis in rats. Recent studies have demonstrated that OM induces apoptosis in a variety of cells; mainly malignant cells^[12]. Apoptosis-inducing activity of OM makes it an attractive antifibrotic agent. However, there is limited evidence for the efficacy of OM in hepatic fibrosis and the underlying mechanism.

In the present study we aimed to investigate whether Arg-Gly-Asp (RGD)-mediated targeting delivery of OM exerted antifibrogenic action with improved efficiency of fibrogenic liver^[13]. *In vitro* experiments showed that uptake of OM in HSCs was enhanced and the apoptotic process was induced. In CCl₄-induced rats, delivery of OM to HSCs with this formulation strategy improved the efficacy of this medication in the treatment of hepatic fibrosis.

MATERIALS AND METHODS

Preparation of OM-RGD liposomes

The lipid phase consisting of a mixture of lecithin and cholesterol in a ratio of 2:1 was dissolved in CHCl₃-MeOH (1:1) followed by evaporation and addition of pleic acid and polysorbate. Lipids were mixed with the aqueous solution containing OM and polyvinylpyrrolidone in phosphate-buffered saline (PBS; pH 7.4).

The mixture was sonicated for 5 min at 50% amplifying strength resulting in a water-in-oil emulsion. After removal of the organic solvent with a rotary evaporator under vacuum, the dispersion of liposomes was formed.

RGD peptide was synthesized by the Chinese Peptide Company (Hangzhou, China). RGD peptide coupling was performed as described previously^[14]. In brief, 4 nmol cyclo-Arg-Gly-Asp (cRGD) peptide per mmol total lipid was added after deacetylation of the peptide in 0.5 mol hydroxylamine solution, and incubated for 1 h at room temperature. Unloaded liposomes and unbound RGD were separated by CL-4B column (Amersham, Piscataway, NJ, United States).

HSC preparation

HSCs were isolated by collagenase perfusion through the portal vein in Sprague-Dawley rats, followed by Nycomed gradient centrifugation. Cells were incubated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (v/v) and 100 U/mL penicillin and streptomycin, and maintained at 37 °C in a humidified incubator (90% humidity) containing 50 mL/L CO₂. HSCs seeded at a density of 10⁴ cells/cm² attained confluence in 6 d and formed a monolayer of closely apposed polygonal cells. The morphology and growth of HSCs were confirmed and evaluated by microscopy.

Cell viability assay

Cell viability was determined through 3-(4,5)-dimethyl thiaziazol(-z-y1)-3,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay depends on the extent to which viable cells convert MTT bromide to an insoluble colored formazan product that can be determined spectrophotometrically. After treatment, cells were harvested and washed in PBS, and 200 mL DMEM without phenol red, containing 5 mg/mL MTT, was added to each cell. Three hours later, the medium was aspirated, and the converted dye was solubilized with isopropanol (0.1 mol/L HCl in isopropanol). The resulting absorbance from each cell was measured at a wavelength of 570 nm with background subtraction at 630 nm.

Flow cytometry

HSCs were treated with different concentration of OM-RGD liposomes for 48 h at a cell density of 2 × 10⁵ cells/mL, and then stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) (Sigma, St Louis, MO, United States). Annexin V-FITC-positive and PI-negative cells were considered to be apoptotic cells.

Transmission electron microscopy

HSCs were cultured with different formulation of OM at 37 °C for 24 h and then harvested by trypsinization and centrifugation for 10 min at 3500 rpm at room temperature. Cells were fixed in 4% (v/v) glutaraldehyde for 4 h at 4 °C. The specimens were washed with sodium

Table 1 Serum level of alkaline phosphatase and ratio of collagen area to liver tissue in rats with CCl₄-induced hepatic fibrosis

Group	n	ALP (U/L)
Normal	10	73.91 ± 5.97
CCl ₄ -induced hepatic fibrosis	10	550.69 ± 43.80 ^a
OM-RGD liposomes	10	272.51 ± 19.55 ^{a,c}
OM liposomes	10	344.47 ± 27.52 ^c
RGD liposomes	10	562.78 ± 40.22
		Collagen area (%)
CCl ₄ -induced hepatic fibrosis	5	7.70 ± 0.60
RGD liposomes	5	8.32 ± 0.42
OM liposomes	5	2.36 ± 0.09 ^e
OM-RGD liposomes	5	0.26 ± 0.09 ^e

^a*P* < 0.05 vs normal; ^c*P* < 0.05 vs CCl₄-induced hepatic fibrosis group; ^e*P* < 0.05 vs oxymatrine (OM) liposomes. ALP: Alkaline phosphatase; RGD: Arg-Gly-Asp.

cacodylate buffer (pH 7.4) followed by post-fixation in 1% osmium tetroxide at 4 °C. Cells were then washed with cacodylate buffer (pH 7.4) and dehydrated with a graded series of acetone. The cells were embedded with 100% resin in a beam capsule. A sample block was sectioned using an ultramicrotome. The sections were placed into a grid and stained with uranyl acetate for 10 min followed by 50% filtered acetone, and finally stained with lead. The stained samples were then viewed under a transmission electron microscope (Phillips, Eindhoven, The Netherlands).

Real-time PCR

Total RNA was extracted from cells using the TRIzol reagent. The amount of each RNA sample was determined by Qubit fluorometer. Reverse transcription was performed in a 20-μL reaction system with 200 ng total RNA using high capacity cDNA reverse transcription kits. Relative quantification of designated genes including *MMP-2*, *TIMP* and type I procollagen were assessed by real-time PCR through the ABI 7900HT system. A housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. Primer sequences used are shown as follows: type I procollagen, 5'-CCTGGCAGAACGGAGATGAT-3', 5'-ACCA CAGCACCATCGTTACC-3'; *MMP-2*, 5'-CTATTCTGT CAGCACTT TGG-3', 5'-CAGACTTTGGTTCTC-CA ACTT-3'; *TIMP-1*, 5'-ACA GCTTT CTGCA ACTCG-3', 5'-CTATAGGTCTTT ACGAAGGCC-3'; *GADPH*, 5'-AC CCCC AATGTATCCGTGT-3', 5'-TA CTCCTTGGAGGCCATGTA-3'. The relative abundance of target mRNA was determined with the comparative cycle threshold method.

Animals and experimental design

Male Sprague-Dawley rats came from the Experimental Animal Center of Beijing Medical University (Beijing, China) and were caged in an environment with regulated temperature (21 ± 1.6 °C), humidity (45% ± 10%), and an alternating 12-h light and dark cycle. The animals had

free access to water and diet throughout the study.

For the chronic liver injury model, animals were injected intraperitoneally with 50% CCl₄ (CCl₄:vegetable oil = 1:1) at a dose of 0.15 mL/100 g body weight and were also divided into four groups: Group A (administered with OM-loaded liposome); Group B (administered with OM-RGD liposome); Group C (hepatic fibrosis); and Group D (blank vector). Injections were given three times weekly for 8 wk. After treatment, the rats received an intravenous injection according to their subgrouping. Groups A and B were given OM liposomes and OM-RGD liposomes, respectively. The rats in Group D were administered with 0.5 mL blank liposomes. The rats were sacrificed 8 wk after injection, and the livers and blood samples were collected for further assessments.

For HSC targeting, CCl₄-treated animals were injected with FITC-labeled OM liposomes and OM-RGD liposomes *via* the tail vein. Hepatocytes and HSCs were isolated from each individual rat 24 h post-injection, as described previously^[15].

Histological analysis

Animals were sacrificed 8 wk after treatment. Liver samples from each group were harvested, fixed with 10% formaldehyde, embedded in olefin, and stained with hematoxylin and eosin (HE) as well as Masson collagen staining.

Statistical analysis

The data are expressed as mean ± SD. Student's *t*-test or Dunnett's *t*-test was used to compare the differences between treated and control groups, and differences were considered significant at *P* < 0.05.

RESULTS

Antifibrogenic effect of OM-RGD liposomes in CCl₄-induced fibrotic liver

We evaluated the therapeutic effect of OM-RGD liposomes on CCl₄-induced liver injury in rats. As shown in Table 1, elevated levels of alkaline phosphatase were reduced by OM. Histopathological analysis revealed that CCl₄-induced hepatic fibrosis was ameliorated by OM (Figure 1A). Excessive collagen deposited in response to CCl₄-induced liver injury was ameliorated by OM (Table 1, Figure 1B). Moreover, the RGD-labeled liposomal formulation exerted a more aggressive therapeutic effect in hepatic fibrosis than did OM in terms of alkaline phosphatase (Table 1), histopathology (Figure 1A), and collagen deposits (Table 1, Figure 1B).

OM-RGD liposomes induced apoptosis in HSCs in vitro

The inhibitory effect of OM-RGD liposomes on the viability of primary HSCs was determined by MTT assay. As shown in Figure 2A, OM-RGD liposomes significantly inhibited the viability of HSCs, whereas OM liposomes exhibited low cytotoxicity in HSCs. The ultrastructure of HSCs treated with different formulation of OM was

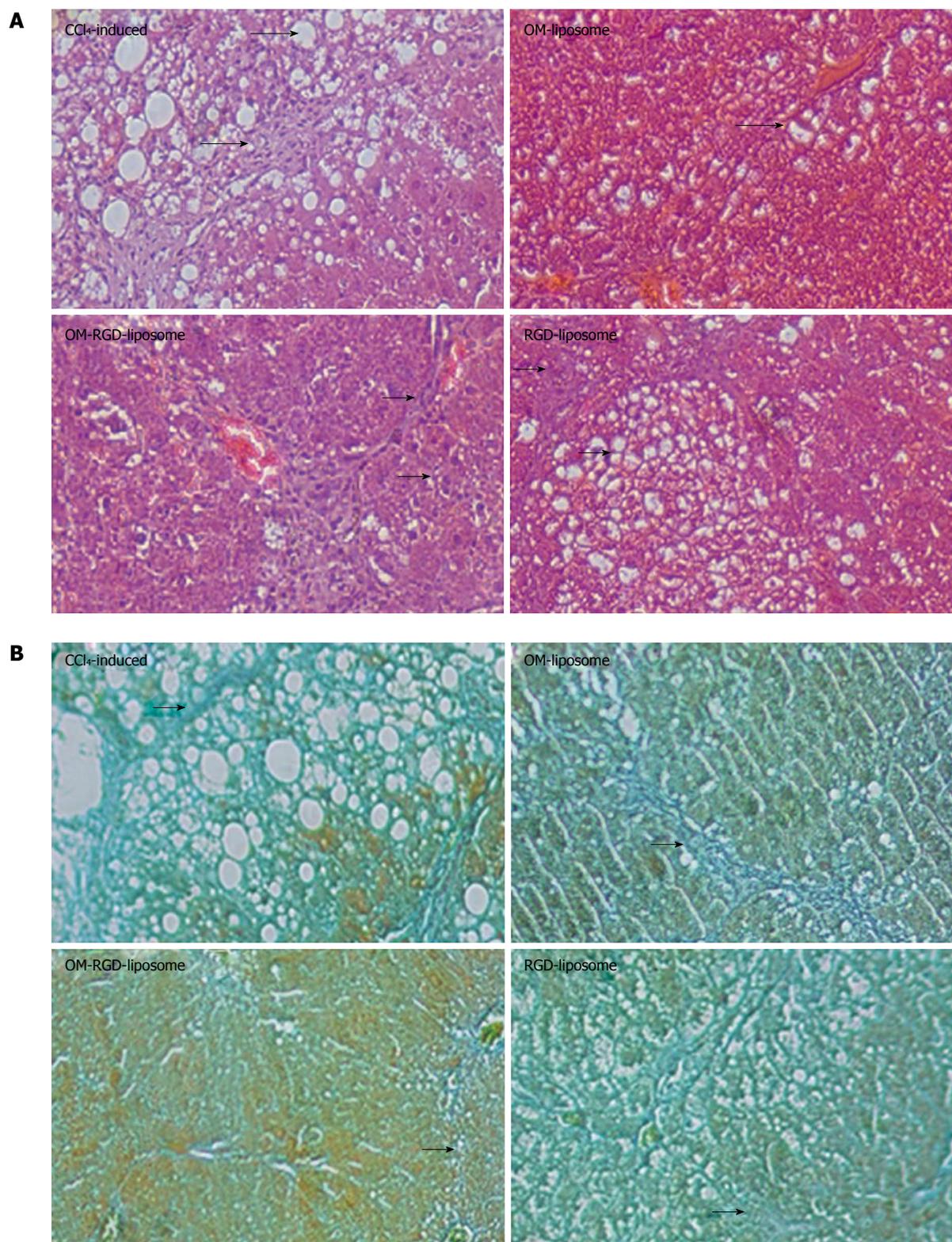


Figure 1 Oxymatrine liposomes attenuated hepatic fibrosis and improved collagen deposition. A: Representative images of liver treated with oxymatrine (OM) in different liposomal formulations in rats with CCl₄-induced hepatic fibrosis. Liver tissues were obtained at 4 wk after treatment and stained with HE; B: Representative histological images of liver treated with OM in different liposomal formulations in rats with CCl₄-induced hepatic fibrosis. Liver tissues were obtained at 4 wk after treatment and stained with Masson stain (original magnification × 100). HE: Hematoxylin and eosin.

examined by transmission electron microscopy (TEM). TEM revealed that treatment with OM-RGD liposomes resulted in typical morphological sign of apoptosis, including cell shrinkage, increased cellular granularity, and

formation of apoptotic bodies (Figure 2B). The apoptotic effect of different formulations of OM was determined by using flow cytometry. Cell cycle analysis revealed that incubation with OM-RGD liposomes resulted

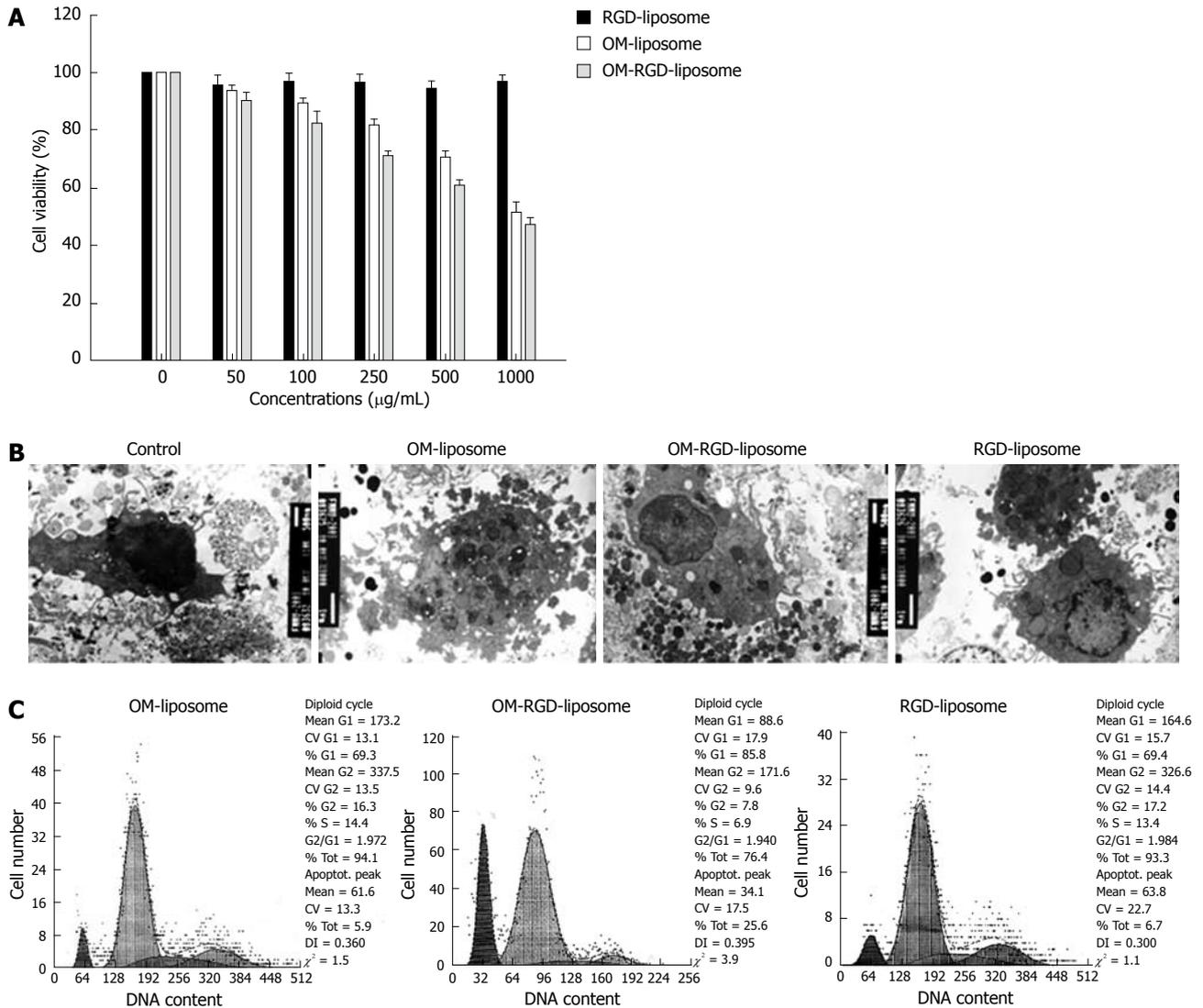


Figure 2 Oxymatrine liposomes induced apoptosis in hepatic stellate cells *in vitro*. **A:** Inhibitory effect of oxymatrine (OM) on hepatic stellate cell (HSC) viability *in vitro*. HSCs were isolated and treated with OM in different liposomal formulations. Cell viability was determined using MTT assay; **B:** Electron micrograph of untreated HSCs demonstrates the normal structure of HSCs. OM-liposome-treated (24 h) HSCs had morphological features of apoptosis: cell shrinkage and apoptotic body formation. OM-RGD-liposome-treated HSCs showed typical morphological features of apoptosis: cell shrinkage and apoptotic body formation. RGD-liposome-treated HSCs showed normal structure; **C:** Cell cycle analysis after induction of apoptosis in HSCs by OM *via* flow cytometry. The cells were incubated with different formulation of OM for 24 h, and stained with PI. MTT: 3-(4,5)-dimethylthiazolozol-2-yl-2,5-diphenyltetrazolium bromide; RGD: Arg-Gly-Asp; PI: Propidium iodide; DI: DNA grading index.

in a significant increase in sub-G₁ phase accumulation that was recognized as a biomarker of apoptosis (Figure 2C). Moreover, the RGD-labeled liposomal formulation had a more aggressive effect on HSCs than that of OM in terms of cell viability (Figure 2) and apoptosis (Figures 3 and 4)

OM-RGD liposomes inhibited fibrosis-related gene expression in CCl₄-induced fibrotic liver injury

We also examined the change in mRNA expression of fibrosis-related genes upon treatment with OM in different liposomal formulations. As shown in Figure 3, mRNA expression of MMP-2, TIMP-1 and type I procollagen was considerably elevated upon CCl₄ induction (*vs* normal, *P* < 0.05). Treatment with OM resulted in significant decreases in mRNA expression of these designated fibro-

sis-related genes and fibrosis-related gene expression (*vs* CCl₄-induced group, *P* < 0.05). Moreover, RGD-labeled liposomal formulation had a more aggressive downregulation of fibrosis-related gene expression than that of OM (*vs* CCl₄-induced hepatic fibrosis group, *P* < 0.05; as compared to OM liposomes, *P* < 0.05).

RGD enhanced OM targeting of HSCs in fibrotic rats

To evaluate the specificity of binding to HSCs in fibrotic liver, OM-RGD liposomes were conjugated with FITC and injected intravenously to rats with CCl₄-induced hepatic fibrosis. HSCs were isolated and examined by fluorescence microscopy. As shown in Figure 4, a significantly high number of FITC-positive HSCs was found in the OM-RGD liposome group compared with the OM liposome group.

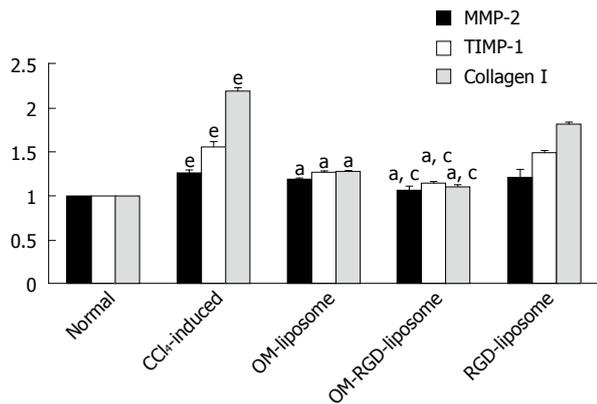


Figure 3 Oxymatrine liposomes inhibited fibrosis-related gene expression. The expression of fibrosis-related gene, such as MMP-2, TIMP-1 and collagen I was evaluated by real-time polymerase chain reaction. ^a*P* < 0.05 vs normal; ^c*P* < 0.05 vs CCl₄-induced hepatic fibrosis; ^{a, c}*P* < 0.05 vs oxymatrine liposomes. MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinase; OM: Oxymatrine; RGD: Arg-Gly-Asp.

DISCUSSION

Increased understanding of the pathogenesis of hepatic fibrosis has led to drug discovery for its treatment. Pre-clinical and clinical studies have reported that hepatic fibrosis is dynamic and possibly reversible^[16,17]. During recent decades, antifibrotic strategies have predominantly focused on eradication of causative factors, for example, clearance of virus^[18]. Since the pathogenesis of fibrosis was clarified recently^[19], researches have focused on agents that could prevent or reverse fibrosis. OM has been reported for its pharmacological potential to treat liver disorders, particularly inhibiting viral infection^[10,11,20,21]. It has been demonstrated to exert antifibrotic action^[22]. In our study, we confirmed that OM could attenuate CCl₄-induced hepatic fibrosis in a rat model, as defined by a significant decrease in the serum level of alkaline phosphatase and improvement of histopathological change.

OM was recently referred to as an antifibrosis agent in clinical and preclinical studies. However, its mechanisms of action were still puzzling. In preclinical studies, it was proved that OM showed prophylactic and therapeutic effects in D-galactosamine-induced rat hepatic fibrosis, partly by protecting hepatocytes and suppressing fibrosis accumulation through acting against lipid peroxidation^[23]. Another study also demonstrated that OM was effective in reducing the production and deposition of collagen in the liver tissue of experimental rats in ways that relate to modulating the fibrogenic signal transduction *via* the p38 mitogen-activated protein kinase signaling pathway^[24]. Moreover, OM could promote the expression of Smad 7 and inhibit the expression of Smad 3 and cAMP-response element binding protein-binding protein in CCl₄-induced hepatic fibrosis in rats, and modulate the fibrogenic signal transduction of the transforming growth factor (TGF)- β -Smad pathway^[25]. Clinical studies have proved that the effect of OM against hepatic fibrosis is

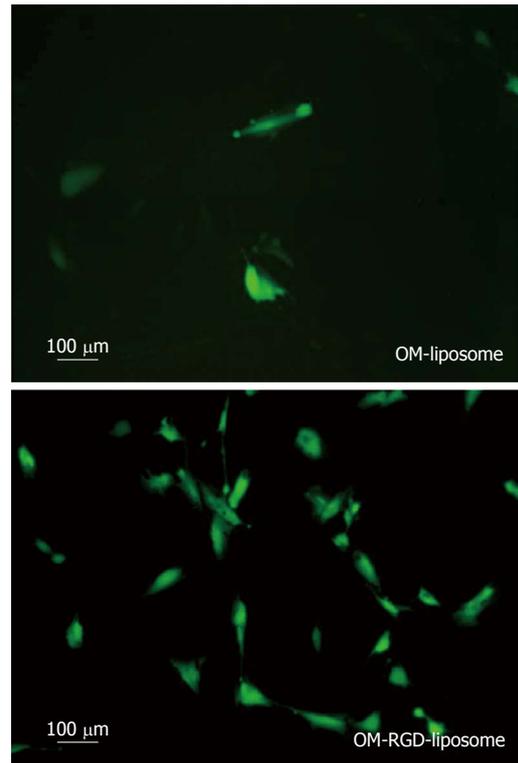


Figure 4 Representative fluorescein isothiocyanate hepatic stellate cells in rat hepatic tissue treated with oxymatrine liposomes and oxymatrine-Arg-Gly-Asp liposomes (original magnification \times 200). OM: Oxymatrine; RGD: Arg-Gly-Asp.

mediated by lowering the levels of TGF- β 1 and tumor necrosis factor- α and increasing the level of interleukin-10 in chronic hepatitis B patients^[26]. OM could also target directly fibrogenic effector cells, which has received much attention^[27]. Various cells are involved in the fibrogenic process. HSCs, the most important fibrogenic cells, are activated during injury and contribute to excessive synthesis of ECM, resulting in deposition of scar or fibrous tissue^[28-31]. OM has been demonstrated to prevent pig-serum-induced liver fibrosis in rats by inhibiting the activation of HSCs and synthesis of collagen^[32]. However, how OM inhibits HSC activation was not determined in that study^[32].

We explored the effect of OM on HSCs *in vitro*. OM inhibited viability and induced apoptosis of HSCs. This might be the underlying mechanism involved in OM therapy of hepatic fibrosis. Furthermore, we also detected fibrosis-related gene expression after OM administration. MMP-2, produced by HSCs, plays an important role in liver fibrogenesis^[33]. TIMPs, especially TIMP-1 and -2 expression and activity, were significantly increased at 8 wk in a rat porcine-serum-induced hepatic fibrosis model^[34]. Furthermore, inhibition of cell viability and type I procollagen expression in rat HSCs could improve recovery from CCl₄-induced liver fibrogenesis in rats^[35]. As shown in our study, mRNA expression of MMP-2, TIMP-1 and type I procollagen was considerably elevated upon CCl₄ induction. Treatment with OM resulted

in significant decreases in mRNA expression of these designated fibrosis-related genes. All the data indicated that OM could attenuate hepatic fibrosis *via* its effect on HSCs, such as inhibiting cell viability, inducing apoptosis and downregulation of fibrosis-related gene expression.

Since OM therapy was dependent on its interaction with HSCs, binding to HSCs became a key factor for its function. Due to a relatively small population of HSCs in the liver and lack of specific membrane receptors, HSC-specific targeting therapy has remained unavailable. Several studies have attempted to use different formulation approaches for targeting HSCs. Beljaars *et al.*^[36] have reported that human serum albumin (HSA) modified with mannose 6-phosphate (M6P) accumulated in HSCs by binding to the M6P-insulin-like growth factor II receptors found on activated HSCs. Modification of HSA with a cyclic peptide that recognizes the collagen type VI receptor has been demonstrated to enhance effectiveness and tissue specificity of antifibrogenic drugs^[37]. Moreover, the affinity of a cyclic peptide, cRGD, for collagen type VI receptor on HSCs was confirmed in both *in vitro* and *in vivo* experiments^[13]. In our study, in order to facilitate OM binding to HSCs, we conjugated liposomes targeted to HSCs in rats with CCl₄-induced hepatic fibrosis. Fluorescence microscopy showed more FITC-positive HSCs in the OM-RGD-liposome group compared with the OM-liposome group. We compared the difference in therapeutic effect of the alternative formulations of OM on liver fibrosis. We demonstrated better results in the OM-RGD-liposome group, as demonstrated by significant decreases in serum alkaline phosphatase and improvement of histopathological changes, compared with the OM-liposome group. Moreover, OM-RGD liposomes showed a more aggressive effect on viability, apoptosis and fibrosis-related gene expression of HSCs, compared with the OM liposomes. The results showed that specific binding of this liposomal formulation to HSCs enhanced the liver protective effect of OM.

In conclusion, we conjugated OM with RGD liposomes and confirmed that this formulation could enhance OM binding to HSCs and the therapeutic effect on hepatic fibrosis induced by CCl₄. We also demonstrated that the therapeutic effects of OM on hepatic fibrosis were partly dependent on inhibiting cell viability, inducing apoptosis, and downregulating fibrosis-related gene expression of HSCs, thus highlighting OM-RGD liposomes as an attractive novel therapy in liver fibrosis.

COMMENTS

Background

Oxymatrine (OM) has been reported to have a beneficial effect on progression of CCl₄-induced hepatic fibrosis in rats, however, its mechanism of action is still uncertain. Hepatic stellate cells (HSCs) have been identified as an important factor in the hepatic fibrotic process. Drugs that could induce HSC apoptosis or death might be the potential strategy for treatment of hepatic fibrosis. Recent studies have demonstrated that OM induces apoptosis in a variety of cells; mainly malignant cells. Thus, the authors performed an assay to demonstrate whether OM could attenuate hepatic fibrosis *via* inducing HSC apoptosis.

Research frontiers

OM was demonstrated to attenuate hepatic fibrosis but its mechanisms of action were still uncertain. Moreover, targeting of HSCs might facilitate the therapeutic effect of OM. The research hotspot is to clarify the mechanism of action of OM in attenuating hepatic fibrosis and how to enhance OM binding to HSCs.

Innovations and breakthroughs

Apoptosis-inducing activity of OM makes it an attractive antifibrotic agent. However, there is limited evidence for the efficacy of OM in hepatic fibrosis and the underlying mechanism of action. The authors demonstrated for the first time that OM could attenuate hepatic fibrosis *via* inhibiting viability and inducing apoptosis of HSCs. Moreover, Arg-Gly-Asp (RGD) could promote OM targeting to HSCs and enhance its effect on hepatic fibrosis.

Peer review

This study aimed to analyze the effects of RGD-peptide-labeled liposomes on CCl₄-induced hepatic fibrosis in rats. This is an interesting approach that clearly improves the efficacy of treatment. The research combined *in vitro* and *in vivo* studies, and data are clear and well presented.

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X-ray repair cross-complementing group 1 polymorphisms and hepatocellular carcinoma: A meta-analysis

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Abstract

AIM: To perform a systematic meta-analysis to investigate the association between X-ray repair cross-complementing group 1 (*XRCC1*) polymorphisms and hepatocellular carcinoma (HCC) risk.

METHODS: Relevant studies extracted from PubMed, Embase, Wanfang, VIP and the Chinese National Knowledge Infrastructure databases up to March 2012 were included in the study. Stata software, version 11.0, was used for the statistical analysis. The odds ratios (ORs) and 95% confidence interval (CI) of the *XRCC1* polymorphisms in HCC patients were analyzed and compared with healthy controls. The meta-analysis was performed using fixed-effect or random-effect

methods, depending on the absence or presence of significant heterogeneity.

RESULTS: Eleven studies with 2075 HCC cases and 2604 controls met our eligibility criteria (four studies, 888 cases and 938 controls for Arg194Trp, four studies, 858 cases and 880 controls for Arg280His, and nine studies, 1845 cases and 2401 controls for Arg399Gln). The meta-analysis revealed no associations between the Arg194Trp and Arg399Gln polymorphisms of the *XRCC1* gene and HCC risk under all contrast models (codominant, dominant and recessive models) in the overall analysis and sensitivity analysis (the studies with controls not in the Hardy-Weinberg equilibrium were excluded). For *XRCC1* Arg280His polymorphism, the overall analysis revealed the significant association between the His/His genotype and the increased risk of HCC (His/His vs Arg/Arg model, OR: 1.96, 95% CI: 1.03-3.75, $P = 0.04$). However, sensitivity analysis showed an altered pattern of result and non-significant association (OR: 2.06, 95% CI: 0.67-6.25, $P = 0.20$). The heterogeneity hypothesis test did not reveal any heterogeneity, and Begg's and Egger's tests did not find any obvious publication bias.

CONCLUSION: The *XRCC1* Arg194Trp and Arg399Gln polymorphisms are not associated with HCC risk. More rigorous association studies are needed to verify the involvement of *XRCC1* Arg280His polymorphism in HCC susceptibility.

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Key words: X-ray repair cross-complementing group 1; Polymorphism; Hepatocellular carcinoma; Meta-analysis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide^[1]. It is accepted that the carcinogenesis of HCC is a multistep process, and multiple factors are involved in this complex process^[2]. Epidemiological studies have indicated that chronic hepatitis B virus (HBV), chronic hepatitis C virus (HCV), heavy cigarette smoking, and alcohol abuse are associated with the risk of HCC^[1]. The progression of HCC might result from a complex interaction of both environmental (including HBV or HCV infection) and genetic factors^[2]. Loss of genomic stability and the gene alterations resulting from endogenous and/or exogenous damage appear to be crucial molecular and pathogenic steps that occur early in the carcinogenesis process of HCC^[2]. Various enzymes and proteins involved in the DNA repair system play a pivotal role in maintaining the genome integrity in cells through the reversal of DNA damage^[3]. The mutations and single-nucleotide polymorphisms (SNPs) in corresponding DNA repair genes may impair their repair or reversal capacity and increase the risk of cancer^[4].

The X-ray repair cross-complementing group 1 (*XRCC1*) gene, located on chromosome 19 (19q13.2), encodes a crucial scaffold protein that is closely associated with the base excision repair (BER) pathway^[5]. The *XRCC1* protein is responsible for the repair of oxidative DNA damage and single-strand breaks through interacting with DNA ligase 3 and the complexes with DNA polymerase and poly (adenosine diphosphate-ribose) polymerase (PARP)^[6,7]. Although more than 300 validated SNPs have been identified and described in the *XRCC1* gene, only three common SNPs have been extensively studied: Arg194Trp (rs1799782, C/T substitution at position 26304 on exon 6), Arg280His (rs25489, G/A substitution at position 27466 on exon 9), and Arg399Gln (rs25487, G/A substitution at position 28152 on exon 10)^[8]. Numerous studies have focused on the association between these *XRCC1* polymorphisms and development of cancer in humans^[9-16]. *XRCC1* SNPs have been shown in the previous meta-analyses to be significantly associated with risk of gastric^[9], breast^[12] and lung^[16] cancer.

Over the past decade, a considerable number of epidemiological studies have focused on the association between *XRCC1* polymorphisms and HCC risk. However, the results remain either controversial or inconclusive. To address these issues, we carried out a systematic review and meta-analysis of all eligible case-control studies

to estimate the risk of HCC associated with the *XRCC1* polymorphisms.

MATERIALS AND METHODS

Literature search

To identify the studies eligible for inclusion in the systematic review and meta-analysis, the following electronic databases were searched: PubMed, Embase, Wanfang (Chinese), VIP (Chinese) and the Chinese National Knowledge Infrastructure (CNKI) (up to March 1, 2012). The following keywords were used: “X-ray repair cross-complementing group 1” or “XRCC1 and haplotype or polymorphism” and “liver cancer” or “hepatocellular carcinoma”. The search was performed without restriction on language and all studies on human subjects were included. Additional studies were identified by a manual search of the references of the original studies. Of the studies with overlapping data published by the same investigators, only the most recent or complete study was included in this meta-analysis.

Inclusion/exclusion criteria

The included studies had to meet all the following criteria: (1) evaluated *XRCC1* polymorphisms and HCC risk; (2) case-control or cohort studies; and (3) contained sufficient published data for estimating an odds ratio (OR) with a 95% confidence interval (CI). The polymorphisms, for which eligible data were reported in at least three published studies, were included into the meta-analysis.

Data extraction

Information was carefully extracted independently by two investigators according to the inclusion criteria. The following data were collected: the first author's surname, year of publication, country of origin, ethnicity, mean age and type of cases and controls, and the number of cases and controls for each genotype of *XRCC1* polymorphisms. Ethnic origins were categorized as Caucasian, Asian, and African. If a study did not present the ethnic origin, or if it was not possible to separate the participants into a mono-ethnic group according to the phenotypes, the group reported was termed “mixed”.

Statistical analysis

The Hardy-Weinberg equilibrium (HWE) in the control groups was calculated in our meta-analysis to determine selective bias in the control population. The χ^2 goodness-of-fit test was used to identify deviation from HWE ($P < 0.05$ was considered significant).

Associations between HCC risk and SNPs in Arg194-Trp, Arg280His, and Arg399Gln were estimated by ORs and 95% CI. The statistical significance of the summary OR was determined with the Z test according to Thakkinian's method^[17] ($P < 0.05$). For each polymorphism, the wild-type allele was set as A and the risk allele as B. The

Table 1 Characteristics of eligible studies included in this study

Ref.	Country (ethnicity)	Genotyping method	Cases/controls	Source of controls	Type of controls	Polymorphisms of <i>XRCC1</i> gene
Tang <i>et al.</i> ^[26]	China (Asian)	PCR-RFLP	150/150	Hospital	Age matched, male and healthy	Arg194Trp, Arg280His, Arg399Gln
Bo <i>et al.</i> ^[30]	China (Asian)	PCR-RFLP	130/130	Hospital	Age matched and healthy	Arg194Trp
Zeng <i>et al.</i> ^[29]	China (Asian)	TaqMan SNP Genotyping	545/515	Hospital	Age, sex and residence matched and without cancer	Arg194Trp, Arg280His, Arg399Gln
Kiran <i>et al.</i> ^[22]	India (Asian)	PCR-RFLP	63/155	Hospital	HBsAg (-), anti-HCV (-), and without renal or hepatic disease	Arg194Trp, Arg280His, Arg399Gln
Wu <i>et al.</i> ^[27]	China (Asian)	PCR-RFLP	100/60	Hospital	Age and sex matched, healthy and HBsAg (-)	Arg280His
Ren <i>et al.</i> ^[25]	China (Asian)	PCR-RFLP	50/92	Hospital	Healthy and HBsAg (-)	Arg399Gln
Borentain <i>et al.</i> ^[20]	France (Caucasian)	Sequencing	56/89	Population	Healthy and without chronic liver disease	Arg399Gln
Kirk <i>et al.</i> ^[23]	Gambia (African)	PCR-RFLP	195/352	Hospital	Age and sex matched, normal α -fetoprotein levels, and without clinical evidence of liver disease	Arg399Gln
Long <i>et al.</i> ^[24]	China (Asian)	PCR-RFLP	140/536	Hospital	Age, sex and ethnicity matched and without cancer	Arg399Gln
Han <i>et al.</i> ^[21]	China (Asian)	PCR-RFLP	69/136	Population	Age, sex and residence matched	Arg399Gln
Yu <i>et al.</i> ^[28]	China (Asian)	PCR-RFLP	577/389	Population	Age and sex matched, HBsAg (+), and without HCC	Arg399Gln

XRCC1: X-ray repair cross-complementing group 1; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; SNP: Single nucleotide polymorphism; HBsAg: Hepatitis B surface antigen; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

A and B allele frequencies were first compared between the case and the control groups. Then, we evaluated the multiple comparisons including BB *vs* AA (codominant model), AB *vs* AA (codominant model), BB *vs* AB, (BB + AB) *vs* AA (dominant model), BB *vs* (AB + AA) (recessive model), and (BB + AA) *vs* AB (complete overdominant model).

The χ^2 -based Q statistic was used to test for the between-study heterogeneity^[18]. When $P < 0.1$ or $I^2 > 50\%$, the heterogeneity was considered statistically significant^[19]. The data were analyzed using a random-effects model if heterogeneity existed. In the absence of heterogeneity, a fixed-effects model was used. Sensitivity analysis was performed to examine the effect of excluded specific studies, such as studies with controls that were not in HWE. The statistical significance of the summary OR was determined with the Z test ($P < 0.05$).

The publication bias was assessed qualitatively by the Begg's rank correlation method and the Egger's weighted regression method ($P < 0.05$). All statistical analyses were performed with Stata software (version 11.0; Stata Corporation, College Station, TX) using two-sided P values.

RESULTS

With the retrieval strategy, 60 potentially relevant papers were extracted (12 from PubMed, 15 from Embase, 16 from Wanfang, 3 from VIP and 14 from CNKI). Forty-seven studies were subjected to a full-text review and excluded according to the selection criteria stated above. Eleven studies were identified that examined the association between the *XRCC1* polymorphisms and HCC risk^[20-30]. Table 1 summarizes the data from these studies,

which included 2075 HCC patients and 2604 control subjects. The HCC was defined according to the clinical pathological examinations. Those with no clinical evidence of HCC served as controls.

These studies focused on three identified polymorphisms of the *XRCC1* gene: Arg194Trp, Arg280His, and Arg399Gln. The genotypes and allelic frequencies of these three *XRCC1* polymorphisms in the eligible studies are listed in Table 2. Three studies which respectively analyzed the Arg194Trp^[30], the Arg280His^[22], and the Arg399Gln polymorphisms^[29] significantly deviated from the HWE ($P < 0.05$).

Arg194Trp

The Arg194Trp polymorphism is located on exon 6 of the *XRCC1* gene and has been investigated in association studies in patients with HCC. A positive association was initially noted by Kiran *et al.*^[22] who reported an excess frequency of the Arg/Trp genotype in an Indian sample of HCC patients *vs* controls. Furthermore, Bo *et al.*^[30] reported significant associations among a Chinese population between HCC and the Arg/Trp and Trp/Trp genotypes. However, another two studies of a Chinese population reported no association between the Arg194Trp polymorphism of the *XRCC1* gene and HCC^[26,31].

In this meta-analysis, four studies focused on the Arg194Trp polymorphism of *XRCC1* gene in an Asian population, including 888 HCC cases and 938 controls. An evaluation of the association between the Arg194Trp polymorphism and HCC risk is presented in Table 3. No significant association was detected between HCC and the Arg/Trp or Trp/Trp genotype (the Arg/Trp *vs* the Arg/Arg model, fixed-effects OR: 1.30, 95% CI:

Table 2 Genotype distribution of X-ray repair cross-complementing group 1 polymorphisms used in this study

Polymorphism	Ref.	Ethnicity	Case			Control			HWE
Arg194Trp			Arg/Arg	Arg/Trp	Trp/Trp	Arg/Arg	Arg/Trp	Trp/Trp	
	Tang <i>et al.</i> ^[26]	Asian	94	41	15	81	58	11	0.88
	Bo <i>et al.</i> ^[30]	Asian	94	31	5	116	12	2	0.02
	Zeng <i>et al.</i> ^[29]	Asian	305	200	40	275	202	38	0.91
	Kiran <i>et al.</i> ^[22]	Asian	8	43	12	27	64	52	0.35
Arg280His			Arg/Arg	Arg/His	His/His	Arg/Arg	Arg/His	His/His	
	Tang <i>et al.</i> ^[26]	Asian	138	11	1	123	26	1	0.76
	Zeng <i>et al.</i> ^[29]	Asian	451	86	8	423	89	3	0.46
	Wu <i>et al.</i> ^[27]	Asian	76	23	1	47	13	0	0.34
	Kiran <i>et al.</i> ^[22]	Asian	19	30	14	91	29	35	0.00
Arg399Gln			Arg/Arg	Arg/Gln	Gln/Gln	Arg/Arg	Arg/Gln	Gln/Gln	
	Tang <i>et al.</i> ^[26]	Asian	41	94	15	84	54	12	0.43
	Zeng <i>et al.</i> ^[29]	Asian	312	196	37	309	169	37	0.04
	Kiran <i>et al.</i> ^[22]	Asian	25	33	5	45	70	27	0.98
	Ren <i>et al.</i> ^[25]	Asian	32	14	4	46	41	5	0.28
	Borentain <i>et al.</i> ^[20]	Caucasian	27	21	8	27	43	19	0.80
	Kirk <i>et al.</i> ^[23]	African	160	31	4	300	48	4	0.19
	Long <i>et al.</i> ^[24]	Asian	72	63	5	362	159	15	0.62
	Han <i>et al.</i> ^[21]	Asian	34	28	7	58	63	15	0.73
	Yu <i>et al.</i> ^[28]	Asian	301	223	53	218	143	28	0.49

HWE: Hardy-Weinberg equilibrium.

0.68-2.48, $P = 0.42$; the Trp/Trp *vs* the Arg/Arg model, fixed-effects OR: 1.03, 95% CI: 0.71-1.49, $P = 0.86$). Furthermore, no significant results were observed in any other genetic models. Sensitivity analysis was performed after excluding the study conducted by Bo *et al.*^[30], because the controls were not in HWE.

Arg280His

The Arg280His allele is located on exon 9 of the XRCC1 gene. A study by Kiran *et al.*^[22] in an Indian population showed that the Arg280His polymorphism of the XRCC1 gene was positively associated with HCC. The Arg/His genotype was associated with a significantly increased risk of HCC. However, three studies in Chinese populations reported no association between the Arg280His polymorphism of the XRCC1 gene and HCC^[28,29,31].

In this study, the four studies on the Arg280His polymorphism of the XRCC1 gene among Asian populations, included 858 HCC cases and 880 controls. Overall, significant association was found for the His/His *vs* Arg/Arg model (fixed-effects OR: 1.96, 95% CI: 1.03-3.75, $P = 0.04$) (Table 3). However, sensitivity analysis after exclusion of the study^[22] with controls not in HWE did not suggest the association (the His/His *vs* Arg/Arg model, fixed-effects OR: 2.06, 95% CI: 0.67-6.25, $P = 0.20$). No significant results were observed in any other genetic models in the overall analysis and sensitivity analysis.

Arg399Gln

The Arg399Gln allele is located on the exon 10 of the XRCC1 gene. Studies by Long *et al.*^[24] and Tang *et al.*^[26] reported an excess frequency of the Arg/Gln genotype of the XRCC1 gene in HCC patients *vs* controls in Chinese populations. However, this observation was not replicated in four other studies^[21,25,30,31] of the Chinese population. A positive association between the Arg/Gln

genotype and a significantly increased risk of HCC in the African population was reported by Kirk *et al.*^[23]. The study by Borentain *et al.*^[20] in a Caucasian population showed an increased frequency of the Arg/Arg genotype in HCC patients *vs* controls. Moreover, Kiran *et al.*^[22] reported significant associations among an Indian population between HCC and the Gln/Gln genotype acting as a protective genotype for HCC.

We retrieved nine studies involving 1845 HCC cases and 2401 controls of different populations (one Caucasian, one African, and seven Asian) reporting detailed allele frequencies^[20-26,28,29]. The overall meta-analysis did not suggest any association between the XRCC1 Arg399Gln polymorphism and HCC susceptibility in all genetic models (Table 3). For example, the ORs of the HCC risks associated with the Arg399Gln polymorphism were 1.07 (random-effects, 95% CI: 0.84-1.34, $P = 0.56$) for the comparison of Arg allele *vs* Gln allele, 1.05 (random-effects, 95% CI: 0.71-1.56, $P = 0.77$) for the comparison of Gln/Gln *vs* Arg/Arg genotype, and 1.13 (random-effects, 95% CI: 0.80-1.57, $P = 0.71$) for the comparison of Arg/Gln *vs* Arg/Arg. The results were consistent after we excluded one study^[29] with controls not in HWE.

By stratifying the meta-analysis by ethnicity, no significant association between XRCC1 Arg399Gln polymorphism and HCC risk was observed in the Asian subgroup, which included 1594 HCC cases and 1960 controls. Sensitivity analysis was performed after one study was excluded^[29]. This did not alter the pattern of the results.

Publication bias

The Begg's rank correlation method and Egger's weighted regression method were used to assess publication

Table 3 Associations between Arg194Trp, Arg280His and Arg399Gln polymorphisms of X-ray repair cross-complementing group 1 gene and hepatocellular carcinoma risk shown in the meta-analysis

	<i>n</i> ¹	Cases/controls	OR (95% CI)	Significance (<i>Z</i> test) ²		Heterogeneity (<i>Q</i> test)		
				<i>Z</i>	<i>P</i>	<i>Q</i>	<i>I</i> ² (%)	<i>P</i>
Arg194Trp								
All	4	888/938						
Trp vs Arg			1.08 (0.73-1.60)	0.39	0.69	13.64	78.0	0.00
Trp/Trp vs Arg/Arg			1.03 (0.71-1.49)	0.17	0.86	2.18	0.0	0.53
Arg/Trp vs Arg/Arg			1.30 (0.68-2.48)	0.79	0.42	17.86	83.2	0.00
Trp/Trp vs Arg/Trp			0.88 (0.41-1.89)	0.31	0.75	9.95	69.9	0.01
Arg/Trp + Trp/Trp vs Arg/Arg			1.24 (0.70-2.20)	0.76	0.44	15.45	80.6	0.00
Trp/Trp vs Arg/Trp + Arg/Arg			0.93 (0.50-1.74)	0.21	0.83	7.45	59.7	0.05
Trp/Trp + Arg/Arg vs Arg/Trp			0.72 (0.36-1.43)	0.92	0.36	23.78	87.4	0.00
All HWE	3	758/808						
Trp vs Arg			0.89 (0.76-1.05)	1.32	0.18	0.55	0.0	0.76
Trp/Trp vs Arg/Arg			0.96 (0.66-1.43)	0.18	0.85	0.39	0.0	0.82
Arg/Trp vs Arg/Arg			0.94 (0.56-1.60)	0.20	0.84	6.57	69.6	0.03
Trp/Trp vs Arg/Trp			0.87 (0.36-2.13)	0.29	0.77	9.95	79.9	0.00
Arg/Trp + Trp/Trp vs Arg/Arg			0.88 (0.72-1.09)	1.12	0.26	2.88	30.6	0.23
Trp/Trp vs Arg/Trp + Arg/Arg			0.83 (0.43-1.58)	0.56	0.57	5.86	65.9	0.05
Trp/Trp + Arg/Arg vs Arg/Trp			0.92 (0.47-1.81)	0.23	0.81	14.09	85.8	0.00
Arg280His								
All	4	858/880						
His vs Arg			1.03 (0.62-1.70)	0.11	0.90	12.73	76.4	0.00
His/His vs Arg/Arg			1.96 (1.03-3.75)	2.06	0.04	0.44	0.0	0.93
Arg/His vs Arg/Arg			1.16 (0.47-2.86)	0.33	0.74	26.36	88.6	0.00
His/His vs Arg/His			1.13 (0.30-4.22)	0.19	0.85	7.01	57.2	0.07
Arg/His + His/His vs Arg/Arg			1.10 (0.52-2.33)	0.25	0.80	20.16	85.1	0.00
His/His vs Arg/His + Arg/Arg			1.23 (0.69-2.21)	0.73	0.46	1.63	0.0	0.65
His/His + Arg/Arg vs Arg/His			0.90 (0.38-2.11)	0.23	0.81	24.71	87.9	0.00
All HWE	3	795/725						
His vs Arg			0.83 (0.49-1.41)	0.67	0.50	5.44	63.2	0.06
His/His vs Arg/Arg			2.06 (0.67-6.25)	1.28	0.20	0.43	0.0	0.80
Arg/His vs Arg/Arg			0.74 (0.43-1.30)	1.03	0.30	5.08	60.6	0.07
His/His vs Arg/His			2.54 (0.80-8.04)	1.59	0.11	0.07	0.0	0.96
Arg/His + His/His vs Arg/Arg			0.78 (0.44-1.36)	0.86	0.39	5.40	63.0	0.06
His/His vs Arg/His + Arg/Arg			2.11 (0.69-6.43)	1.32	0.18	0.36	0.0	0.83
His/His + Arg/Arg vs Arg/His			1.34 (0.78-2.31)	1.07	0.28	4.95	59.6	0.08
Arg399Gln								
All	9	1845/2401						
Gln vs Arg			1.07 (0.84-1.34)	0.57	0.56	33.12	75.8	0.00
Gln/Gln vs Arg/Arg			1.05 (0.71-1.56)	0.29	0.77	14.84	46.1	0.06
Arg/Gln vs Arg/Arg			1.13 (0.80-1.57)	0.71	0.47	39.18	79.6	0.00
Gln/Gln vs Arg/Gln			0.93 (0.71-1.21)	0.52	0.60	6.32	0.0	0.61
Arg/Gln + Gln/Gln vs Arg/Arg			1.11 (0.80-1.54)	0.63	0.52	41.34	80.6	0.00
Gln/Gln vs Arg/Gln + Arg/Arg			1.00 (0.78-1.28)	0.03	0.97	7.87	0.0	0.44
Gln/Gln + Arg/Arg vs Arg/Gln			0.85 (0.63-1.15)	1.02	0.30	32.13	75.1	0.00
All HWE	8	1300/1886						
Gln vs Arg			1.06 (0.79-1.41)	0.40	0.69	32.61	78.5	0.00
Gln/Gln vs Arg/Arg			1.06 (0.65-1.73)	0.25	0.80	14.61	52.1	0.04
Arg/Gln vs Arg/Arg			1.10 (0.73-1.68)	0.49	0.62	38.85	82.0	0.00
Gln/Gln vs Arg/Gln			0.96 (0.70-1.30)	0.25	0.79	6.15	0.0	0.52
Arg/Gln + Gln/Gln vs Arg/Arg			1.09 (0.72-1.64)	0.43	0.66	40.82	82.9	0.00
Gln/Gln vs Arg/Gln + Arg/Arg			1.02 (0.77-1.37)	0.19	0.84	7.72	9.3	0.35
Gln/Gln + Arg/Arg vs Arg/Gln			0.86 (0.60-1.24)	0.77	0.44	31.84	78.0	0.00
Asian	7	1594/1960						
Gln vs Arg			1.12 (0.87-1.44)	0.89	0.37	25.85	76.8	0.00
Gln/Gln vs Arg/Arg			1.13 (0.86-1.48)	0.92	0.35	10.48	42.7	0.10
Arg/Gln vs Arg/Arg			1.22 (0.83-1.78)	1.04	0.29	33.10	81.9	0.00
Gln/Gln vs Arg/Gln			0.92 (0.69-1.21)	0.58	0.56	5.83	0.0	0.44
Arg/Gln + Gln/Gln vs Arg/Arg			1.20 (0.83-1.73)	0.98	0.32	33.84	82.3	0.00
Gln/Gln vs Arg/Gln + Arg/Arg			1.02 (0.79-1.33)	0.20	0.83	5.96	0.0	0.42
Gln/Gln + Arg/Arg vs Arg/Gln			0.81 (0.57-1.13)	1.21	0.22	28.55	79.0	0.00
Asian HWE	6	1049/1445						
Gln vs Arg			1.12 (0.81-1.54)	0.69	0.49	24.80	79.8	0.00
Gln/Gln vs Arg/Arg			1.16 (0.69-1.97)	0.59	0.55	9.89	49.4	0.07
Arg/Gln vs Arg/Arg			1.22 (0.74-2.00)	0.78	0.43	32.23	84.5	0.00
Gln/Gln vs Arg/Gln			0.95 (0.68-1.32)	0.31	0.76	5.70	12.3	0.33

Arg/Gln + Gln/Gln <i>vs</i> Arg/Arg	1.20 (0.74-1.94)	0.75	0.45	32.66	84.7	0.00
Gln/Gln <i>vs</i> Arg/Gln + Arg/Arg	1.06 (0.78-1.46)	0.41	0.67	5.69	12.1	0.33
Gln/Gln + Arg/Arg <i>vs</i> Arg/Gln	0.80 (0.51-1.26)	0.93	0.35	27.84	82.0	0.00

¹Number of studies; ²Random-effects model was used when *P* value for heterogeneity test $P < 0.1$ or $I^2 > 50\%$; otherwise, fixed-effects model was used. HWE: Hardy-Weinberg equilibrium; OR: Odds ratio.

bias. There was no evidence of publication bias for these polymorphisms.

DISCUSSION

The *XRCC1* protein is a key molecule of BER in the DNA repair process, which plays a key role in the integrity and stability of the genome and the pathogenesis and progression of human cancers^[31]. Polymorphisms that can alter *XRCC1* expression and function may contribute to the risk of cancers. Several studies have been conducted in recent years to evaluate the association between the *XRCC1* SNPs and HCC risk predisposition in different ethnic populations, but the results have been conflicting^[20-30].

In the present study, we performed a systematic review and meta-analysis to examine the association between three *XRCC1* gene polymorphisms and HCC risk. These three polymorphisms of the *XRCC1* gene result in nonconservative amino-acid changes at evolutionary conserved regions: a C to T substitution in codon 194 of exon 6 (Arg to Trp), a G to A substitution in codon 280 of exon 9 (Arg to His), and a G to A substitution in codon 399 of exon 10 (Arg to Gln)^[32]. Although the functional effects of these nonsynonymous polymorphisms in *XRCC1* are not well known, the nature of the amino acid substitutions may cause functional changes in the *XRCC1* protein and impair DNA repair efficiency or accuracy, which could be implicated in the risk of cancer. However, previous meta-analysis showed inconsistent results in the association between the polymorphisms of the *XRCC1* gene and the risk of cancers.

These previous meta-analysis found that the carriers of homozygous Trp/Trp variant genotype of the Arg-194Trp polymorphism had an increased risk of gastric cancer^[9] and other cancers, especially lung cancer and esophageal cancer, in the Chinese mainland population^[11]. However, no associations were observed between the Arg194Trp polymorphism with skin^[15], colorectal^[33], and prostate cancer^[34], and nasopharyngeal carcinoma^[10]. The Arg280His polymorphism was associated with an approximate 3.5-fold increase in skin cancer risk in homozygote codominant and recessive models^[15]. However, the Arg280His polymorphism was not found to be a statistically significant risk factor for gastric cancer^[9] and nasopharyngeal carcinoma^[10]. The Gln/Gln genotype of the Arg399Gln polymorphism was associated with an increased risk of prostate cancer^[34] and nasopharyngeal carcinoma^[10], but was not correlated with skin^[15], gastric^[9], and colorectal^[33] cancer susceptibility for all genetic models. For prostate cancer, Wei *et al.*^[34] concluded that the Gln allele of the Arg399Gln polymorphism might be a low-

penetrant risk factor for prostate cancer only in Asian men, but was not related to overall prostate cancer risk.

It is difficult to interpret the reasons for these inconsistent results; nonetheless, several factors may influence the function of these polymorphisms of the *XRCC1* gene in various ways, including variation in the exposure of different populations to carcinogens and variation in different types of DNA damage associated with the initiation of different cancers. Further research is needed to clarify this inconsistency.

There has been no previous meta-analysis of the effect of the Arg194Trp and Arg280His polymorphisms of the *XRCC1* gene on the risk of HCC. Our data suggested that the Arg194Trp polymorphism might not be a risk factor for HCC. For the *XRCC1* Arg280His polymorphism, our meta-analysis on the available studies showed that the His/His genotype was significantly associated with increased HCC risk. However, the sensitivity analysis after exclusion of the study with controls not in HWE did not suggest this association. Our meta-analysis does not strongly support the association between the His/His genotype of the *XRCC1* Arg280His polymorphism and the increased risk in HCC. Although there is some evidence of association between the *XRCC1* Arg280His polymorphism and HCC, the above finding deserves further and more rigorous association studies. A previous meta-analysis study found that the Arg399Gln polymorphism had no association with HCC^[13], which is similar to our current finding. Recently, another variant in the *XRCC1* gene located in the 5'-untranslated region, -77 T to C, was identified^[35] and indicated as a genetic determinant for developing breast cancer^[14]. However, there is a lack of association study between the -77 T/C polymorphism of the *XRCC1* gene and HCC risk.

Similar to other systematic reviews and meta-analyses, there were some limitations in this study. Firstly, the sample sizes in the overall and subgroup analyses were small. Secondly, only two published studies included in this meta-analysis focused on the Arg399Gln polymorphism and its relationship with HCC in Caucasian and African populations. Thirdly, the sources of heterogeneity that existed among the studies for most polymorphisms were not addressed. Finally, this meta-analysis was based on unadjusted data, whereas a more precise analysis could be performed if individual data were available. Additional well-designed, more-detailed studies with larger populations and different ethnicities are needed to further evaluate the associations.

In conclusion, The *XRCC1* Arg194Trp and Arg399Gln polymorphisms are not associated with HCC risk. More rigorous association studies are needed to clarify the involvement of *XRCC1* Arg280His polymorphism in

HCC susceptibility. No publication biases regarding these three evaluated polymorphisms were found in this meta-analysis.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide. The X-ray repair cross-complementing group 1 (XRCC1) gene encodes a crucial scaffold protein that is responsible for the repair of oxidative DNA damage and single-strand breaks. Many studies have explored the association between the XRCC1 polymorphisms and HCC risk, but the results remain either controversial or inconclusive. To address these issues, the authors carried out a systematic review and meta-analysis of all eligible case-control studies to estimate the association between the XRCC1 polymorphisms and the risk of HCC.

Research frontiers

To date, a number of studies have assessed the association between the XRCC1 polymorphisms and HCC risk among different populations; however, the results have been inconsistent and inconclusive.

Innovations and breakthroughs

This meta-analysis suggested that none of the Arg194Trp and Arg399Gln polymorphisms of XRCC1 were significantly associated with a risk of HCC. More rigorous association studies are needed to verify the involvement of XRCC1 Arg280His polymorphism in HCC susceptibility.

Applications

This meta-analysis showed that the Arg399Gln and Arg194Trp polymorphisms of the XRCC1 gene did not alter the susceptibility to HCC. The findings may provide valuable information about the etiology of HCC for both researchers and clinicians.

Peer review

This manuscript was a meta-analysis to analyze the association between X-ray repair cross-complementing group 1 polymorphisms and HCC. This is a scientifically interesting topic. Although the results show that this is a negative study, it is very important to systematically review these relevant reports.

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Metabolic syndrome and gallstone disease

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Abstract

AIM: To investigate the association between metabolic syndrome (MetS) and the development of gallstone disease (GSD).

METHODS: A cross-sectional study was conducted in 7570 subjects (4978 men aged 45.0 ± 8.8 years, and 2592 women aged 45.3 ± 9.5 years) enrolled from the physical check-up center of the hospital. The subjects included 918 patients with gallstones (653 men and 265 women) and 6652 healthy controls (4325 men and 2327 women) without gallstones. Body mass index (BMI), waist circumference, blood pressure, fasting plasma glucose (FPG) and serum lipids and lipoproteins levels were measured. Colorimetric method was used to measure cholesterol, high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C). Dextrose oxidizing enzyme method was used to measure FPG. Subjects were asked to complete a questionnaire that enquired about the information on

demographic data, age, gender, histories of diabetes mellitus, hypertension, and chronic liver disease and so on. Metabolic syndrome was diagnosed according to the Adult Treatment Panel III (ATP III) criteria. Gallstones were defined by the presence of strong intraluminal echoes that were gravity-dependent or attenuated ultrasound transmission.

RESULTS: Among the 7570 subjects, the prevalence of the gallstone disease was 12.1% (13.1% in men and 10.2% in women). BMI, waist circumference, systolic blood pressure, diastolic blood pressure, fasting blood glucose and serum triglyceride (TG) in cases group were higher than in controls, while serum high-density lipid was lower than in controls. There were significant differences in the waist circumference, blood pressure, FPG and TG between cases and controls. In an age-adjusted logistic regression model, metabolic syndrome was associated with gallstone disease. The age-adjusted odds ratio of MetS for GSD in men was 1.29 [95% confidence interval (CI), 1.09-1.52; $P = 0.0030$], and 1.68 (95% CI, 1.26-2.25; $P = 0.0004$) in women; the overall age-adjusted odds ratio of MetS for GSD was 1.42 (95% CI, 1.23-1.64; $P < 0.0001$). The men with more metabolic disorders had a higher prevalence of gallstone disease, the trend had statistical significance ($P < 0.0001$). The presence of 5 components of the MetS increased the risk of gallstone disease by 3.4 times ($P < 0.0001$). The prevalence of GSD in women who had 5 components of MetS was 5 times higher than in those without MetS. The more the components of MetS, the higher the prevalence of GSD ($P < 0.0001$). The presence of 5 components of the MetS increased the risk of gallstone disease by 4.0 times.

CONCLUSION: GSD appears to be strongly associated with MetS, and the more the components of MetS, the higher the prevalence of GSD.

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Key words: Gallstone disease; Obesity; Hypertension;

Dyslipidemia; Metabolic syndrome

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INTRODUCTION

China is one of the fastest developing countries. Rapid economic development and industrialization have brought about changes in traditional diets and increasingly sedentary lifestyles. Metabolic syndrome (MetS) is defined as a cluster of multiple cardiovascular risk factors, including central obesity, elevated fasting plasma glucose, high blood pressure, lower high-density lipid (HDL), and higher serum triglyceride (TG) levels. The prevalence of MetS has been increasing gradually in China. In 1992, the overall prevalence of MetS in China was 13.3% (12.7% in men and 14.2% in women)^[1]. By 2000, the prevalence of MetS had elevated to 15.1%; 13.6% in men and 16.6% in women^[2]. In 2009, a population-based cross-sectional survey in China showed that the crude and age-standardized prevalence of MetS was 31.5% and 30.5%, respectively^[3]. Numerous studies have indicated that MetS is closely associated with some common diseases, such as diabetes, hypertension, cardiovascular diseases, cancer, and gallstone disease. Consequently, the increasing prevalence of MetS may potentially associate with the increased prevalence of these diseases. Studies about the association between gallstone disease and MetS suggested that MetS is a risk factor for gallstone disease (GSD)^[4], and some studies concluded that GSD might be a component of MetS^[4,5] although it needs to be validated by more evidences.

GSD represents a significant burden for health care worldwide^[6] and is one of the most common disorders among the patients admitted to the emergency rooms with abdominal discomfort, epigastric pain, nausea, vomiting, loss of appetite, *etc*^[7]. Ethnicity and family traits are recognized as contributing factors^[8]. GSD is known to affect 60%-70% of native Americans and a proportionately smaller number of individuals of mixed hispanic/native American origin^[9]. The incidence of GSD is at least 10% among white adults in Western countries^[10], but it is lower in African Americans and East Asians^[9]. GSD is also on the rise and becoming a major health problem in China^[11,12]; according to the reported estimates that the prevalence of GSD increased from 4.3% in 1989 to as high as 10.7% in 1995^[12]. Risk factors associated with

cholelithiasis include female gender, age, obesity, diabetes, hyperlipidemia, rapid appetite loss, hepatitis C, cirrhosis, and high caloric intake^[9,13,14]. The association between GSD and MetS has been a focus of some recent studies. To the best of our knowledge, the prevalence of the disease and the association between the development of GSD and MetS are not fully elucidated. Moreover, there is currently only minimal data regarding the relationship between GSD and MetS in apparently healthy Asian people. This study aimed to establish if there is an association between the presence of MetS and the development of GSD. MetS is known to be strongly associated with lifestyle, and if MetS is proved to be related to gallstone, we may reduce the prevalence of gallstones through lifestyle interventions.

MATERIALS AND METHODS

Data resource and data collection

We conducted a cross-sectional study in 7570 subjects enrolled from the Physical Check-Up Center of the Sir Run Run Shaw Hospital. Among them, there were 4978 men, aged 45.0 ± 8.8 years, and 2592 women, aged 45.3 ± 9.5 years. The study protocol was approved by the Ethics Committee of the hospital. All the subjects signed the informed consent. And 918 (653 men and 265 women) subjects were found to have gallstones. The gallstone cases and controls consisted of a series of consecutive asymptomatic subjects. Exclusion criteria included histories of cholecystectomy, pancreatitis, sequela of clonorchis sinensis infection, gallbladder polyps, silt, dimly disease or gallbladder wall thickening, chronic kidney disease, pregnancy, and major gastrointestinal surgeries. Blood samples were collected via venipuncture from the study participants after they had fasted overnight for laboratory tests. Fasting plasma glucose (FPG), TG, total cholesterol, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) concentrations were measured using the AEROSET analyze system (ADDO™, America). Colorimetric method was used to measure cholesterol, HDL-C and LDL-C. Dextrose oxidizing enzyme method was used to measure FPG. Ultrasonographic examinations were also done.

Questionnaire

Subjects were asked to complete a questionnaire that enquired for information on demographic data, age, gender, marital status, address, telephone, histories of diabetes mellitus, hypertension, chronic liver disease, hyperlipidemia, systemic diseases, gastrointestinal surgery (vagotomy gastrectomy for peptic ulcer, ileal resection for inflammatory bowel disease, or any other disease or cause), gravidity, and the use of oral contraceptives, any other medications, and family history.

Physical examination

Body weight of the subjects, dressed in light clothing

Table 1 Demographic and clinical characteristics of the study subjects (mean \pm SD) *n* (%)

Variables	Cases (<i>n</i> = 918)	Controls (<i>n</i> = 6652)	<i>P</i> values
Age (yr)	48.5 \pm 9.1	44.7 \pm 9.0	< 0.0001
Height (cm)	165.6 \pm 7.9	165.6 \pm 7.7	0.2791
Weight (kg)	72.6 \pm 11.3	69.4 \pm 12.2	< 0.0001
Body mass index (kg/m ²)	26.3 \pm 3.0	25.2 \pm 3.4	< 0.0001
Waist circumference (cm)	91.6 \pm 9.4	87.8 \pm 10.7	< 0.0001
Systolic blood pressure (mmHg)	123.6 \pm 14.3	119.8 \pm 14.5	< 0.0001
Diastolic blood pressure (mmHg)	74.1 \pm 9.8	72.2 \pm 10.4	< 0.0001
Fasting blood glucose (mmol/L)	5.39 \pm 1.37	5.11 \pm 1.04	< 0.0001
High density lipid (mg/L)	44.6 \pm 10.9	46.0 \pm 0.58	0.0004
Triglyceride (mg/L)	201.1 \pm 183.3	183.7 \pm 182.7	0.0069
Male	653 (71.1)	4325 (65.0)	0.0003
Larger waist circumference	673 (73.3)	3918 (58.9)	< 0.0001
Higher blood pressure	401 (43.7)	2072 (31.2)	< 0.0001
Higher FPG	146 (15.9)	600 (9.0)	< 0.0001
Lower high-density lipid	407 (44.3)	2842 (42.8)	0.3551
Higher triglyceride	473 (51.5)	3044 (45.8)	0.0010

Student *t* test was applied to compare the differences between cases and controls for all continuous variables. Larger waist circumference denotes waist circumference \geq 80 cm for females or \geq 90 cm for males; higher blood pressure denotes systolic blood pressure \geq 130 or diastolic blood pressure \geq 85 mmHg or drug treatment; raised fasting plasma glucose (FPG) denotes FPG \geq 5.6 mmol/L or drug treatment; raised triglyceride denotes triglyceride \geq 1.70 mmol/L or drug treatment; lower HDL-C denotes HDL < 1.29 mmol/L for females or < 1.03 mmol/L for males or drug treatment. χ^2 test was used to compare the differences between cases and controls for all category variables. HDL: High blood pressure; HDL-C: High-density lipoprotein cholesterol.

and without shoes, was measured to the nearest 0.10 kg. Height was measured to the nearest 0.5 cm. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared (kg/m²). Waist circumference (at the nearest 0.1 cm) was measured at the midpoint between the lower border of the rib cage and the iliac crest. Three blood pressure readings were obtained at 1-min intervals, and the second and third systolic and diastolic pressure readings were averaged and used in the analyses.

Diagnostic criteria

MetS was diagnosed using the Adult Treatment Panel III (ATP III) criteria. According to the ATP III criteria, MetS was defined as the presence of any three of the following five traits: (1) Abdominal obesity, defined as a waist circumference in men \geq 90 cm and in women \geq 80 cm; (2) Serum triglycerides \geq 150 mg/dL (1.7 mmol/L) or medicinal treatment for elevated TG; (3) Serum HDL cholesterol < 40 mg/dL (1.03 mmol/L) in men and < 50 mg/dL (1.29 mmol/L) in women or medication for low HDL-C; (4) Blood pressure \geq 130/85 mmHg or medication for high blood pressure; and (5) Fasting plasma glucose (FPG) \geq 100 mg/dL (5.6 mmol/L) or

Table 2 Association of metabolic syndrome with human gallstone *n* (%)

MetS status	Gallstone status		OR (95% CI)	<i>P</i> values
	Cases	Controls		
Male				
Non-MetS	343 (11.6)	2600 (88.4)	1.29 (1.09-1.52)	0.0030
MetS	310 (15.2)	1725 (84.8)		
Female				
Non-MetS	169 (8.4)	1852 (91.6)	1.68 (1.26-2.25)	0.0004
MetS	96 (16.8)	475 (82.2)		
Total				
Non-MetS	512 (10.3)	4452 (89.7)	1.42 (1.23-1.64)	< 0.0001
MetS	406 (15.6)	2200 (84.4)		

Age-adjusted logistic regression model was used to test the associations between metabolic syndrome (MetS) and human gallstone. OR: Odds ratio; CI: Confidence interval.

medication for elevated blood glucose^[15].

The diagnosis of GSD was established on the basis of the results of abdominal ultrasound (US) using a 3.5-MHz transducer. US was conducted by an experienced radiologist, who was unaware of the objectives of the study and blinded to laboratory values. Gallstones were defined by the presence of strong intraluminal echoes that were gravity-dependent or that attenuated ultrasound transmission (acoustic shadowing)^[16].

Statistical analysis

All statistical analyses were conducted using SPSS version 13.0 (SPSS Inc, Chicago, Ill). The results were expressed as the mean \pm SD. Binary variables were summarized by N and percentage. Student *t* test was applied to compare the differences between cases and controls for all continuous variables. χ^2 test was used to compare the differences between cases and controls for all category variables. Age-adjusted logistic regression model was used to test the association between MetS and human gallstones.

RESULTS

Of the 7570 examined subjects, the prevalence of the GSD was 12.1% (13.1% in men and 10.2% in women). The results of univariate analysis of various factors and its relationship with GSD are shown in Table 1. In comparison with subjects without GSD, those with GSD were significantly older and had a higher waist circumference (WC), BMI, systolic blood pressure, diastolic blood pressure, fasting blood glucose and TG. Moreover, subjects with GSD had a significantly lower HDL cholesterol level than those without GSD. The incidence of larger waist circumference, higher blood pressure, increased FPG and TG was obviously higher in cases group than in the controls.

In age-adjusted logistic regression analyses (Table 2), MetS was significantly associated with the risk of GSD irrespective of the sex of the subjects. The age-adjusted odds ratio of MetS for GSD was 1.29 [95% Confidence

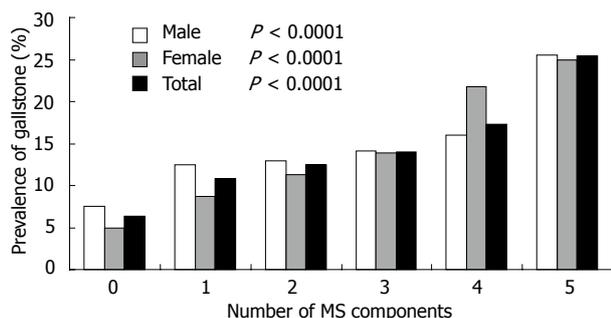


Figure 1 Trend test of the prevalence of gallstone disease and the number of metabolic syndrome components in males, females and total study subjects respectively. Trend test of the prevalence of gallstone disease and the number of metabolic syndrome components in males, in females and in total study subjects. MS: Metabolic syndrome.

interval (CI), 1.09-1.52; $P = 0.0030$] in men, and 1.68 (95% CI, 1.26-2.25; $P = 0.0004$) in women; and the overall age-adjusted odds ratio of MetS for GSD was 1.42 (95% CI, 1.23-1.64; $P < 0.0001$).

We also analyzed the association between the prevalence of GSD and the number of MetS components. Figure 1 shows that the more the components of MetS, the higher the prevalence of GSD in men, and the trend was significant ($P < 0.0001$). The presence of 5 components of the MetS increased the risk of gallstone disease by 3.4 times ($P < 0.0001$). We found the same trend in the women. The prevalence of GSD in women who had 5 components of MetS, was 5 times that of those without MetS component. The overall prevalence of GSD, and the trend was also significant ($P < 0.0001$).

DISCUSSION

In China, the study about the prevalence of GSD is rare, and the available studies are not sufficient in sample size or lack of appropriate statistical methods. We designed the cross-sectional study with a large sample of Chinese population. We found that the prevalence of the GSD was 12.1% (13.1% in men and 10.2% in women), which was slightly higher than the figure presented in a previous hospital-based study conducted at the West China Hospital, Sichuan University, which was reported to be 10.7% (9.9% in men and 11.6% in women)^[17]. A population-based screening study conducted in Taiwan in 2006 reported that the overall prevalence of GSD was 5.0% (4.6% in men, 5.4% in women), without significant gender differences^[18]. The apparently higher prevalence rate in our study may contribute to the Westernized lifestyle of our subjects who were of middle-to-high income class.

According to most of the previous epidemiologic studies, women have a higher prevalence of GSD than men in the Western world, and estrogen is considered to be an obvious factor for the gender difference^[19]. However, our findings showed that the prevalence of GSD was higher in men than in women. Actually, gender as a risk factor for cholelithiasis still remains controversial. While the majority of studies conducted in the West have

concluded that women are more likely to develop cholelithiasis^[20,21] than men, studies among Asian patients have failed to identify a gender-related difference^[22,23]. In fact, Liu *et al.*^[24] found a higher incidence of cholelithiasis in men than in women below 50 years of age, but a higher incidence in women than in men in age groups above 50 years. And Hung *et al.*^[25] indicated that menopause is a risk factor for cholelithiasis in women. Moreover, female predominance is less evident in Asia where the pigmented stone diseases are more common^[26]. So the male predominance with GSD in our study may be attributed to the fact that our subjects were of Asian ethnicity and most of them were aged below 50 years, meanwhile most of our female subjects were premenopausal women.

Older age is another significant risk factor for GSD^[27,28], so we used age-adjusted logistic regression model to test the associations between MetS and human gallstone. It has been reported that the presence of MetS as an insulin resistance phenotype was associated with an increased prevalence of gallstones^[29]. In our age-adjusted logistic regression analysis, MetS was associated with an increased risk of GSD.

Obesity is a major cornerstone of MetS, in our study, the presence of a high waist circumference was common in patients with GSD. A population-based follow-up study on GSD in Kinmen also showed that greater waist circumference was associated with the development of GSD among type 2 diabetics^[30]. Cojocar *et al.*^[29] found that waist circumference and BMI were significantly associated with a higher risk of cholesterol gallstone. Obesity is a major risk factor for developing GSD because it can increase hepatic secretion of cholesterol^[31].

Although dyslipidemia is very common in MetS, no conclusive evidence links dyslipidemia and GSD. A Korean study demonstrated that HDL cholesterol level was significantly lower in subjects with GSD; however, they did not find any component of dyslipidemia related to MetS that could be correlated with GSD formation^[19]. A cross-sectional study in a check-up unit in a university hospital in Mexico City described the influence of low HDL cholesterol on developing GSD (OR = 2.32)^[4]. In our study, subjects with GSD had lower HDL cholesterol and higher TG, but there was only difference in the incidence of higher TG between cases and controls. The relationship between HDL and GSD remains unclear. In most patients with higher TG, an association with overweight and insulin resistance is often observed based on the supersaturated (cholesterol) bile and diminished gallbladder motility, both contributing to gallstone formation^[14]. Phase separation of cholesterol crystals from supersaturated bile is considered the key event in cholesterol gallstone formation. It is a basal framework of the interactions between the sterol, bile salts and phospholipids in aqueous solutions. Biliary bile acid and phospholipids are important to solubilize cholesterol^[32]. Phospholipid transfer protein (PLTP) transfers lipids from low-density lipoproteins to high-density lipoproteins. It was found that an inhibitory effect of haptoglobin over PLTP activ-

ity in hyperlipidemic plasma may contribute to the regulation of reverse cholesterol transport^[33]. Huang *et al.*^[34] revealed a hitherto unrecognized role of protein kinase C β (PKC β) in the fine tuning diet-induced cholesterol and bile acid homeostasis, thus identifying PKC β as a major physiological regulator of both triglyceride and cholesterol homeostasis. Moreover, polymorphisms in the gene encoding are also found to increase the gallstone risk. Such as the cholesterol transporter ABCG5-G8 and phospholipid floppase ABCB4^[32].

Considering the obvious association between gallstone disease and MetS in this study, the fact that higher blood pressure was associated with MetS and GSD appears reasonable. Systolic blood pressure and diastolic blood pressure were higher in patients with GSD as compared with the controls. A study in Taiwan documented that cholelithiasis in Asian obese patients is significantly associated with increased diastolic blood pressure^[35]. Blood pressure $\geq 130/85$ mmHg was significantly associated with a higher risk of cholesterol gallstone^[36]. The mechanism why higher blood pressure increased the risk of GSD still remains unclear. Some scholars considered that this association could be explained by the action of insulin in hypertension. To validate the mechanism, we will further study the relationship between hypertension and GSD. We have designed a study to investigate the association between BP and GSD, as well as the impact of medication for hypertension on GSD.

Previous studies indicated that diabetes mellitus was a risk factor for GSD^[37-39]. GSD appeared strongly associated with fasting glycemia^[29]. We noted that there was a positive correlation between prevalence of gallstone with higher FPG. The possible mechanisms for this association may be as follows: hyperglycemia inhibits bile secretion from the liver and disturbs gallbladder contraction^[40]; hyperglycemia may affect gallbladder motility^[41]; or some factors modifying the crystal nucleation and mucous secretion in bile^[42].

Finally, GSD appears to be strongly associated with MetS. The result is consistent with the hypothesis that insulin resistance plays an important role in the pathogenesis of GSD. Animal experiments demonstrated that mice with isolated hepatic insulin resistance created by liver-specific disruption of the insulin receptor [Liver insulin receptor knockout (LIRKO) mice] are markedly predisposed towards cholesterol gallstone formation. After only one week on a lithogenic diet, 36% of LIRKO mice developed gallstones and 100% had gallstones by 12 wk^[43]. Chang *et al.*^[6], showed that insulin resistance was positively associated with gallstones in non-diabetic Korean men, and this occurred regardless of obesity. Taking into account this association, some authors raised the question whether administration of lipid-lowering drug is a therapeutic option for GSD? Ezetimibe was shown to have a beneficial effect against cholelithiasis in both animal and humans^[44], it is, therefore, possible to suggest that a clinical trial designed to investigate the potential efficiency of ezetimibe for reducing biliary cholesterol

saturation and insulin resistance in populations with a predisposition to cholelithiasis should be now warranted.

In conclusion, GSD is common in China, and the present study shows an obvious association between MetS and GSD, and the more the metabolic components of MetS, the higher the prevalence of the GSD. But the mechanism for the association remains unclear, further research is needed to clarify how BP influences the formation of GSD, whether medication for dyslipidemia benefits the GSD patients, and whether we can reduce the prevalence of GSD through lifestyle interventions.

COMMENTS

Background

China is one of the fastest developing countries. The rapid economic growth and industrialization has brought about changes in traditional diets and lifestyles. The prevalence of metabolic syndrome (MetS) is increasing. But there have been few studies about the prevalence of gallstone disease (GSD), and the association between the development of GSD and MetS is not fully elucidated.

Research frontiers

The authors designed a cross-sectional study with a large sample of Chinese subjects to evaluate the association between MetS and the development of GSD. They concluded that GSD is strongly associated with MetS.

Innovations and breakthroughs

This study demonstrated a strong association between GSD and MetS, but the prevalence of GSD was higher in men than in women. This finding is not consistent with the results from most previous epidemiologic studies. The reason for the discrepancy may be that the subjects in this study are of Asian ethnicity and most of the subjects are below 50 years of age, meanwhile most of the female subjects are premenopausal women.

Applications

There is only minimal data regarding the relationship between GSD and MetS in apparently healthy Asian population. The findings of this study will make it possible to reduce the prevalence of gallstones with appropriate administration of medication and/or lifestyle interventions.

Peer review

This article is well written. This study demonstrated a strong association between GSD and MetS. The presentation of results is logic and the discussion is comprehensive.

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Eosinophilic esophagitis-endoscopic distinguishing findings

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Abstract

Eosinophilic esophagitis (EE) is the most frequent condition found in a group of gastrointestinal disorders called eosinophilic gastrointestinal diseases. The hypothetical pathophysiological mechanism is related to a hypersensitivity reaction. Gastroesophageal reflux disease-like complaints not ameliorated by acid blockade or occasional symptoms of dysphagia or food impaction are likely presentations of EE. Due to its unclear pathogenesis and unspecific symptoms, it is difficult to diagnose EE without a strong suspicion. Although histological criteria are necessary to diagnosis EE, there are some characteristic endoscopic features. We present the case of a healthy 55-year-old woman with dysphagia and several episodes of esophageal food impaction over the last six months. This case report stresses the most distinguishing endoscopic findings-mucosa rings, white exudative plaques and linear furrows-that can help in the prompt recognition of this condition.

Key words: Distinguishing findings; Dysphagia; Eosinophilic esophagitis; Gastro esophageal reflux disease; Histological criteria

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INTRODUCTION

Eosinophilic gastrointestinal diseases (EGD) are rare conditions of growing interest due to their increasing diagnostic frequency in well developed countries^[1]. Eosinophilic esophagitis (EE) is the most common EGD, and its clinical presentation varies extensively making the diagnosis difficult and clinical suspicion fundamental. Although not entirely clear, given that EE correlates with other atopic disorders and has a good response to corticoid treatment, it seems that its pathophysiological mechanism is related to a hypersensitivity reaction^[1].

In this case report, through several expressive images, we highlight the set of endoscopic features which helped in the early recognition of EE.

CASE REPORT

A 55-year-old woman with no previous medical history presented with dysphagia and several episodes of esophageal food impaction over the last six months. Upper gastrointestinal (GI) endoscopy revealed scattered white plaques in the proximal esophagus (Figure 1A), a whitish exudate coating the mucosa in the distal part of the esophagus (Figure 1B), and characteristic images of concentric transient rings and linear furrows (Figure 1C).

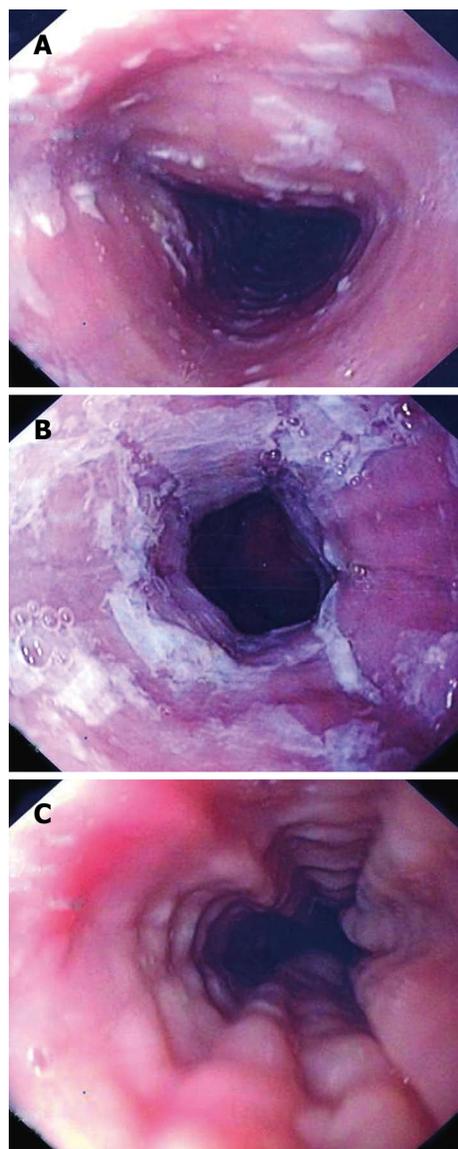


Figure 1 A 55-year-old woman presented with dysphagia and several episodes of esophageal food impaction over the last six months. A: Scattered white plaques in the proximal esophagus; B: Whitish exudate coating the mucosa in the distal part of the esophagus; C: Concentric transient rings and linear furrows on esophagoscopy.

Biopsy specimens showed dense eosinophilic infiltrates, > 20 eosinophils/high power field (HPF) and microabscesses (Figure 2A and B). Gastroesophageal reflux disease (GERD) was excluded when no improvement was observed following the administration of a proton pump inhibitor (PPI). The patient started treatment with a budesonide inhaler twice daily (instructions to swallow) and experienced symptom relief.

DISCUSSION

EE is part of a group of disease known as the eosinophilic gastrointestinal disorders. The pathogenesis of EE is not yet understood, although it appears to be related

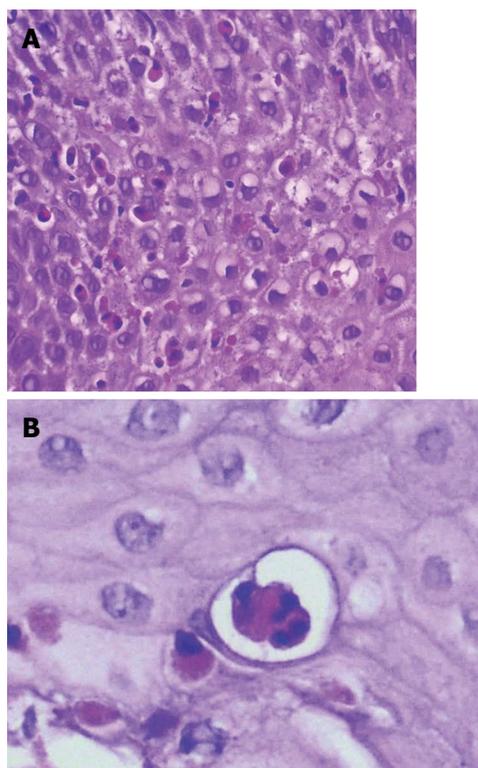


Figure 2 Histological findings in esophageal biopsy specimen. A: Dense eosinophilic infiltrates; B: Microabscesses on esophageal microscopy.

to a hypersensitivity reaction. Some studies suggest that increased mucosa permeability allows contact with potential allergenic digestion products leading to a consequent immunologic response^[2]. EE tends to be a chronic disorder with intermittent or persistent symptoms, usually GERD-like complaints which are not ameliorated by acid blockade with PPI. Additionally, patients may present with symptoms of dysphagia or food impaction. Due to its unspecific esophageal symptoms, clinical suspicion is critical in the diagnosis of EE. Although endoscopy may be normal in one third of cases, images of mucosal rings, white exudative plaques and esophageal strictures are characteristic findings of this pathology. Nevertheless, multiple biopsies should be performed in different esophageal locations, as well as in the stomach and duodenum as the diagnosis of EE relies on histological criteria-one HPF must contain, at least, 15 intraepithelial eosinophils. Additional histological features include eosinophilic microabscesses^[1,3].

To date, there are no large randomized controlled trials on EGD treatment. The majority of data are from smaller studies where corticosteroids play a role in the treatment of these disorders. Generally, oral or topical corticoid therapy is given to the patient for at least eight weeks followed by a gradual taper. The symptoms usually recur, suggesting the need for continuous therapy. Some case reports show evidence of better symptom control following maintenance treatment with mast cell inhibitors or leukotriene receptor antagonists, however,

larger trials are needed^[2,3].

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Dehiscence following successful endoscopic closure of gastric perforation during endoscopic submucosal dissection

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in an *en-bloc* resection. Intensive conservative management was conducted following ESD, however, an endoscopic examination five days after ESD revealed dehiscence of the perforation requiring an emergency laparotomy.

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Key words: Early gastric cancer; Endoscopic closure; Endoscopic submucosal dissection; Gastric perforation; Laparotomy

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Abstract

Gastric perforation is one of the most serious complications that can occur during endoscopic submucosal dissection (ESD). In terms of the treatment of such perforations, we previously reported that perforations immediately observed and successfully closed with endoclips during endoscopic resection could be managed conservatively. We now report the first case in our medical facility of a gastric perforation during ESD that was ineffectively treated conservatively even after successful endoscopic closure. In December 2006, we performed ESD on a recurrent early gastric cancer in an 81-year-old man with a medical history of laparotomy for cholelithiasis. A perforation occurred during ESD that was immediately observed and successfully closed with endoclips so that ESD could be continued resulting

INTRODUCTION

Endoscopic submucosal dissection (ESD) is now an accepted therapy for node-negative early gastric cancer (EGC). Compared to endoscopic mucosal resection (EMR), ESD is often preferred because this technique provides a higher rate of successful *en-bloc* resections^[1-7], however, the risk of complications, particularly perforations, is also higher with ESD. The incidence of perforations occurring during ESD has been reported to range from 3.5% to 6.1%^[2-8]. In terms of the treatment of gastric perforations during endoscopic resection, we previously reported that perforations immediately observed

and successfully closed with endoclips during endoscopic resection could be treated conservatively without the need for an emergency laparotomy^[8]. We now report the first case in our medical facility of a gastric perforation during ESD of EGC which was ineffectively treated conservatively, even though it was immediately detected and successfully closed with endoclips. Laparotomy was performed five days after ESD due to dehiscence following endoscopic closure.

CASE REPORT

An 81-year-old man with a medical history of laparotomy for cholelithiasis performed in 1987 was admitted to our hospital in December 2006 for the endoscopic treatment of a recurrent EGC. He had previously undergone an initial ESD for EGC at the lesser curvature of the lower gastric body in 1999. Pathological findings revealed a well-differentiated mucosal adenocarcinoma without lymphovascular involvement. The lateral margin could not be properly evaluated, however, because an incision had inadvertently been made in the lesion during ESD. After the initial ESD, endoscopic examinations were performed periodically and a recurrent tumor 20 mm in size was detected at the site of the ESD scar in October 2006 (Figure 1A).

In December 2006, ESD was performed on the recurrent tumor and an oval perforation 5 mm in length occurred during the procedure at the site of extensive fibrosis from the initial ESD scar (Figure 1B). The perforation was immediately observed and successfully closed with endoclips (HX-610-090; Olympus Medical Systems Corp., Tokyo, Japan) (Figure 1C) so that the ESD could then be continued resulting in an *en-bloc* resection. Pathological examination revealed a well to moderately differentiated mucosal adenocarcinoma with ulcer scar from the initial ESD without lymphovascular involvement (Figure 1D, E and F). The lateral and vertical margins were negative. Following ESD, the patient was intensively managed with nasogastric suction, fasting and intravenous antibiotic therapy. The patient's abdominal symptoms were unremarkable with no physical indications of diffuse peritonitis observed, although the patient had a fever and high C-reactive peptide level during the observation period. Despite intensive conservative management, the patient's fever persisted and his C-reactive peptide level remained elevated at 20 mg/dL, thus we performed an endoscopic examination five days after ESD which revealed that the perforation site previously closed with endoclips had split open (Figure 2A). Since the open perforation site could not be closed endoscopically, an emergent laparotomy was performed on the same day. Surgical findings indicated that the perforation of the gastric wall extending to the omental bursa was 30 mm × 10 mm in length and the perforation site was not covered with adipose tissue (Figure 2B). Pigmentation from bile and localized findings of inflammation were observed in the omental bursa probably caused by adhesions from the previous laparotomy for cholelithiasis performed in 1987. Distal gastrectomy was performed instead of omental implantation because

the gastric wall at the perforation site was fragile and the split-open perforation was relatively large in size. The patient recovered well postoperatively and was discharged 11 d after surgery with no recurrence detected as of December 2010.

DISCUSSION

We previously reported that gastric perforations which occurred during endoscopic resection could be managed conservatively with complete endoscopic closure based on the analysis of 117 patients with such perforations at our facility^[8]. In our analysis, endoscopic closure with endoclips was successful in 115 of the 117 patients (98.3%) and conservative management for those 115 patients was uneventful. That is, none of the patients with successful endoscopic closure required an emergency laparotomy. In the present case, however, conservative management proved to be ineffective despite the fact that successful endoscopic closure was performed during ESD. An endoscopic examination conducted five days after ESD revealed that the perforation site previously closed successfully with endoclips had split open necessitating an emergency laparotomy.

The gastric lesion in our patient was a recurrent tumor following an initial ESD, and the existence of fibrosis at the site of ESD scar was considered to be one of the factors associated with incomplete healing of the perforation. However, on referring to our previous analysis of 46 patients who received ESDs for locally recurrent EGCs after prior endoscopic resections, perforations occurred during ESD in four patients (8.7%) with all four patients fully recovering following successful endoscopic closure with endoclips^[9]. Therefore, based on those earlier reported results, we cannot conclude that the existence of fibrosis in connection with local recurrence was the only reason for failure of the healing process in the present perforation case. We speculate that there may have been another contributing cause, specifically the patient's medical history of laparotomy for cholelithiasis. It is thought that the reason the perforation site did not heal after successful endoscopic closure was related to the adhesions resulting from the previous laparotomy. Adipose tissue did not cover the perforation site after endoscopic closure due to these adhesions, thus the perforation site was unable to heal properly. We believe that the patient's unremarkable abdominal symptoms were also related to these adhesions as they created a closed space in the abdominal cavity thus localizing the peritonitis resulting from the gastric perforation.

Our experience in this case indicates that gastric perforations which occur during ESD cannot always be treated conservatively even after successful endoscopic closure using endoclips, particularly if the patient has a medical history of laparotomy. Consequently, we must keep this possibility in mind when performing ESD on an EGC patient with a medical history of laparotomy. In addition, we need to remember that perforation patients with a medical history of laparotomy do not always show

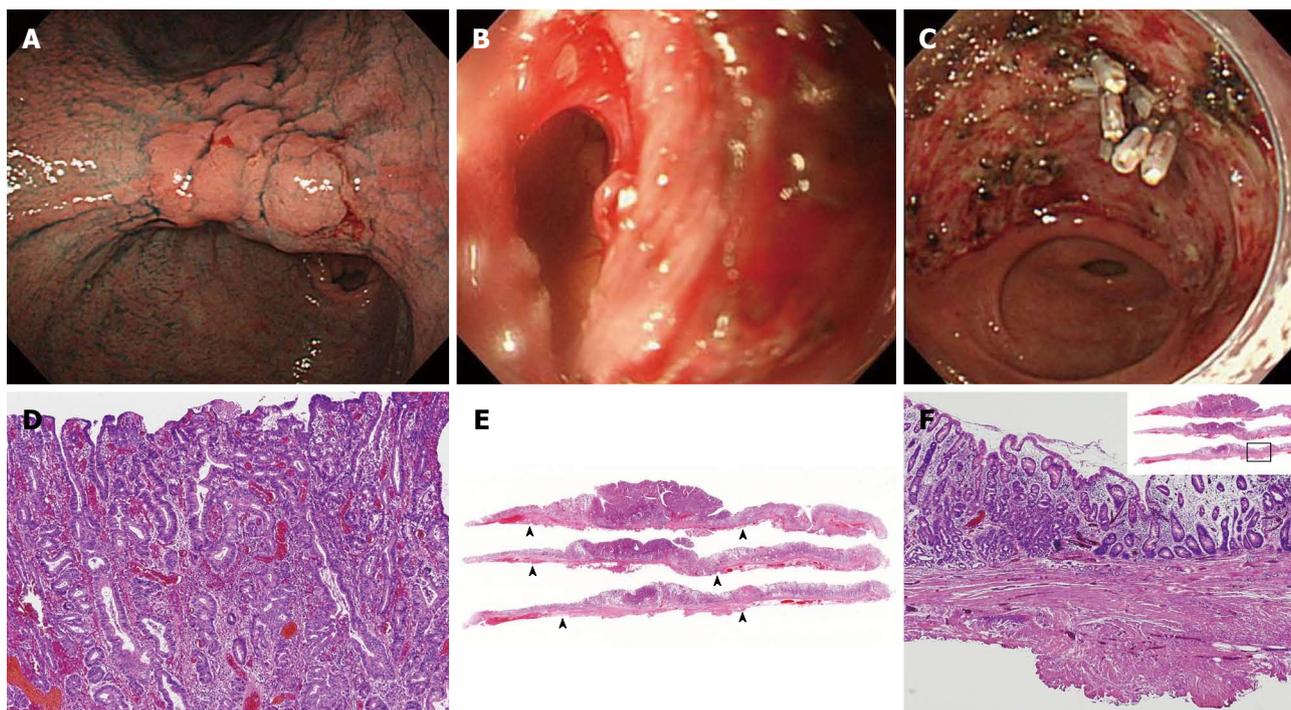


Figure 1 Endoscopic submucosal dissection of a recurrent tumor in December 2006. A: Pre-treatment endoscopy. A recurrent tumor 20 mm in size was detected at the site of the initial endoscopic submucosal dissection (ESD) scar by surveillance endoscopy; B: Endoscopic treatment. ESD was performed on the recurrent tumor, and an oval perforation 5 mm in length occurred at the site of extensive fibrosis during ESD; C: Endoscopic closure of the gastric perforation. The perforation was immediately observed and successfully closed with endoclips so that ESD could be continued resulting in *en-bloc* resection; D: Microscopic examination. A well to moderately differentiated mucosal adenocarcinoma without lymphovascular involvement was shown (hematoxylin and eosin stain, $\times 40$); E, F: Histopathologically, an ulcer scar from the initial ESD (UI-III)s was also shown [between arrowheads (E)] [hematoxylin and eosin stain, $\times 1$ (E), $\times 20$ (F)].

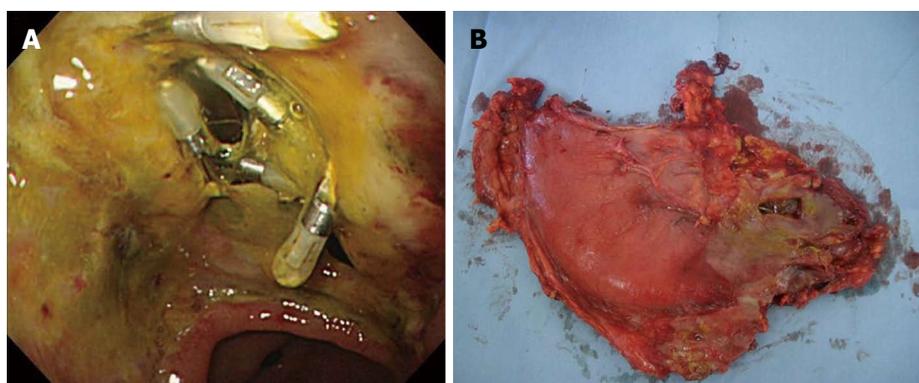


Figure 2 Dehiscence following successful endoscopic closure of the gastric perforation. A: Endoscopy five days after endoscopic submucosal dissection (ESD). The split-open perforation previously closed successfully with endoclips during ESD was revealed; B: Surgical findings. A perforation of the gastric wall extending to the omental bursa 30 mm \times 10 mm in length was seen. The perforation site was not covered with adipose tissue.

remarkable abdominal symptoms and physical findings of diffuse peritonitis.

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Autoimmune pancreatitis complicated by gastric varices: A report of 3 cases

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Abstract

We present three cases of autoimmune pancreatitis (AIP) complicated by gastric varices. Case 1: A 57-year-old man was diagnosed with AIP complicated by gastric varices and splenic vein obstruction. Splenomegaly was not detected at the time of the diagnosis. The AIP improved using steroid therapy, the splenic vein was reperfused, and the gastric varices disappeared; case 2: A 55-year-old man was diagnosed with AIP complicated by gastric varices, splenic vein obstruction, and splenomegaly. Although the AIP improved using steroid therapy, the gastric varices and splenic vein obstruction did not resolve; case 3: A 68-year-old man was diagnosed with AIP complicated by gastric varices, splenic vein obstruction, and splenomegaly. The gastric varices, splenic vein obstruction, and AIP did not improve using steroid therapy. These three cases suggest that gastric varices or splenic vein obstruction without splenomegaly may be an indication for steroid therapy in patients with AIP because the complications will likely become irreversible over time.

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INTRODUCTION

Autoimmune pancreatitis (AIP) is accepted worldwide as a distinctive type of pancreatitis, and the number of patients with AIP is increasing^[1-5]. However, there are few reports of AIP complicated by gastric varices, and the effect of steroid therapy on the gastric varices is unknown. We present three cases of autoimmune pancreatitis complicated by gastric varices.

CASE REPORT

Case 1

A 57-year-old man was admitted to the hospital due to back pain and jaundice. He had a history of diabetes mellitus and no history of habitual alcohol consumption. Laboratory studies revealed liver dysfunction (total bilirubin 3.1 mg/dL, aspartate aminotransferase 275 IU/L, alanine aminotransferase 557 IU/L, and alkaline phosphatase 2822 IU/L), hyperglycemia (236 mg/dL), elevated hemoglobin A1c (7.9%), and elevated IgG4 (176.4 mg/dL). Tumor markers, a complete blood count, electrolyte plasma levels, coagulation tests, amylase levels, lipase levels, and kidney function were all within the nor-

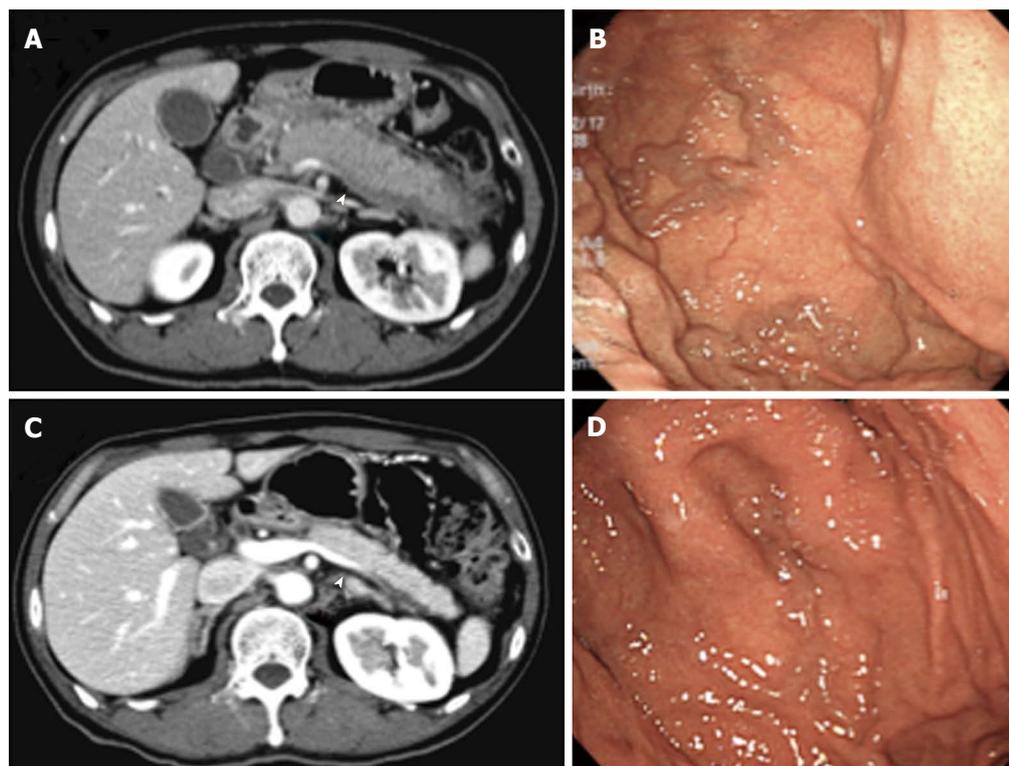


Figure 1 Disappearance of gastric varices following steroid therapy. A: Computed tomography showed a diffusely enlarged pancreas with a capsule-like rim, obstructed splenic vein (arrowhead), and dilated common bile duct; B: Esophagogastroduodenoscopy showed gastric varices in the fundus of the stomach; C: The splenic vein was reperused (arrowhead), and autoimmune pancreatitis improved following steroid therapy; D: Gastric varices disappeared following steroid therapy.

mal limits. Computed tomography (CT) of the abdomen revealed a diffusely enlarged pancreas with a capsule-like rim, an obstructed splenic vein, and a dilated common bile duct (Figure 1A). Endoscopic retrograde cholangiopancreatography (ERCP) revealed irregular narrowing of the main pancreatic duct and stricture of the lower common bile duct. Esophagogastroduodenoscopy (EGD) revealed gastric varices in the fundus of the stomach (Figure 1B).

According to the 2006 Clinical Diagnostic Criteria of The Japan Pancreas Society, the patient was diagnosed with AIP complicated by splenic vein obstruction and gastric varices. Endoscopic biliary drainage by stent placement was performed to alleviate the obstructive jaundice, followed by the oral administration of 30 mg/d prednisolone for 2 wk. The dose was tapered by 5 mg every 2 wk to a maintenance dose of 5 mg/d. Two weeks after the initial treatment, a CT scan showed that the enlarged pancreas had improved and that the splenic vein was reperused (Figure 1C). Six mo after the initial therapy, EGD showed that the gastric varices had disappeared (Figure 1D). Twenty-one mo after admission to the hospital, the patient was followed in the clinic with a maintenance dose of 5 mg/d prednisolone.

Case 2

A 55-year-old man was admitted to the hospital following the incidental detection of gastric fundal varices on EGD during a complete physical examination (Figure 2A). He

had no previous illnesses and no history of habitual alcohol consumption. The patient was asymptomatic, and nothing abnormal was detected on physical examination. A CT scan revealed a locally enlarged pancreatic tail with a capsule-like rim around the lesion, an obstructed splenic vein, and splenomegaly (Figure 2B). ERCP was performed, revealing irregular narrowing of the main pancreatic duct in the pancreatic tail (Figure 2C). Laboratory studies showed an elevated IgG4 (239.1 mg/dL). Tumor markers, a complete blood count, electrolyte plasma levels, coagulation tests, amylase levels, lipase levels, and kidney and liver functions were all within the normal limits.

According to the 2006 Clinical Diagnostic Criteria, the patient was diagnosed with AIP complicated by splenic vein obstruction, gastric varices, and splenomegaly. Oral administration of 30 mg/d prednisolone for 4 wk was used to induce remission, and the dose was tapered by 5 mg every 2 wk to a maintenance dose of 5 mg/d. Two weeks after the initial treatment, a CT scan showed that the enlarged pancreas tail had improved and that the capsule-like rim had disappeared (Figure 2D). However, the splenic vein was not reperused. Ten mo after the initial therapy, EGD showed no improvement of the gastric varices. One year after hospital admission, the patient was followed in the clinic with a maintenance dose of 5 mg/d prednisolone.

Case 3

A 68-year-old man was admitted to the hospital due

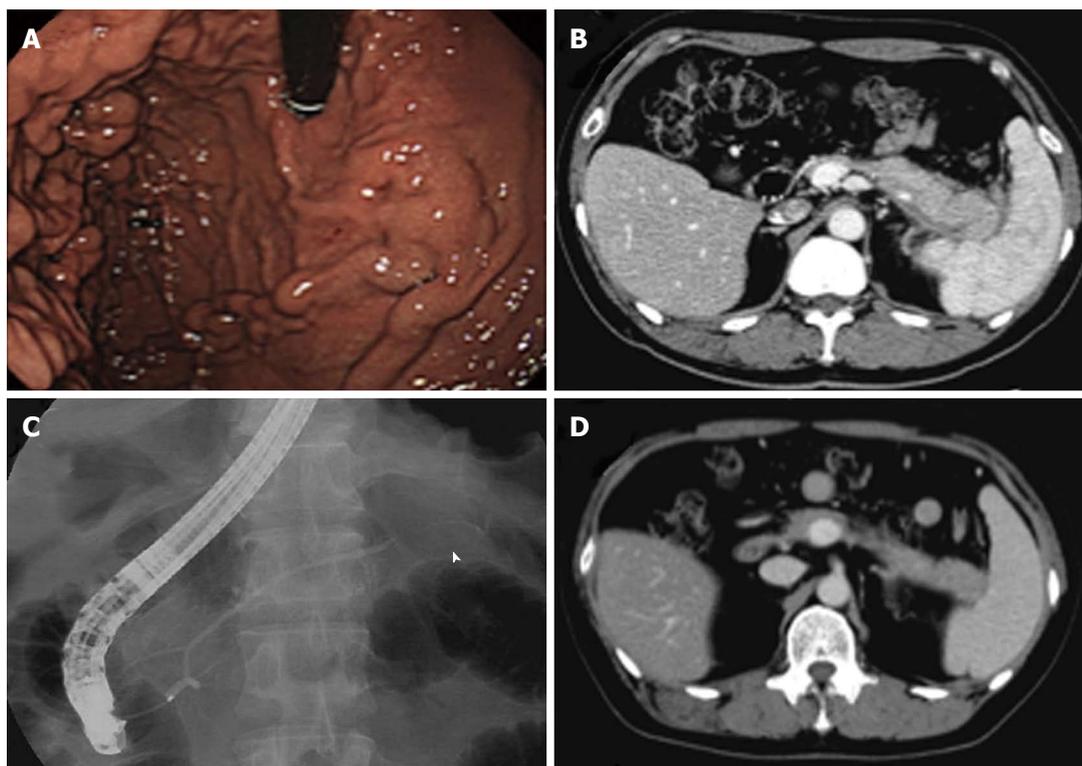


Figure 2 The splenic vein was not reperfused, although the autoimmune pancreatitis improved. A: Esophagogastroduodenoscopy showed gastric varices in the fundus of the stomach; B: Computed tomography showed a locally enlarged pancreatic tail with a capsule-like rim, an obstructed splenic vein, and splenomegaly; C: Endoscopic retrograde cholangiopancreatography showed irregular narrowing of the main pancreatic duct in the pancreatic tail (arrowhead); D: Autoimmune pancreatitis improved following steroid therapy, but the splenic vein was not reperfused.

to hematemesis. An emergency EGD was performed, revealing gastric ulcer bleeding at the gastric notch and incidentally detected gastric varices in the fundus of the stomach (Figure 3A). The gastric ulcer was successfully treated with endoscopic coagulation and administration of a proton pump inhibitor. Additional investigations were performed to ascertain the cause of the gastric varices. The patient had a past history of diabetes mellitus and no history of habitual alcohol consumption. A CT scan revealed a slightly enlarged pancreas with a capsule-like rim around the lesion, a pancreatic stone in the pancreatic tail, an obstructed splenic vein, and splenomegaly (Figure 3B and C). Magnetic resonance cholangiopancreatography (MRCP) revealed irregular narrowing of the main pancreatic duct in the pancreatic head and body, slight dilation of the main pancreatic duct in the pancreatic tail, stricture of the hilar bile duct and lower bile duct, and dilation of the right intra-hepatic bile duct (Figure 3D). ERCP showed the same findings as MRCP. Laboratory studies revealed elevated levels of IgG4 (186 mg/dL), hemoglobin A1c (8.0%), carcinoembryonic antigen (8.2 ng/mL), CA19-9 (38.7 U/mL), and alkaline phosphatase (345 IU/L). The complete blood count, electrolyte plasma levels, coagulation tests, amylase levels, lipase levels, and kidney function were all within the normal limits.

Although the slight dilation of the distal main pancreatic duct was atypical of AIP, the slightly enlarged pancreas, the irregular narrowing of the main pancreatic duct in

the pancreatic head and body, and the elevated levels of IgG4 met the 2006 Clinical Diagnostic Criteria. The patient was diagnosed with AIP complicated by splenic vein obstruction, gastric varices, splenomegaly, and sclerosing cholangitis. Oral administration of 30 mg/d prednisolone for 4 wk was used to induce remission, and the dose was tapered by 5 mg every 2 wk to a maintenance dose of 5 mg/d. However, a CT scan showed no improvement of the pancreatic lesion, and EGD showed no improvement of the gastric varices. Because steroid therapy was not effective, maintenance therapy was discontinued 5 mo after the initial treatment. One year after hospital admission, the patient was followed in the clinic without treatment.

DISCUSSION

There are few reports of autoimmune pancreatitis complicated by gastric varices. The effects of steroid therapy on the varices is unknown^[6]. However, the reported 8%^[7] frequency of splenic vein obstruction in patients with chronic pancreatitis indicates that it is not a rare complication. Splenic vein obstruction causes a localized form of portal hypertension, known as sinistral portal hypertension, which leads to the formation of gastric varices along the fundus and the greater curvature of the stomach due to increased blood flow through the short gastric veins or the gastroepiploic vein^[8]. From 1999 to 2011, our hospital treated 20 consecutive patients with AIP who fulfilled either the 2006 Clinical Diagnostic Criteria

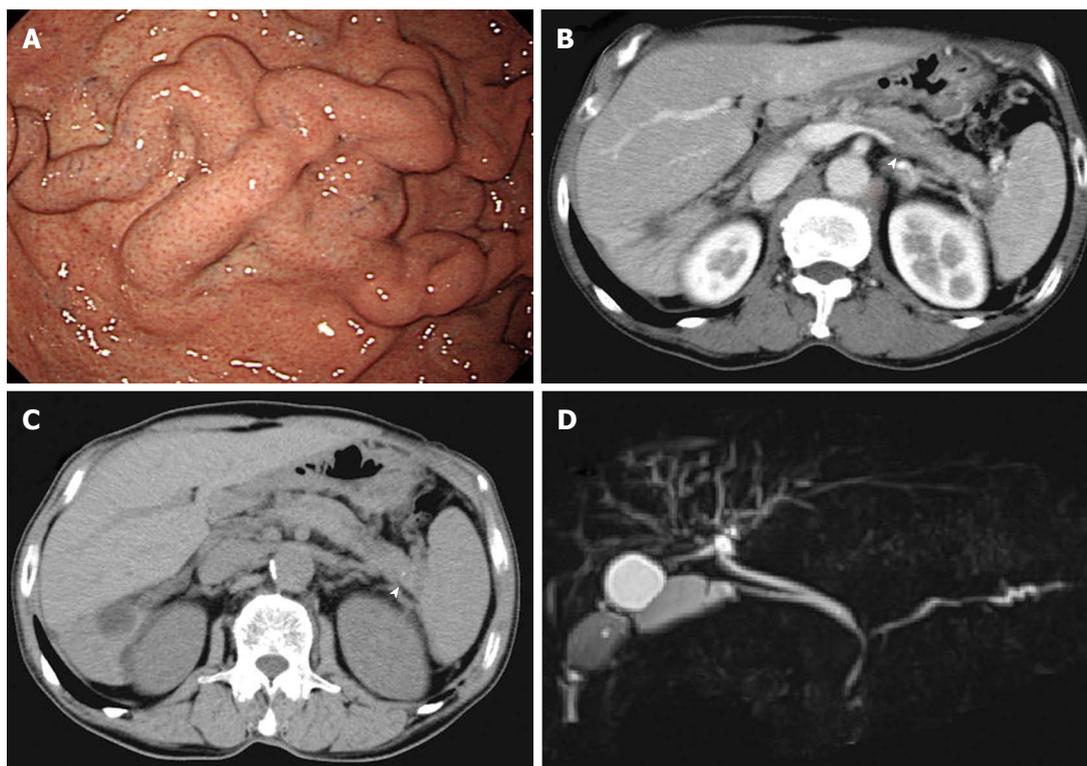


Figure 3 Autoimmune pancreatitis that did not improve with steroid therapy. A: Esophagogastroduodenoscopy showed gastric varices in the fundus of the stomach; B: Computed tomography (CT) showed a slightly enlarged pancreas with a capsule-like rim, an obstructed splenic vein (arrowhead), and splenomegaly; C: CT showed a pancreatic stone in the pancreatic tail (arrowhead); D: Magnetic resonance cholangiopancreatography showed irregular narrowing of the main pancreatic duct in the pancreatic head and body, slight dilation of the main pancreatic duct in the pancreatic tail, stricture of the hilar bile duct and lower bile duct, and dilation of the right intra-hepatic bile duct.

of The Japan Pancreas Society or the Asia diagnostic criteria. Splenic vein obstruction was confirmed in 4 of the patients (20%) using CT, and 3 of the 4 patients (15%) had gastric varices.

The clinical course of these three cases varied depending on the presence of splenomegaly. In case 1, splenomegaly was not detected at the time of diagnosis. Gastric varices disappeared as the AIP improved using steroid therapy; In case 2, splenomegaly was detected at the time of diagnosis. The gastric varices did not improve, although the AIP improved using steroid therapy; In case 3, splenomegaly was detected at the time of diagnosis. Neither the gastric varices nor the AIP improved with steroid therapy. These three cases suggest that gastric varices complicating AIP without splenomegaly may improve using steroid therapy.

The pathogenetic hypothesis of splenic vein obstruction has been related to many factors: compression by a pseudocyst or an enlarged pancreatic parenchyma and secondary involvement of the vein by surrounding edema, cellular infiltration, and the fibroinflammatory process^[9]. Whether the splenic vein obstruction is formed by mechanical force or inflammatory infiltration, the obstruction will become an irreversible thrombosis over time. Although the AIP improved in case 2, the splenic vein obstruction, which had likely progressed into a thrombosis, was irreversible. A gastrosplenic shunt or other collateral veins that drain into the systemic circulation

were not detected on CT scans in any of the three cases. Development of congestive splenomegaly may have been dependent on the length of time the patients had been affected with sinistral portal hypertension. These three cases indicate the need to reperfuse the obstructed splenic vein before the development of splenomegaly, otherwise the obstruction becomes irreversible. According to a nationwide survey by the Research Committee of Intractable Pancreatic Diseases conducted by the Ministry of Health, Labor and Welfare of Japan, the remission rate of steroid-treated AIP is 98%^[10]. However, rare cases of steroid-refractory AIP can occur. Kamisawa *et al*^[11] reported the development of pancreatic atrophy in 5 out of 23 patients with AIP. Takayama *et al*^[12] reported that AIP has the potential to be a progressive disease with pancreatic stones. These reports suggest that recurrent cases of AIP can turn into chronic pancreatitis-like lesions during long-term follow-up and become refractory to steroid therapy. In case 3, the AIP was refractory to steroid therapy. Because the enlargement of the pancreas was not prominent and a pancreatic stone was detected, the lesion was likely the result of recurrent inflammation of AIP.

The role of a prophylactic splenectomy in asymptomatic patients with splenic vein obstruction and gastric varices remains controversial. Badley concluded that the benefit of preventing possible bleeding of the varices outweighs the risk of postsplenectomy sepsis^[13], although

Bernades *et al*^[7] reported that the risk of variceal bleeding is lower than previously reported. In cases 2 and 3, we did not perform a prophylactic splenectomy or partial splenic embolization because the patients opted for a watchful waiting approach.

The indications for steroid therapy in patients with AIP are symptoms such as obstructive jaundice, abdominal pain, back pain and the presence of symptomatic extrapancreatic lesions^[1]. However, some patients with AIP improve spontaneously^[1,14]. The treatment of asymptomatic patients with AIP remains controversial. Based on the potential risk and benefits, these three cases suggest that patients with AIP and gastric varices or splenic vein obstruction without splenomegaly should be treated with steroids before pancreatic lesions or splenic vein obstructions become irreversible. Because this study only included three cases, it is necessary to collect data on more patients with AIP complicated by gastric varices to effectively evaluate this hypothesis.

In conclusion, we treated 3 cases of autoimmune pancreatitis complicated with gastric varices. Gastric varices or splenic vein obstruction without splenomegaly may be an indication for steroid therapy in patients with AIP.

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Ischemic colitis during interferon-ribavirin therapy for chronic hepatitis C: A case report

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Abstract

Ischemic colitis is a rare complication of interferon administration. Only 9 cases in 6 reports have been described to-date. This report describes a case of ischemic colitis during pegylated interferon and ribavirin treatment for chronic hepatitis C, and includes a review of the relevant literature. A 48-year-old woman was treated with pegylated interferon α -2a and ribavirin for chronic hepatitis C, genotype 1b. After 19 wk of treatment, the patient complained of severe afebrile abdominal pain with hematochezia. Vital signs were stable and serum white blood cell count was within the normal range. Abdominal computed tomography showed diffuse colonic wall thickening from the splenic flexure to the proximal sigmoid colon, which is the most vulnerable area for the development of ischemic

colitis. Colonoscopy revealed an acute mucosal hyperemic change, with edema and ulcerations extending from the proximal descending colon to the sigmoid colon. Colonic mucosal biopsy revealed acute exudative colitis. Polymerase chain reaction and culture for *Mycobacterium tuberculosis* were negative and the cultures for cytomegalovirus, *Salmonella* and *Shigella* species were negative. After discontinuation of interferon and ribavirin therapy, abdominal pain and hematochezia subsided and, following colonoscopy showed improvement of the mucosal ulcerations. Ischemic colitis cases during interferon therapy in patients with chronic hepatitis C reported so far have all involved the descending colon. Ischemic colitis is a rarely encountered complication of interferon administration in patients with chronic hepatitis C and should be considered when a patient complains of abdominal pain and hematochezia.

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Key words: Ischemia; Hepatitis C; Interferon

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INTRODUCTION

Pegylated interferon (IFN) combined with ribavirin is currently the standard treatment for chronic hepatitis C (CHC) and can achieve sustained virological response

in approximately 55% of genotype-I hepatitis C virus (HCV) infections^[1]. The side effects of this combination treatment result in premature treatment termination in 10%-15% and dose adjustment in 32%-42% of patients^[1]. Therefore, physicians need to be well aware of both the common and uncommon side effects of treatment. Ischemic colitis is a rare complication of interferon administration^[2]. Only 9 cases in 6 reports (one in Japanese^[3] and 5 in English^[2,4-7]) have been described to date. This report describes a case of ischemic colitis during pegylated interferon and ribavirin treatment for CHC and includes a review of the relevant literatures.

CASE REPORT

A 48-year-old woman was admitted for the evaluation of acute abdominal pain and hematochezia. The patient had been treated with combination therapy of pegylated IFN- α 2a (180 μ g/wk) (Pegasys; Roche, Basel, Switzerland) and ribavirin (1000 mg/d) (LG Ribavirin; LG Life Sciences, Seoul, Korea) for CHC, genotype Ib. A qualitative HCV test was initially obtained using reverse transcription-polymerase chain reaction (RT-PCR) (Abbott Laboratories, Abbott Park, IL, United States), and the serum HCV viral load was 1.67×10^6 viral copies/mL. The serum HCV RNA level decreased to undetectable levels after 12 wk of treatment. The treatment process was uneventful until 16 wk of therapy. At week 16, the patient complained of mild epigastric discomfort and nausea, which resolved spontaneously a few days later. Three weeks later, at week 19 of therapy, the patient complained of severe abdominal pain with hematochezia, but she remained afebrile. Small amounts of bloody stool were passed 2-3 times per day. The patient denied recent travel or additional medications such as antibiotics or non-steroidal anti-inflammatory drugs. The patient had no history of diabetes mellitus, hypertension, inflammatory bowel disease, atrial fibrillation, valvular heart disease, coronary artery disease, or hypercoagulable conditions. She was a non-smoker, and her family history was unremarkable.

Physical examination revealed direct tenderness on the left lower quadrant of the abdomen. Blood pressure was 114/76 mmHg, pulse 80 beats/min, respiration rate 20/min, and body temperature 37.9 °C. Complete blood count revealed a hematocrit of 26.9%, hemoglobin 8.7 gm/dL, platelets 115 000/ μ L, and white blood cell count 4700/ μ L with differential counts within the normal range. C-reactive protein was slightly elevated at 0.7 mg/dL (normal < 0.4 mg/dL). Serum electrolytes, liver profile, renal function tests, amylase, and lipase were within normal limits. The coagulation profile was within the normal range, with a prothrombin time of 11.2 s and an activated partial thromboplastin time of 23.9 s. Upper endoscopy was performed 6 mo prior to admission, which documented a healing stage small gastric ulceration at the antrum. *Helicobacter pylori* infection was documented by histological examination and was successfully eradicated

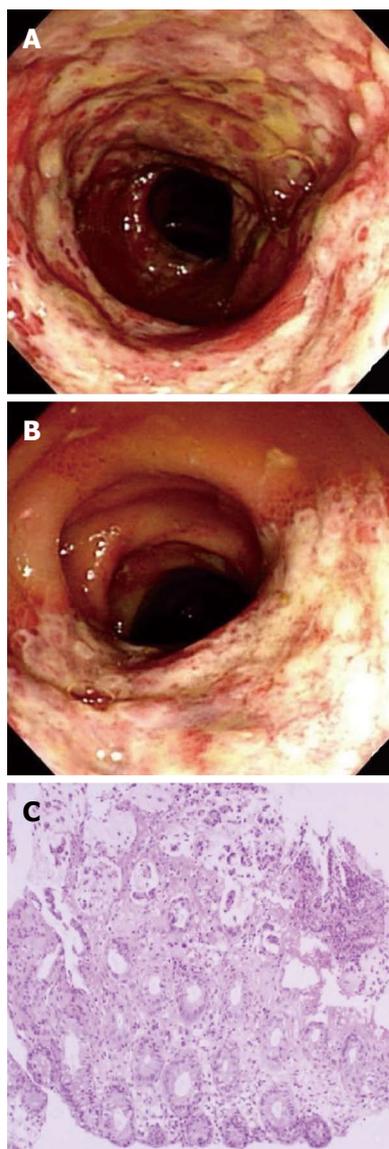


Figure 1 Colonoscopy images and pathology. A: Mucosal hyperemic change with edema, erosion, and ulcerations and hemorrhagic friable mucosa on the sigmoid colon; B: Segmental ulceration was seen on the proximal descending colon; C: Pathological examination of the descending colon showed acute exudative colitis. Epithelial detachment was observed (hematoxylin and eosin stain, $\times 40$).

with antibiotic therapy at that time. At admission, upper endoscopy was negative for an active bleeding source, and colonoscopic examination revealed acute hyperemic mucosal changes with edema, erosion, and ulcerations. Acute inflamed, friable mucosal changes extended from the sigmoid colon (Figure 1A) to the proximal descending colon (Figure 1B). The rectum showed relatively normal mucosa. Colonic mucosal biopsy revealed acute exudative colitis compatible with a diagnosis of ischemic colitis (Figure 1C). On the biopsy specimen, staining for cytomegalovirus showed no positive cells, and staining for acid-fast bacilli was negative. Tuberculous nucleic acid was undetectable by polymerase chain reaction. A serum anti-Streptolysin O test was negative. Bacteriologic culture studies of stool and colonic fluid were negative

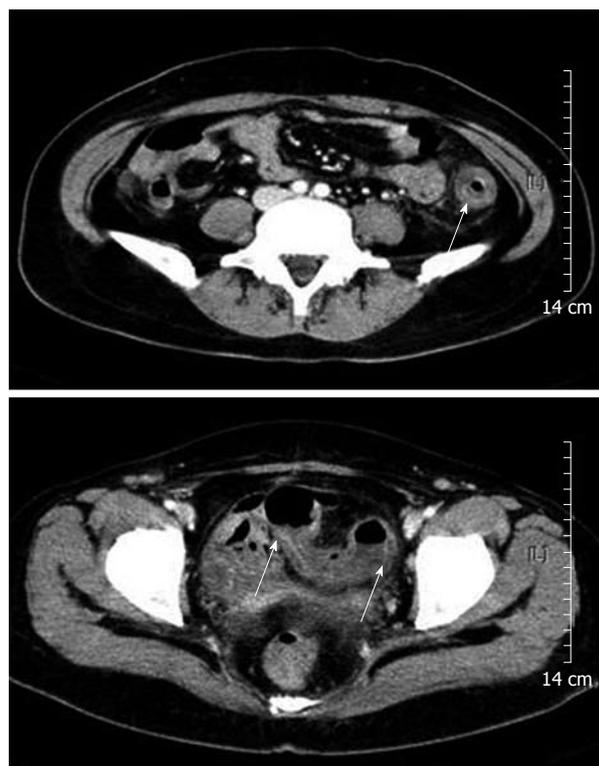


Figure 2 Abdominal computed tomography. A: Circumferentially layered wall thickening and pericolic fat infiltration at the descending colon (arrow); B: Circumferential wall thickening and pericolic fat infiltration at the proximal sigmoid colon (arrows).

for *Mycobacterium*, *Salmonella*, and *Shigella* species. Abdominal computed tomography showed circumferential wall thickening and pericolic fat infiltration from the descending colon to the proximal sigmoid colon, a well-known predisposing area for the development of ischemic colitis (Figure 2). The findings from colonoscopy, computed tomography, and pathology were all compatible with the diagnosis of ischemic colitis.

After IFN and ribavirin treatment were discontinued, the patient's abdominal pain decreased and hematochezia resolved. One week later, subsequent colonoscopy showed marked improvement in ulceration and mucosal edema. Serum HCV RNA remained undetectable for up to 3 mo after cessation of treatment, but reappeared 1 year later. The reappearance of serum HCV RNA suggests that the antiviral treatment for CHC was not successful.

DISCUSSION

In current standard combination therapy of pegylated IFN and ribavirin in CHC patients, more than 50% of sustained virological response is expected. However, a large proportion of the patients are not eligible for the treatment or drop out early during the treatment due to side effects.

The side effects of IFN and ribavirin combination therapy are mostly associated with IFN administration, while hemolytic anemia is attributable to ribavirin^[1]. Side

Table 1 Characteristics of ischemic colitis associated with interferon treatment in chronic hepatitis C patients

Ref.	Age/gender, diagnosis	Treatment and duration	Location of ischemic colitis
Okanoue <i>et al</i> ^[2]	47/F, CHC	IFN- α , 4 wk	Rectum to descending colon
	55/F, CHC	IFN, 23 wk	Rectum to descending colon
	65/M, CHC	IFN, 5 wk	Rectum to descending colon
Horigome <i>et al</i> ^[3]	53/M, CHC	IFN- α , 12 wk	Sigmoid colon to descending colon
Tada <i>et al</i> ^[6]	65/M, CHC	IFN- α , 4 wk	Descending colon
	55/F, CHC	IFN- α , 24 wk	Descending colon
Leung <i>et al</i> ^[7]	44/M, CHC	Pegylated IFN- α , 34 wk	Sigmoid colon to splenic flexure
Wenner <i>et al</i> ^[4]	7/F, CHC with CRF	IFN- α , 7 wk	Descending colon
Punnam <i>et al</i> ^[5]	51/M, CHC	Pegylated IFN- α , 12 wk	Mid-transverse to proximal descending colon

CHC: Chronic hepatitis C; CRF: Chronic renal failure; IFN- α : Interferon- α ; F: Female; M: Male.

effects associated with IFN treatment have been described not only in CHC, but also in chronic hepatitis B^[8], chronic myeloid leukemia^[9], metastatic renal cell carcinoma^[10], and multiple myeloma^[11]. Flu-like symptoms such as general malaise, fever, arthralgia, headache, and hematologic abnormalities such as leukopenia and thrombocytopenia are frequently observed early adverse effects of IFN- α administration^[2]. Although the severity of these symptoms is directly related to the dose and frequency of IFN administration, remarkable individual variation has been observed^[2]. Among the various gastrointestinal side effects of IFN- α , ischemic colitis is rarely reported during treatment of CHC, occurring in less than 1% of patients^[12]. Other than ischemic colitis, microscopic colitis^[7] and ulcerative colitis^[13] have also been reported.

Nine cases of ischemic colitis related to IFN therapy in CHC have been reported in 6 studies, and the most common symptoms were abdominal pain and lower gastrointestinal bleeding such as melena and hematochezia^[2-7]. The descending colon was most frequently involved in all ischemic colitis cases associated with CHC (Table 1). In reported cases, hematochezia and melena appeared between week 4 and 34 of IFN treatment, and the mean duration of IFN treatment before the occurrence of ischemic colitis was 14 wk. The characteristics of this case correspond well with those of previously reported cases in terms of symptoms, location, and period of duration.

The etiology of ischemic colitis is not yet clearly elucidated. The mechanism of IFN-induced ischemic colitis is thought to be associated with immune modulation of cytokine networks. Sparano *et al*^[10] documented the role of interleukin-2 (IL-2) and IFN- α in the development of colonic ischemia. They suggested that a disordered coagulation cascade played a role in the pathogenesis of colonic ischemia, assuming that derangement of coagulation is

mediated by tumor necrosis factor or its interactions with other cytokines^[10]. IFN therapy also induces an increase in plasma-activated complement C5a, a potent intravascular aggregator of granulocytes, favoring the development of microthrombi in small vessels. IFN-induced vasculitic reactions, mediated by the deposition of immune complexes in the vasculature, may also play a pathogenic role in ischemia of various organs^[14,15]. Thrombogenic effects of activated cytokines and immune complex-mediated vasculitic reactions may play a role in the pathogenesis of IFN-induced ischemic colitis^[14]. The potential role of cytokines for the development of IFN induced ischemic colitis needs to be investigated further.

In conclusion, physicians should be vigilant of the various common and uncommon complications of both pegylated IFN and ribavirin. Ischemic colitis is a rarely encountered complication of pegylated IFN administration but it should be considered when a patient complains of abdominal pain and hematochezia.

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Spontaneous hemoperitoneum from hepatic metastatic trophoblastic tumor

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common appearances of hepatic metastases. For SP resulting from hepatic metastatic tumors, surgical intervention is still the predominant therapeutic method, but the prognosis is very poor.

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Key words: Hemoperitoneum; Hepatic metastases; Trophoblastic tumor; Computed tomography; Treatment

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Abstract

Spontaneous hemoperitoneum (SP) is defined as the presence of blood within the peritoneal cavity that is unrelated to trauma. Although there is a vast array of etiologies for SP, primary hepatocellular carcinoma and hepatic adenoma are considered to be the most common causes. Hepatic metastatic tumor associated with spontaneous rupture is rare. SP from hepatic metastatic trophoblastic tumor may initially present with a sudden onset of abdominal pain. Abdominal computed tomography (CT) plays an important role in establishing the diagnosis of SP, indicating its origin and etiology, and determining subsequent management. Herein, we report an uncommon case of hemoperitoneum from spontaneous rupture of a hepatic metastatic trophoblastic tumor in a young female patient. Interestingly, the contrast-enhanced CT findings demonstrated hypervascular hepatic masses with persistent enhancement at all phases, which were completely different from the

INTRODUCTION

Spontaneous hemoperitoneum (SP) is defined as the presence of blood within the peritoneal cavity that is unrelated to trauma. Primary hepatocellular carcinoma (HCC) and hepatic adenoma are considered to be the most common causes; however, hepatic metastatic tumors associated with SP are rare. Those metastatic tumors may include colon, lung, renal cell and testicular neoplasms, and Wilm's tumor, but few cases of metastatic trophoblastic tumor responsible for HP have been reported, especially in the absent evidence of primary malignant tumor. Computed tomography (CT) plays an important role in establishing the diagnosis of SP. Metastatic liver tumors display variant appearances in routine CT scan and are often classified as hypervascular, hypovascular and nonvascular metastases on dynamic contrast-enhanced CT scan. Most of the hepatic metastatic tumors present with hypovascular appearances, namely mild enhancement in both the portal

phase and delayed phase. But in our patient with hepatic trophoblastic tumor, there were typically unusual presentations, which showed almost identical enhancement patterns with abdominal aorta in all phases. To our knowledge, there are few reports of similar cases focusing on uncommon CT manifestations in hepatic metastatic trophoblastic tumor. We report an uncommon case of hemoperitoneum from spontaneous rupture of a hepatic metastatic trophoblastic tumor in a young female patient below.

CASE REPORT

A 24-year-old girl was admitted to our emergency department because of sudden onset of abdominal pain, and emergent non-enhanced CT scan showed intrahepatic multiple lesions associated with rupture and bleeding into the peritoneal cavity (Figure 1). Three months ago, she underwent splenic repair, and her family could not tell about the specific cause even after she was discharged from the hospital. She had a history of pregnancy and abortion two years ago. Physical examination on admission was unremarkable except for mild epigastric tenderness and longitudinal abdominal scar. At exploratory laparotomy, there was dark red blood in the abdominal cavity. After cleaning of the blood, multiple cystic masses bulging from the surface of the liver were found, one of which was ruptured and then removed for pathological analysis. Her serum beta human chorionic gonadotropin (β -HCG), alpha-fetoprotein (AFP) and cancer antigen-125 (CA-125) levels were 94 mIU/mL (reference range: 0-6 mIU/mL), 1.01 ng/mL (reference range: < 7.0 ng/mL) and 46.86 ng/mL (reference range: < 3.4 ng/mL), respectively. Post-operative contrast-enhanced CT of abdomen showed multiple hepatic hypervascular masses keeping the similar enhancement with abdominal aorta in all phases (the diameter of the masses ranged from 0.8 cm to 2.8 cm) and arteriportal shunts (Figures 2, 3A and B). CT scan of the chest and pelvis revealed no abnormalities. Histopathological examination confirmed the tumor to be predominantly composed of mononuclear epithelioid cells with pink cytoplasm and large, irregularly shaped nuclei with prominent nucleoid (Figure 4). Immunostaining was positive for placental alkaline phosphatase (Figure 5), while negative for HepPar-1, smooth muscle actin. Hepatic metastatic trophoblastic tumor was diagnosed by histopathological examination and immunohistochemical analyses. No abnormality was found in the all-round gynecological checkup, especially in the uterus. The patient refused to chemotherapy and died a month later despite of receiving supportive treatment. Her family refused the autopsy.

DISCUSSION

SP, an uncommon cause of acute abdominal pain, is defined as the presence of blood within the peritoneal cavity that is unrelated to trauma. Its occurrence may



Figure 1 Axial computed tomography showing multiple hypodense nodules in the liver and hemoperitoneum around the liver and spleen.



Figure 2 Contrast-enhanced computed tomography scan showing multiple hypervascular nodules similar to the enhancement of abdominal aorta in the arterial phase.

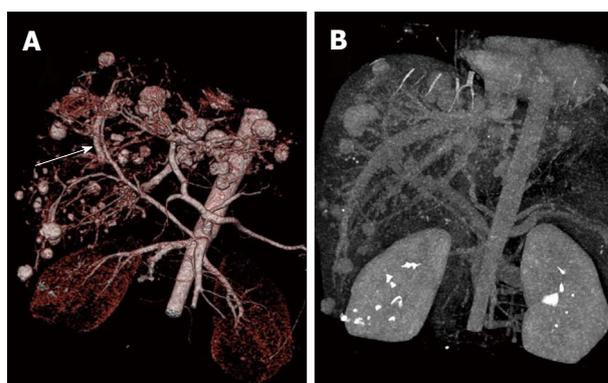


Figure 3 Volume rendering images showing multiple hypervascular nodules similar to saccular aneurysmal dilation and the presence of arteriportal shunts (white arrow). A: Multiple hypervascular nodules similar to saccular aneurysmal dilation; B: Presence of arteriportal shunts.

be catastrophic. There are various causes for SP, such as hepatic, splenic, gynecological and vascular involvement, and altered coagulation status^[1,2]. Therefore, underlying causes must be identified if the patient survives the initial events. As for hepatic causes, previously undetected liver lesion may be a relatively common cause of spontaneous rupture leading to hemoperitoneum, and unnoticeable

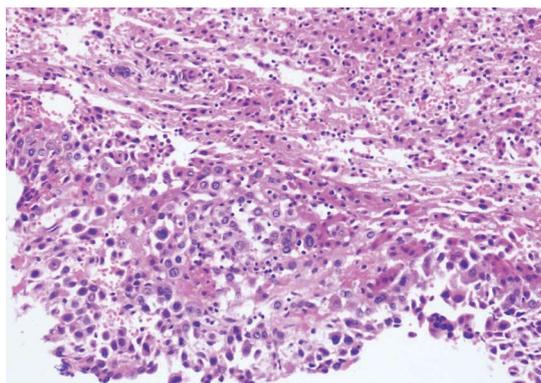


Figure 4 Histopathology showing the tumor composed of mononuclear epithelioid cells with large, irregularly shaped nuclei (hematoxylin and eosin stain × 200).

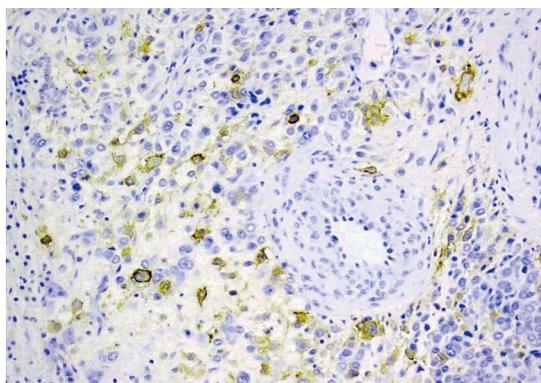


Figure 5 Immunohistochemical analysis was positive for placental alkaline phosphatase (× 200).

minor trauma may be the precipitating factor. Hepatic adenoma and HCC developed from cirrhosis are the most common causes for SP^[3,4], however, hepatic metastatic tumor associated with SP is rare, as most of them tend to be less invasive, and have less blood vessels and less propensities to penetrate the liver capsule as compared with HCC. In our case, hepatic metastatic tumor associated with SP was due to the rupture of hepatic metastasis of a trophoblastic tumor with hypervascularity, which was seldom found responsible for SP in clinical practice.

The mechanism for the rupture of hepatic metastasis remains unclear, but the large lesion adjacent to the liver capsule is at the greatest risk due to direct pressure against the capsular surface, especially in increased intra-abdominal pressure. Acute hemoperitoneum as a result of hemorrhage from liver metastasis is an uncommon but a terminal event. In a large report of 70 patients with SP, metastatic disease was only found responsible for one case^[5]. Those metastatic lesions may include colon, lung, renal cell, testicular tumors, and Wilm's tumor^[6-8]. So, a thorough search should be made for evidence of a primary tumor that may have metastasized to the liver. However, hepatic involvement from trophoblastic tumor accounts for only 10% of the patients and occurs in the late course of the disease. Regression of a primary tumor

after it has metastasized is not uncommon, and one-third of cases manifests as complications of metastatic diseases^[9].

The available imaging modalities used for the diagnosis of SP include sonography, CT and magnetic resonance imaging. Meticulous imaging technique and careful observation of key imaging features are important for accurate characterization of the organ origin of the spontaneous bleeding. Currently, CT scan is the most frequently used modality in evaluating the patients with suspected hemoperitoneum^[2,10,11]. Emphasis should be laid on detecting hemoperitoneum, then localizing the source of bleeding and finally detecting the primary cause. On CT imaging, the appearance of blood within the peritoneum varies depending on the site and the extent of bleeding, and the age of blood. If the time interval between bleeding and imaging is several hours, high attenuation clot may be seen. Over the next few days, the attenuation of the blood decreases, which becomes similar to simple fluid after 2-3 wk. High attenuation clots may appear at the site of the bleeding and suggest a clue as to the site of the bleeding origin. Once the presence of hemoperitoneum has been identified or active bleeding is controlled, a comprehensive search should be made further for an underlying cause. This may start with a careful evaluation of the liver and spleen, because they are the most common organs responsible for spontaneous bleeding. If the SP is derived from a hepatic or splenic cause, the peritoneal blood may be centered around the responsible lesion^[12,13], as described in our case. Although hepatic metastases can come from many locations of the body, SP secondary to metastases exhibit similar findings on non-enhanced CT scan.

Dramatic advance in multi-detector technology has significantly improved the accuracy of CT and dynamic contrast-enhanced CT scan which can identify imaging characteristics of intrahepatic lesions, especially the rare appearances from uncommon disease entity. Metastatic tumors of liver present with intra-liver occupied lesions on unenhanced CT scan, including multi-nodules similar to the "bull's eye" sign, homogenized low-density mononodule, equal- or high-density mass and cystic lesion. Appearances of dynamic contrast-enhanced CT scan for these disease entities may have variant enhancement patterns according to the vascularity of the primary tumor and are often classified as hypervascular, hypovascular and nonvascular metastases. Most of hepatic metastatic tumors show mild enhancement in the portal phase and delayed phase consistent with hypovascular lesions, while there were typically unusual presentations in our patient, which showed the synchronized enhancement with abdominal aorta in all phases. The similar case is extremely rare in the reported literature. Therefore, for a female patient in child-bearing age who presents with SP and unusual contrast-enhanced CT findings similar to our patient's, we should be alert to the possibility of the spontaneous rupture of metastatic trophoblastic disease in the liver, even though the primary lesion is not found on that

occasion.

Prognosis of patients with rupture hepatic metastasis of trophoblastic disease is extremely poor, and the choice of treatment depends on the tumor size, tumor location, severity of bleeding and the general condition of the patient. Surgery and embolisation with or without chemotherapy are the mandatory therapeutic choices^[14,15]. Because of the severe condition of our patient, transcatheter arterial chemotherapeutic embolization is abortive.

In conclusion, we described the CT findings of SP secondary to rupture of hepatic metastatic trophoblastic disease in a female patient. Unusual enhancement patterns on contrast-enhanced CT scan will further enhance our recognition of the SP from hepatic metastatic trophoblastic disease and help differentiate it from other hypervascular hepatic masses.

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Non-steroidal anti-inflammatory drugs-induced small intestinal injury and probiotic agents

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Abstract

Intestinal bacteria play a role in the development of non-steroidal anti-inflammatory drugs (NSAID)-induced small intestinal injury. Agents such as probiotics, able to modify the gut ecology, might theoretically be useful in preventing small intestinal damage induced by NSAIDs. The clinical studies available so far do suggest that some probiotic agents can be effective in this respect.

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Key words: Non-steroidal anti-inflammatory drugs; Small intestine; Intestinal bacteria; Probiotics

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TO THE EDITOR

Park *et al*^[1,2] in their interesting editorial^[1] about small intestinal injury induced by non-steroidal anti-inflammatory drugs (NSAIDs), discussed the possible role of the intestinal flora in the pathogenesis of the enteric damage, but, oddly enough, when listing the pharmacological agents theoretically useful as protective or therapeutic medicines, they omitted to mention probiotics. Live micro-organisms could prevent NSAID-induced small intestinal damage by both modifying intestinal microbial ecology and modulating the local immune function.

Various studies have addressed the issue. While a pilot study in human volunteers failed to demonstrate any effect of *Lactobacillus GG* in preventing indomethacin-induced alterations of intestinal permeability^[3], a subsequent experimental study demonstrated that in rats pretreated with *Lactobacillus casei*, strain Shirota significantly prevents the development of indomethacin-induced enteropathy^[4], although the mechanism responsible for this phenomenon remains not completely clear.

In a recent trial, patients treated for three months with low-dose enteric-coated aspirin (100 mg daily) were randomized to receive either co-administration of *Lactobacillus casei* or no additional treatment^[5]. Capsule endoscopy, performed before and after treatment, showed a significant decrease ($P = 0.039$) in the number of mucosal breaks and in the endoscopic score in the probiotic group as compared with controls.

In a randomized, double-blind, cross-over placebo-controlled study in healthy volunteers, the probiotic mixture VSL # 3 was found to prevent the increase in faecal concentration of the inflammatory marker calprotectin during intake of indomethacin 50 mg daily^[6].

The role of bacteria in the development of small intestinal lesions during NSAID administration seems indirectly confirmed, but the recent experimental observations showed that proton pump inhibitors significantly worsen intestinal ulcerations and bleeding in naproxen- and celecoxib-treated rats and this is related to substantial

shifts in enteric microbial population (e.g., a marked reduction in *Actinobacteria* and *Bifidobacteria*)^[7].

All in all, it appears that probiotics can represent promising agents in the prevention of NSAID-induced small intestine injury, although additional studies are needed to better clarify this point. However, the efficacy of any single probiotic strain should be evaluated separately, due to the differences in the biological effects and mode of actions of the various agents currently available.

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 Kuala Lumpur, Malaysia

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 Cancers Symposium
 San Francisco, CA 3000,
 United States

January 19-21, 2012
 2012 Gastrointestinal Cancers
 Symposium
 San Francisco, CA 94103,
 United States

January 20-21, 2012
 American Gastroenterological
 Association Clinical Congress of
 Gastroenterology and Hepatology
 Miami Beach, FL 33141,
 United States

February 3, 2012
 The Future of Obesity Treatment
 London, United Kingdom

February 16-17, 2012
 4th United Kingdom Swallowing
 Research Group Conference
 London, United Kingdom

February 23, 2012
 Management of Barretts
 Oesophagus: Everything you need
 to know
 Cambridge, United Kingdom

February 24-27, 2012
 Canadian Digestive Diseases Week
 2012
 Montreal, Canada

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 International Conference on
 Nutrition and Growth 2012
 Paris, France

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 and Endoscopic Surgeons Annual
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 San Diego, CA 92121, United States

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 World Congress on
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 Omaha, NE 68197, United States

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 Mayo Clinic Gastroenterology and
 Hepatology
 Orlando, FL 32808, United States

March 26-27, 2012
 26th Annual New Treatments in
 Chronic Liver Disease
 San Diego, CA 92121, United States

March 30-April 2, 2012
 Mayo Clinic Gastroenterology and
 Hepatology
 San Antonio, TX 78249,
 United States

March 31-April 1, 2012
 27th Annual New Treatments in
 Chronic Liver Disease
 San Diego, CA 92121, United States

April 8-10, 2012
 9th International Symposium on
 Functional GI Disorders
 Milwaukee, WI 53202, United States

April 13-15, 2012
 Asian Oncology Summit 2012
 Singapore, Singapore

April 15-17, 2012
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 Colorectal Cancer Congress 2012
 Prague, Czech

April 18-20, 2012
 The International Liver Congress
 2012
 Barcelona, Spain

April 19-21, 2012
 Internal Medicine 2012
 New Orleans, LA 70166,
 United States

April 20-22, 2012
 Diffuse Small Bowel and Liver
 Diseases
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April 22-24, 2012
 EUROSON 2012 EFSUMB Annual

Meeting
 Madrid, Spain

April 28, 2012
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 Kiev, Ukraine

May 3-5, 2012
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 Amman, Jordan

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 Digestive Diseases Week
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May 17-21, 2012
 2012 ASCRS Annual Meeting-
 American Society of Colon and
 Rectal Surgeons
 Hollywood, FL 1300, United States

May 18-19, 2012
 Pancreas Club Meeting
 San Diego, CA 92101, United States

May 18-23, 2012
 SGNA: Society of Gastroenterology
 Nurses and Associates Annual
 Course
 Phoenix, AZ 85001, United States

May 19-22, 2012
 2012-Digestive Disease Week
 San Diego, CA 92121, United States

June 2-6, 2012
 American Society of Colon and
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 San Antonio, TX 78249,
 United States

June 18-21, 2012
 Pancreatic Cancer: Progress and
 Challenges
 Lake Tahoe, NV 89101, United States

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 PancreasFest 2012
 Pittsburgh, PA 15260, United States

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 Como, Italy

September 6-8, 2012
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 Prague, Czech

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 American College of
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 Dnepropetrovsk, Ukraine

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 San Francisco, CA 94101,
 United States

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 of Liver Diseases
 Boston, MA 02298, United States

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 Hollywood, FL 33028, United States

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In press

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

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

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

No author given

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

Volume with supplement

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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 biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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

Author(s) and editor(s)

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

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- 15 Morse SS. Factors in the emergence of infectious dis-

eases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

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- 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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Write as mean \pm SD or mean \pm SE.

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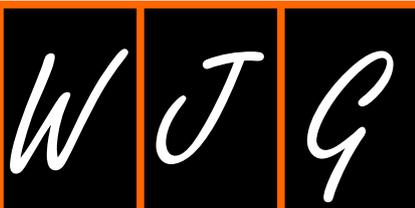
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ABOUT COVER Dr. Lian-Sheng Ma, the Editor-in-Chief from Baishideng Publishing Group Co., Limited, attended the Digestive Disease Week (DDW2012) in San Diego from May 19 to 22, 2012, during which he browsed 4272 posters. On May 28, Dr. Ma emailed to the potential authors to invite them to submit high-quality papers to the *World Journal of Gastroenterology (WJG)*. Until June 30, we had received a total of 109 papers. After peer-review, 54 papers were accepted and the rest 55 papers were rejected. The 28 accepted papers were published on August 28, 2012 free of charge.

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Pancreatico-biliary endoscopic ultrasound: A systematic review of the levels of evidence, performance and outcomes

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Abstract

Our aim was to record pancreaticobiliary endoscopic ultrasound (EUS) literature of the past 3 decades and evaluate its role based on a critical appraisal of published studies according to levels of evidence (LE). Original research articles (randomized controlled trials, prospective and retrospective studies), meta-analyses, reviews and surveys pertinent to gastrointestinal EUS were included. All articles published until September 2011 were retrieved from PubMed and classified according to specific disease entities, anatomical subdivisions and therapeutic applications of EUS. The North of England evidence-based guidelines were used to determine LE. A total of 1089 pertinent articles were reviewed. Published research focused primarily on solid pancreatic neoplasms, followed by disorders of the extrahepatic biliary tree, pancreatic cystic lesions, therapeutic-interventional EUS, chronic and acute pancreatitis. A uniform observation in all six categories of articles was the predominance of LE III studies followed by LE IV, II b, II a, I b and I a, in descending order. EUS remains the most accurate method for detecting small (< 3 cm) pancreatic tumors,

ampullary neoplasms and small (< 4 mm) bile duct stones, and the best test to define vascular invasion in pancreatic and peri-ampullary neoplasms. Detailed EUS imaging, along with biochemical and molecular cyst fluid analysis, improve the differentiation of pancreatic cysts and help predict their malignant potential. Early diagnosis of chronic pancreatitis appears feasible and reliable. Novel imaging techniques (contrast-enhanced EUS, elastography) seem promising for the evaluation of pancreatic cancer and autoimmune pancreatitis. Therapeutic applications currently involve pancreaticobiliary drainage and targeted fine needle injection-guided antitumor therapy. Despite the ongoing development of extra-corporeal imaging modalities, such as computed tomography, magnetic resonance imaging, and positron emission tomography, EUS still holds a leading role in the investigation of the pancreaticobiliary area. The major challenge of EUS evolution is its expanding therapeutic potential towards an effective and minimally invasive management of complex pancreaticobiliary disorders.

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Key words: Endoscopic ultrasound; Fine needle aspiration; Contrast harmonic endoscopic ultrasound; Pancreatic tumors; Pancreatic cysts; Acute pancreatitis; Chronic pancreatitis; Bile duct stones; Duct drainage

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INTRODUCTION

A unique property of endoscopic ultrasonography (EUS) is the detailed imaging of organs in close proximity to the digestive tract. This has been long documented in the investigation of the pancreaticobiliary area. Since its early days, EUS proved an accurate imaging modality for the pancreas and the extrahepatic biliary tree^[1-3]. The introduction of curvilinear-array echoendoscopes and the potential of tissue sampling by EUS-guided fine needle aspiration (EUS-FNA) highly upgraded the diagnostic value of EUS and enabled its evolution to an advanced interventional technique with a wide range of applications.

A prolific trend of pancreaticobiliary EUS-related studies was recently reported, rendering pancreas and extrahepatic biliary tree the major fields of modern EUS research^[4]. This review aimed to record the entire body of literature accumulated over the past 3 decades and to present a comprehensive perspective of current EUS indications, applications and test performance in the pancreatic and extrahepatic biliary tree pathology. Our objectives were then to perform a critical appraisal of published articles, based on the classification of studies according to levels of evidence (LE), in order to assess the scientific progress made in this field and to further inform policy regarding areas that need further research and improvements.

LITERATURE SEARCH

Based on our previous research on EUS literature of the period 1980-2010^[4,5], all articles relevant to pancreaticobiliary diseases were extracted. The PubMed search was extended up to September 2011, to retrieve all additional publications. Moreover, the bibliographies of reviewed articles were scrutinized to obtain any other reference that eluded the primary search.

Original research articles [randomized controlled trials (RCTs), prospective and retrospective studies], meta-analyses, reviews and surveys pertinent to gastrointestinal EUS were included. Studies enrolling up to 15 patients were categorized as case series. Editorials, commentaries, letters, case reports, non-English language articles, abstracts, and articles in which EUS did not represent the principal study matter were not considered for review.

In regard to data collection, priority was assigned to the study subject, design and methods, the type and year of publication and the number of patients enrolled. All articles were classified in six major categories based on specific disease entities, anatomical subdivisions and therapeutic applications of EUS: (1) solid pancreatic tumors; (2) cystic pancreatic lesions; (3) chronic pancreatitis; (4) acute pancreatitis; (5) extrahepatic biliary tree; and (6) therapeutic EUS. The content of each study was further analyzed to identify relevant clinical issues such as the diagnostic and staging accuracy of the technique, technical properties of EUS procedures and the role of EUS-FNA.

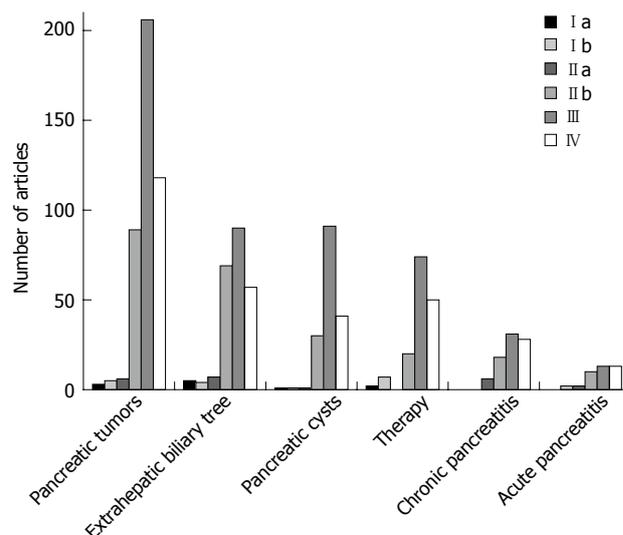


Figure 1 Distribution of papers in pancreaticobiliary endoscopic ultrasonography according to the levels of evidence.

Levels of evidence were stratified according to the North of England evidence-based guidelines^[6,7]. LE I a: Evidence obtained from meta-analysis of randomized controlled trials; LE I b: Evidence obtained from at least one RCT; LE II a: Evidence obtained from at least one well designed controlled study without randomization; LE II b: Evidence obtained from at least one other type of well-designed quasi-experimental study; LE III: Evidence obtained from well-designed non-experimental descriptive studies such as comparative studies, correlation studies, and case studies; LE IV: Evidence obtained from expert committee reports or opinions, or clinical experiences of respected authorities.

A total of 1089 pertinent articles were retrieved (Figure 1). A detailed classification of the studies, according to the main subject-matters and subclasses, and the corresponding LE, is presented in Table 1. Published research focused primarily on solid pancreatic neoplasms (418 studies overall), followed by disorders of the extrahepatic biliary tree (230 studies), pancreatic cystic lesions (165 studies), therapeutic-interventional EUS (153 studies), chronic and acute pancreatitis (83 and 40 studies respectively). A uniform observation in all six categories of articles was the predominance of LE III studies followed by LE IV, II b, II a, I b and I a, in descending order. Strong evidence trials (LE I b, II a) and meta-analyses (I a) were mainly recorded on pancreatic tumor diagnosis and staging, EUS-FNA of solid and cystic pancreatic lesions, common bile duct (CBD) stone detection and on the role of EUS-guided celiac plexus neurolysis (CPN). Novel therapeutic applications, like EUS-guided pancreaticobiliary drainage and pancreatic tumor therapy, are recently being increasingly addressed in well-designed studies (LE II b, III), but data are still limited reflecting lack of maturation of these techniques. Due to the abundance of existing data, a focused description of well-evidenced issues is highlighted below, in a point-by-point form.

Table 1 Levels of evidence per subject: Pancreatic disorders, extrahepatic biliary tree, endoscopic ultrasonography-guided therapy

	I a	I b	II a	II b	III	IV	Total
Solid pancreatic tumors							
Diagnosis, differential diagnosis	1	0	1	10	34	45	86
EUS - FNA, TCB, echobrush	0	4	1	28	48	11	92
Staging	2	0	0	17	30	18	67
Neuroendocrine tumors	0	0	0	4	28	15	47
Molecular markers	0	0	1	5	16	3	25
Screening	0	0	1	5	6	7	19
Elastography, CH-EUS	0	0	1	13	7	4	25
Other	0	1	1	7	37	11	57
Total	3	5	6	89	206	115	418
Pancreatic cysts							
Diagnosis, differential diagnosis	0	0	0	2	26	31	59
EUS-FNA, TCB, brushing	1	0	1	10	17	2	31
IPMN	0	1	0	5	27	6	39
Molecular markers	0	0	0	7	3	0	10
EUS follow-up, clinical outcome	0	0	0	0	5	0	5
Other	0	0	0	6	13	2	21
Total	1	1	1	30	91	41	165
Chronic pancreatitis							
Diagnosis	0	0	6	14	20	21	61
Autoimmune pancreatitis	0	0	0	2	7	2	11
Other	0	0	0	2	4	5	11
Total	0	0	6	18	31	28	83
Acute pancreatitis							
Diagnosis	0	0	1	4	7	10	22
CBD stones	0	0	0	4	1	1	6
Other	0	2	1	2	5	2	12
Total	0	2	2	10	13	13	40
Extrahepatic biliary tree							
Diagnosis (in general)	1	1	0	12	8	9	29
CBD stones	3	3	7	21	6	16	56
Cholangio-Ca	0	0	0	3	9	11	23
Gallbladder	0	0	0	6	18	4	28
IDUS	0	0	0	12	11	7	30
Periampullary neoplasms	0	0	0	7	19	5	31
Other	1	0	0	8	19	5	33
Total	5	4	7	69	90	57	230
EUS-guided therapy							
Fluid collection drainage, necrosectomy	0	2	0	8	31	14	55
Biliary duct drainage	0	0	0	2	23	15	40
EUS-CPN, CPB	2	3	0	4	4	11	24
Pancreatic tumor therapy	0	1	0	6	9	6	22
Pancreatic duct drainage	0	0	0	0	4	1	5
Other	0	1	0	0	3	3	7
Total	2	7	0	20	74	50	153

EUS-FNA: Endoscopic ultrasound-fine needle aspiration; TCB: Trucut biopsy; CH-EUS: Contrast harmonic EUS; IPMN: Intraductal papillary mucinous neoplasm; CBD: Common bile duct; IDUS: Intraductal ultrasound; CPN: Celiac plexus neurolysis; CPB: Celiac plexus block.

SOLID PANCREATIC TUMORS

Diagnosis

The only meta-analysis available, comparing the test performance of different diagnostic modalities, reports a sensitivity of combined positron emission tomography/computerized tomography (PET/CT) (90.1%) higher than PET (88.4%) and EUS (81.2%) alone, but a specificity of EUS (93.2%) higher than PET (83.1%) and PET/CT (80.1%) in diagnosing primary pancreatic

carcinoma^[8] (I a).

In a systematic review of the period 1986-2004, EUS was found more sensitive (range: 93%-100%) than CT (50%-89%), especially for the detection of pancreatic tumors smaller than 3 cm^[9] (I a).

Early comparative studies indicate that the sensitivity and specificity of EUS (94%-99% and 100%, respectively) is higher than transabdominal ultrasound (US) (67% and 40%), CT (69%-77% and 53%-64%) and magnetic resonance imaging (MRI) (83% and 100%), for demonstrating the presence of a pancreatic neoplasm. This is even more obvious in small pancreatic tumors of 3 cm and less. The sensitivity of detecting tumors less than 3 cm was 93% for EUS, 53% for CT, and 67% for MRI imaging. However, as with other imaging procedures, EUS was not able to differentiate reliably malignant from inflammatory pancreatic masses (accuracy 76% for malignancy and 46% for focal inflammation)^[10,11] (II b).

The presence of a dilated pancreatic duct is related to a 65% prevalence of malignancy, compared to a prevalence of 17% in its absence^[12] (II a).

Data from retrospective studies demonstrates a negative predictive value (NPV) for EUS as high as 100%, suggesting that a normal EUS of the pancreas in the setting of subtle radiologic findings, serologic abnormalities, and/or nonspecific symptoms definitively rules out the presence of pancreatic cancer^[13,14] (III).

Molecular markers

Broad panel microsatellite loss and K-ras point mutation analysis can reliably be performed on EUS-FNA samples from pancreatic masses, improving the diagnostic accuracy and differentiation between malignant and benign pancreatic masses^[15] (II a). When K-ras mutation analysis is combined with cytopathology, sensitivity, specificity, positive predictive value (PPV), NPV and overall accuracy reach 88%, 100%, 100%, 63% and 90%, respectively^[16] (II b).

Mucin expression pattern of EUS-FNA aspirates could serve as a potential biological marker for malignant lesions^[17] (II b), and combination of routine cytology with positive fluorescence *in situ* hybridization and K-ras analyses may help the discrimination of atypical FNA samples to benign and malignant^[18] (III).

Mutation status of K-ras, p53 and allelic losses at 9p and 18q are not prognostic markers in patients with pancreatic cancer. None of these markers was identified as an independent factor of survival prognosis^[19] (II b).

Staging

Although EUS is the best test to define vascular invasion in pancreatic and peri-ampullary cancers, the specificity (90%) is high but the sensitivity (73%) is not as high as previously suggested^[20] (I a).

EUS is superior to angiography in the preoperative assessment of vascular involvement for patients with pancreatic carcinoma (sensitivity 86% *vs* 21%, respectively; specificity and accuracy 71% and 81% *vs* 71% and

38%, respectively)^[21] (II b).

No decisive comparative data between EUS and MRI exists. The most recent report shows that EUS and MRI have marginal correlation for staging, especially in the case of advanced tumors. Therefore, both tests should be performed for accurate staging^[22] (II b).

Comparison of EUS vs CT

Helical CT is more accurate in assessing the extent of primary tumor (73%), locoregional extension, vascular invasion, distant metastases, tumor node metastasis stage and tumor resectability, whereas EUS is more accurate in assessing tumor size and lymph node involvement^[23] (II b).

For portal vein and superior mesenteric vein invasion, multislice CT (MSCT) seems superior to EUS (sensitivity 88% *vs* 50%, respectively and specificity 92% *vs* 83%). For resectability, there is no significant difference and agreement is good among the two techniques. Therefore MSCT is the imaging method of choice and routine EUS should be reserved for tumors with borderline resectability on MSCT^[24] (II b).

Compared with MSCT, EUS is superior for tumor detection and staging but similar for nodal staging and resectability of preoperatively suspected non-metastatic pancreatic cancer^[25] (II b).

EUS-FNA

The sensitivity, specificity, PPV and NPV of EUS-FNA for solid pancreatic masses reach 95%, 100%, 100% and 85%, respectively. Patients with suspicious and atypical EUS-FNA aspirates deserve further clinical evaluation^[26,27] (III).

In patients with negative CT-guided biopsies, EUS-FNA yields 90% sensitivity for malignancy, 50% specificity and 84% accuracy. Corresponding values for EUS-FNA in patients with negative endoscopic retrograde cholangiopancreatography (ERCP) tissue sampling are as high as 94%, 67% and 92%, respectively^[28] (II b). A more recent RCT comparing CT/US-FNA and EUS-FNA concludes that EUS-FNA is numerically (though not quite statistically) superior to CT/US-FNA for the diagnosis of pancreatic malignancy^[29] (I b).

A lower sensitivity for EUS-FNA is observed in patients with chronic pancreatitis (CP) than in those without CP (73.9% *vs* 91.3%). While patients with CP had a higher NPV (88.9% *vs* 45.5%), no significant differences were observed for specificity (100% *vs* 93.8%), PPV (100% *vs* 99.5%), and accuracy (91.5% *vs* 91.4%) between those with and without CP^[30] (II b).

False positive EUS-FNA cytology was recently recorded in a large cohort trial, i.e., 1.1% when only “positive” cytology findings were interpreted as malignant and 3.8% when both suspicious and positive cytology findings were interpreted as malignant. False positive cases occurred primarily as a result of cytological misinterpretation in the setting of CP^[31].

No statistically significant differences exist in the diagnostic yield of EUS-FNA between 22G *vs* 25G and 22G

vs 19G needles, nor in the handling of different commercially available needle assemblies^[32,33] (I b).

EUS-FNA sampling with suction of solid masses increases the number of pathology slides, the sensitivity and negative predictive value, without increasing the overall bloodiness of samples^[34] (I b). Neither trained EUS performers nor cytotechnologists seem able to provide a reliable assessment of pancreatic FNA adequacy by using gross visual inspection of the specimen on a slide^[35]. On the other hand, rapid on-site cytopathology performed by an attending cytopathologist shows excellent agreement with the final cytological evaluation^[36] and may reduce the number of passes, ensure specimen adequacy and lower the number of inadequate samples^[37] (II b).

EUS-FNA of solid pancreatic masses is safe when performed by experienced endosonographers. The frequency of post EUS-FNA pancreatitis may be underestimated by retrospective analysis (0.64% when data was prospectively collected *vs* 0.26% in retrospective cohorts)^[38] (II b). A higher incidence of pancreatitis after pancreatic EUS-FNA (2%, with some more cases of silent hyperamylasemia) was recorded in a prospective controlled trial^[39] (II a).

Neuroendocrine tumors

EUS is reliable for the localization of pancreatic neuroendocrine tumors (NETs) (sensitivity, 82% and specificity, 95%) and highly accurate in estimating the actual size of these tumors (deviation within 2 mm between EUS and surgical pathology). Compared to angiography, EUS is significantly more sensitive for tumor localization (sensitivity, 82% *vs* 27%)^[40] (II b).

In patients with multiple endocrine neoplasia type 1 (MEN1) syndrome undergoing screening EUS, pancreatic NETs are identified in 17% of cases before the development of significant biochemical abnormalities^[41] (II b). The frequency of nonfunctioning pancreatic endocrine tumors is higher (55%) than previously thought and pancreatic EUS should be performed once MEN1 is diagnosed, to monitor disease progression^[42] (II b).

In patients with suspected pancreatic NETs undergoing EUS and MSCT, the sensitivity of EUS is greater than MSCT (92% *vs* 63%, respectively), particularly for insulinomas (84% *vs* 32%, respectively) and for lesions smaller than 2 cm. EUS may detect in up to 91% of cases missed by MSCT^[43] (II b).

Screening

A EUS-based strategy of screening for individuals at high risk for pancreatic cancer is feasible and safe. The incidence of clinically relevant findings at first screening is high, reaching 7%-10% for early asymptomatic cancer and 16% for premalignant intraductal papillary mucinous neoplasms (IPMN)-like lesions. Nevertheless, whether screening improves survival remains to be determined, as does the optimal screening interval with EUS. Moreover, without the ability to further quantify these patients' risk, the most effective strategy in clinical and economical

terms may be doing nothing^[44-46] (II a).

In familial pancreatic cancer a EUS/magnetic resonance cholangiopancreatography (MRCP) based screening program leads to the detection of potential precursor lesions of pancreatic cancer. However, the yield of an extensive screening program is low, especially since the tumorigenic value of low grade pancreatic intraepithelial neoplasia is not yet defined. Taking into account the enormous psychological stress for the tested individual and the high costs, a general pancreatic cancer screening in high-risk individuals is not justified^[47] (II b).

New imaging techniques

Detection of a hypoenhancing and inhomogeneous mass with contrast harmonic EUS (CH-EUS) accurately identifies patients with pancreatic adenocarcinoma. CH-EUS increases the detection of malignant lesions in difficult cases (patients with chronic pancreatitis or biliary stents) and helps to guide EUS-FNA. A hyper-enhancing pattern can be used to rule out adenocarcinoma^[48] (II b).

Hypoenhancement in CH-EUS yields higher sensitivity (89% *vs* 72%), lower specificity (88% *vs* 100%) and comparable accuracy (88.5% *vs* 86%) than EUS-FNA, for the diagnosis of pancreatic adenocarcinoma^[49] (II b).

Quantitative analysis of contrast-enhanced EUS seems to improve the efficacy of the technique. The diagnostic accuracy, based on contrast imaging pattern (84%) and time-intensity curves (TICs) (88%), was higher than that based on B-mode imaging (83%) and dynamic CT (81%). EUS in combination with TIC demonstrated increased sensitivity, specificity, and accuracy up to 96%, 93%, and 95%, respectively^[50] (III).

EUS elastography is useful for the differential diagnosis of solid pancreatic masses and allows an objective evaluation of tissue stiffness^[51] (II b). The operating characteristics of the technique for detecting malignancy are: sensitivity 93%, specificity 66%, PPV 92%, NPV 69% and overall accuracy 85%^[52,53] (II b).

The sensitivity, specificity, and accuracy of combined contrast-enhanced power Doppler and real-time sonoelastography in differentiating hypovascular hard masses suggestive of pancreatic carcinoma were 76%, 95% and 83%, respectively, with a PPV and NPV of 96% and 71%, respectively^[54] (II b).

PANCREATIC CYSTS

Diagnosis

Certain morphologic features have been used to discriminate specific types of pancreatic cysts. A cystic lesion with accompanying parenchymal changes, in the absence of intracystic septation or mural nodule, is compatible with a pseudocyst. Multiple microcysts (< 3 mm) within a cystic lesion and a honeycomb appearance are typical of serous cystadenomas, but macrocystic types can also be found. Mucinous cystadenomas usually present with septations of variable thickness, a visible wall, and peripheral calcifications in up to 15% of cases. A communication

between the cyst and the main pancreatic duct is strongly suggestive of IPMN^[55-57] (III).

There is no universally accepted morphologic parameter to reliably predict the malignancy of pancreatic cysts by EUS. The size of cysts is generally considered suspicious for malignancy above the diameter of 3 cm; moreover, a cyst growth rate of more than 2 mm/year has been proposed to indicate a higher risk of malignancy (5-year risk 45.5% *vs* 1.8% for cysts with lower growth rate). The presence and size of mural nodules within the cysts is also predictive of malignancy in IPMNs^[58-60] (III).

There is little more than chance interobserver agreement (IA) among experienced endosonographers for the diagnosis of neoplastic versus non-neoplastic (fair IA), specific type (moderately good IA for serous cystadenomas, but fair for other types of pancreatic cysts), and EUS features (IA ranged from slight to moderately good) of pancreatic cystic lesions. Accuracy rates of EUS for the diagnosis of neoplastic versus non-neoplastic lesions range from 40% to 93%^[61] (II b).

Interobserver agreement for the presence of mural nodules seems good among experts and fair in the semi-expert and novice groups. With respect to specific overall diagnosis, agreement is moderate in the expert group, poor among the semi-experts, and fair among novices^[62] (II b).

Preoperative assessment by intraductal ultrasound (IDUS) has an 85% diagnostic accuracy for tumor extension of IPMN compared with 50% for other imaging methods. Preoperative IDUS is useful in determining the type of surgery and the extent of resection, especially in main-duct IPMN^[63] (I b).

EUS-FNA

EUS-FNA based cytology has overall low sensitivity (63%) but good specificity (88%) in differentiating mucinous cystic lesions (MCLs) from non-mucinous lesions (NMCLs). Further research is required to improve the overall sensitivity of EUS-FNA-based cytology to diagnose MCLs^[64] (I a).

The decision to proceed with non-operative management should not be based on a negative or non-diagnostic FNA alone, as 67% of negative and 92% of non-diagnostic specimens may be associated with malignant or premalignant pathology at surgical pathology^[65] (II b).

Complications of EUS-FNA are encountered in 2.2% of patients overall, and comprise pancreatitis, abdominal pain, retroperitoneal bleed, infection and bradycardia. Type of cyst, size, presence of septations or mass, and same-day ERCP are not predictors of complications^[66] (III).

Two small studies suggest that cytology brushings are more likely to provide an adequate mucinous epithelium specimen than standard FNA and could aid the diagnosis of cystic pancreatic lesions in a selective group of patients^[67] (II a). EUS brushing increases cellular diagnosis of pancreatic cystic lesions compared to fluid analysis, mainly in mucinous lesions. However, its use is not rec-

ommended in patients under anticoagulation therapy even after withdrawal of anticoagulants, as a fatal complication has been reported^[68] (II b). Whether this approach is superior to cyst fluid analysis has not been substantiated by data. The cost of the combined needle and the brush should also be taken into consideration.

Molecular markers

The accuracy of carcinoembryonic antigen (CEA) (79%) is significantly higher compared to EUS morphology (51%) or cytology (59%). No combination of tests provides greater accuracy than CEA alone. Of tested markers, cyst fluid CEA is the most accurate test available for the diagnosis of mucinous cystic lesions of the pancreas^[69] (II b).

Malignant cysts may be differentiated from premalignant cysts on the basis of fluid CEA level, DNA quality, number of mutations and on the sequence of mutations acquired. Early k-ras mutation followed by allelic loss was the most predictive of a malignant cyst (sensitivity, 91%; specificity, 93%)^[70,71] (II b).

There is poor agreement between CEA and molecular analysis (DNA quantity, k-ras mutation, and 2 or more allelic imbalance mutations) for the classification of mucinous cysts. Based on the final pathologic diagnosis, CEA has a sensitivity of 82% compared with 77% for molecular analysis. When CEA and molecular analysis are combined, 100% sensitivity can be achieved^[72] (II b).

Follow-up, clinical outcome

Most branch duct-IPMN asymptomatic patients who have no mural nodules on EUS can be managed without surgery. However, careful attention should be paid to disease progression and the development of pancreatic cancer during follow-up^[73] (II b).

Ductal adenocarcinoma of the pancreas distinct from IPMN may develop in patients with branch duct IPMNs during follow-up. The 5-year rate of ductal carcinoma has been reported to reach 6.9%, with annual incidence of 1.1%. In the same series, cancer developed in IPMN in 3% of branch duct IPMNs during follow-up^[74] (II b).

There is considerable variation in size estimates of pancreatic cysts by different imaging modalities. Median size differences between studies are: between EUS and CT (i.e., absolute value of size determined by EUS minus size determined by CT): 4 mm (range: 0-25 mm), between EUS and MRI: 4 mm (range: 0-17 mm), between CT and MRI: 3 mm (range: 2-20 mm). Median size differences for surgical pathology specimens compared to imaging estimates were as follows: EUS and pathology: 9.5 mm (range: 0-20 mm), CT and pathology: 5 mm (range: 0-21 mm), MRI and pathology: 5.5 mm (range: 2-44 mm). Clinicians should take into account these variations when making management decisions and prefer a single modality during follow-up^[75] (II b).

CHRONIC PANCREATITIS

Diagnosis

Various EUS features have been found to predict changes

of chronic pancreatitis (CP). Parenchymal features comprise calcifications with shadowing, echogenic foci without shadowing, focal regions of reduced echogenicity (lobularity), hyperechoic strands and the presence of cysts within the gland. Ductal features include main pancreatic duct (MPD) calculi, dilation or irregular contour of the MPD, increased thickness/echogenicity of the MPD wall, and side branch dilation. EUS is highly sensitive and specific (> 85%) depending on the number of criteria present. CP is likely (PPV > 85%) when more than 2 criteria (for CP in general) and more than 6 criteria (for moderate to severe CP) are present. Moderate to severe CP is unlikely (NPV > 85%) when fewer than 3 criteria are present^[76,77] (II a).

An attempt to quantify the individual weight of widely accepted EUS features classifies them in major (hyperechoic foci with shadowing, MPD calculi and parenchymal lobularity with honeycombing) and minor criteria (cysts, dilated MPD or side branches, irregular MPD contour, hyperechoic MPD wall, strands, non-shadowing hyperechoic foci and non-contiguous lobularity). The diagnosis of CP is labeled as “most consistent with”, “suggestive of”, “indeterminate for” and “normal” depending on the number of major and minor criteria visualized^[78] (IV).

EUS is as sensitive and effective as ERCP in the detection of CP, particularly when only mild disease is present. However, EUS findings have limited specificity (60%), particularly in patients with mild disease^[79] (II b).

Reports on the concordance of EUS and endoscopic pancreatic function test are inconclusive for patients with suspected early CP, but the combination of the two techniques can add complimentary information and reach a sensitivity of 100%^[80,81] (II b).

EUS may be more sensitive but equally specific, compared with MRCP, in diagnosing CP. The combination of EUS and MRCP has a sensitivity of 98% for either EUS or MRCP and a specificity of 100% for both EUS and MRCP^[82] (II b).

There is good intra-observer agreement in the interpretation of EUS features of CP. The intra-observer agreement seems better than the published inter-observer agreement for EUS features of CP and better than the published intra-observer agreement for ERCP^[83] (II b).

EUS-FNA

EUS-FNA with cytology is safe and improves the negative predictive value (from 75% to 100%) of EUS. Negative EUS-FNA findings rule out CP, but cytology alone does not improve the specificity of EUS findings (from 60% to 67%). Further improvements in tissue sampling and analysis are necessary to support routine use of FNA in patients with CP^[78] (II b).

Transgastric EUS-trucut biopsy of suspected non-focal CP infrequently demonstrates histologic CP in clinically suspected disease. Because of potential complications (acute pancreatitis) and limited diagnostic yield, this technique is not currently recommended for evaluation of these patients^[84] (II b).

Autoimmune pancreatitis

A diffusely hypoechoic, enlarged pancreas, together with chronic inflammatory cells in aspirated cytological specimens, is supportive of the diagnosis of autoimmune pancreatitis (AIP)^[85]. The presence of stromal fragments of high cellularity with a lymphoid infiltrate, in conjunction with clinical and radiology findings, could potentially establish the diagnosis of AIP and exclude carcinoma, thus preventing pancreatic resection^[86] (II b).

Diffuse or focal hypoechoic areas, diffuse or focal enlargement of pancreas, bile duct wall thickening, lymphadenopathy, and peri-pancreatic hypoechoic margins are significantly more frequent in AIP than in pancreatic cancer. All these features may resolve after steroid treatment^[87] (III).

In AIP patients, CH-EUS demonstrates a unique vascularization pattern which enables discrimination between AIP and lesions caused by pancreatic cancer. Lesions caused by AIP and the surrounding pancreas typically demonstrate hyper-vascularization, whereas lesions caused by pancreatic cancer present hypo-vascularized^[88] (III).

EUS elastography shows a typical and unique finding of homogenous stiffness of the whole organ, and this distinguishes AIP from the circumscribed mass lesion in ductal adenocarcinoma^[89] (III).

ACUTE PANCREATITIS

In selected patients with acute pancreatitis (AP), EUS can safely replace diagnostic ERCP and select patients eligible for therapeutic ERCP with a higher success rate^[90]. EUS may prevent ERCP in 71% of patients with AP and offers a complication-free alternative, whereas sphincterotomy is associated with bleeding in up to 22% of cases^[91] (I b).

EUS seems superior to MRCP (51% *vs* 20%) in the evaluation of AP. Cholelithiasis and biliary sludge (24%) are the most frequent EUS diagnoses, and pancreas divisum (8%) is the most frequent MRCP diagnosis. Only in 6% of cases does MRCP identify additional features in patients etiologically undiagnosed using EUS. The EUS yield is lower in patients with a previous cholecystectomy (11% *vs* 60%)^[92] (II a).

In univariate analysis, the presence of peri-pancreatic edema, parenchymal inhomogeneity, CBD dilation and ascites is associated with severe pancreatitis. In multivariate analysis, only the presence of peri-pancreatic edema in EUS correlates with the severity of AP according to the Atlanta criteria. EUS may be a new useful imaging modality for the prediction of severity of AP with prognostic significance in the early phase of AP^[93] (III).

EXTRAHEPATIC BILIARY TREE

CBD stones

EUS has excellent overall sensitivity (94%) and specificity (95%) for the diagnosis of choledocholithiasis^[94]. EUS performance is superior in detecting CBD stones com-

pared to CBD malignancy (sensitivity 78%, specificity 84%)^[95] (I a).

By performing EUS first, ERCP may be safely avoided in two-thirds of patients with CBD stones. A EUS-based selection of patients for therapeutic ERCP significantly reduces the complication rate^[96] (I a).

For patients with intermediate probability of CBD stones, EUS is more sensitive than ERCP in detecting stones smaller than 4 mm (90% *vs* 23%). A management strategy based on EUS (with selective ERCP in patients with confirmed stones) is safer and not associated with an excess of endoscopic procedures (can spare ERCP in up to 75% of patients) compared with a strategy based on ERCP alone^[97-99] (I b).

With respect to sensitivity, specificity and accuracy, there is no statistically significant difference between EUS and MRCP for the detection of choledocholithiasis^[100,101] (I a). However, the sensitivity of MRCP seems to diminish in the setting of small (< 6 mm) CBD stones, while EUS remains highly sensitive even for small stones^[102] (I a).

The diagnostic accuracy of catheter-probe extra-ductal ultrasonography is comparable to that of conventional EUS for the detection of CBD stones^[103] (II a).

Approximately half (57%) of patients with echogenic CBD material visible on IDUS actually have biliary crystals on bile microscopy. The size of the echogenic material is the only significant factor associated with bile microscopy positivity with an optimal size value of 1.4 mm (sensitivity and specificity 71% and 75%, respectively)^[104] (II b).

CBD neoplasms

EUS is significantly more sensitive than US or CT (100% *vs* 80% and 83%, respectively) in making a positive diagnosis of obstruction. EUS is also significantly more accurate than US and CT (97% *vs* 49% and 66%) in diagnosing the cause of the obstruction and in the loco-regional staging of malignant obstructions (75% *vs* 38% and 62%)^[105] (II b).

A comparison between ERCP and EUS for tissue diagnosis of biliary strictures depicts higher sensitivity for ERCP-based techniques in the subgroup biliary tumors (ERCP 75% *vs* EUS 25%), whereas EUS-guided biopsy is superior for pancreatic masses (EUS 60% *vs* ERCP 38%)^[106] (II B).

EUS-FNA is valuable for tissue diagnosis of undetermined hilar strictures. It is technically feasible without significant risks, when other diagnostic tests are inconclusive and is able to change preplanned management in about half of the patients. Accuracy, sensitivity, and specificity are 91%, 89% and 100%, respectively^[107] (II b).

EUS and EUS-FNA are sensitive (overall 73%) for the diagnosis of cholangiocarcinoma and very specific (97%) in predicting unresectability. The sensitivity of EUS-FNA is significantly higher in distal (81%) than in proximal (59%) lesions^[108] (II b).

IDUS is a valuable adjunct to ERCP-guided tissue sampling that increases the ability to distinguish malig-

nant from benign strictures, but cannot assess the lymph node spread of malignant strictures^[109] (II b). When used in conjunction, IDUS increases the accuracy of ERCP for the characterization of biliary strictures from 58% to 90%^[110] (II b).

Ampullary lesions

Early observational studies reported high detection rates (96%-100%) and staging accuracy of EUS with respect to duodenal or CBD wall involvement, invasion of the pancreas and portal vein, and spread to regional lymph nodes. Accuracy rates for cancer extent was 78% for ampullary carcinoma and 81% for CBD carcinoma, when compared with surgical findings^[111,112] (III).

In patients with biliary symptoms, EUS can reliably visualize and characterize a malignant lesion as the first diagnostic tool (detection rate 82%, overall sensitivity of 92% and specificity of 75%) and may be considered the basis for subsequent diagnostic steps^[113] (III).

EUS is more accurate than CT and MRI in local tumor staging of ampullary neoplasms (EUS 78%, CT 24%, MRI 46%). No significant difference in nodal staging exists between the three imaging modalities (EUS 68%, CT 59%, MRI 77%). EUS T-staging accuracy decreases from 84% to 72% in the presence of a trans-papillary endo-biliary stent. This is most prominent in T2/T3 carcinomas and may result in underestimating the need for a Whipple resection because of tumor understaging^[114] (II b).

EUS is more sensitive and specific than CT for tumor and nodal staging of ampullary cancer, and the association of CT to EUS findings does not improve the final test performance characteristics of EUS^[115] (II b).

THERAPEUTIC EUS

EUS-guided celiac plexus neurolysis and block

Alcohol-based EUS-guided celiac plexus neurolysis (EUS-CPN) is a safe and effective technique for patients with pancreatic cancer and pain intractable to narcotic analgesics. The pooled proportion of patients that experience pain relief is 80%. On the other hand, in patients with pain due to CP the outcomes of EUS-CPN are inferior (59% clinical benefit) and better techniques or injected materials are needed to improve the response^[116] (I a).

Steroid-based EUS-guided celiac plexus block (EUS-CPB), although superior to the percutaneous fluoroscopy-guided approach^[117], proves moderately adequate in managing abdominal pain in patients with chronic pancreatitis, and warrants improvement in patient selection and refinement of the technique^[118] (I a).

Pancreatic collection drainage and necrosectomy

Technical success is significantly greater for EUS-guided pancreatic pseudocyst drainage compared to the “blind” endoscopic approach, even after adjusting for luminal compression-bulging. Short-term clinical outcomes seem to favor the EUS-guided technique, yet long-term results

are similar. EUS should be considered the first-line treatment modality for endoscopic drainage of non-bulging lesions^[119,120] (I b).

EUS-guided endoscopic trans-gastric necrosectomy of infected necrosis in acute pancreatitis appears to be a feasible and relatively safe treatment option in patients who are not critically ill. Emergency surgery as the initial treatment can be avoided in the majority of cases. Complications may include minor bleeding after balloon dilation and later development of recurrent pseudocysts because of the “disconnected-duct syndrome”^[121,122] (III).

Biliary and pancreatic duct drainage

The overall success rate of EUS-guided cholangiography *via* an intrahepatic (trans-papillary, trans-gastric) or extrahepatic (trans-papillary, trans-enteric) route approaches 85%-90%, with complication rates in the range of 10%-16%. Based on intention-to-treat analysis, similar success rates of over 70% can be achieved by both types of approach^[123,124] (III).

EUS-guided biliary drainage with one-step placement of a fully-covered self-expanding metal stent seems to be a feasible, safe, and effective alternative to percutaneous trans-hepatic biliary drainage in cases of malignant biliary obstruction when ERCP is unsuccessful^[125] (II b).

EUS-guided pancreaticogastrostomy or pancreaticobulbostomy appears to be an effective (technical success 90%, clinical success 70%) and relatively safe (major complications in 5.5%, including bleeding, severe AP and perigastric collection) procedure. It is indicated for the management of pain secondary to pancreatic ductal hypertension due to chronic pancreatitis or post-Whipple resection anastomotic strictures, in patients with inaccessible main pancreatic ducts by a trans-papillary route. Nevertheless, stent dysfunction may occur in up to 55% of patients after mid- to long-term follow-up, the procedure is technically demanding and careful pre-therapeutic evaluation is required^[126,127] (III).

Pancreatic cyst ablation

EUS-guided ethanol lavage results in a greater decrease in pancreatic cyst size (43%) compared with saline solution lavage (11%), with a similar safety profile. Overall CT-defined complete pancreatic cyst ablation reaches 33%^[128] (I b).

EUS-guided ethanol injection and lavage, followed by injection of paclitaxel, appears to be a safe method for treating pancreatic cysts; 62% of patients may have complete resolution. Small cyst volume predicts complete resolution^[129] (II b).

Pancreatic cancer therapy

High technical and clinical success rates (90%) have been reported for EUS-guided fiducial placement in patients with locally advanced and recurrent pancreatic cancer. The complication rate (mild pancreatitis in 2%), as well as the rate of migration from the initial injection site, seem low^[130,131] (II b).

EUS-guided brachytherapy by implantation of radioactive seeds into unresectable pancreatic tumors could yield a “partial” objective tumor response in 27% of patients, “minimal” response in 20% patients, and “stable disease” in 33% of patients, during a median follow-up period of 10.6 mo. Up to 30% of patients may experience clinical benefit, mostly due to reduction in pain, but this lasts for a limited time. Local complications (pancreatitis and pseudocyst formation) occur in 20% of patients^[132]. Worse clinical outcomes (partial remission in 13.6% and stable disease in 45.5%) were reported in more recent series^[133] (II b).

EUS-guided injection of the oncolytic virus ON-YX-015 into unresectable pancreatic carcinomas by the trans-gastric route with prophylactic antibiotics is feasible and generally well tolerated either alone or in combination with gemcitabine^[134] (II b).

A single administration of cytoimplant (allogeneic mixed lymphocyte culture) immunotherapy by EUS-guided fine needle injection appears to be feasible and not associated with substantial toxicity^[135] (III).

CONCLUSION

Despite the ongoing development of other cross-sectional imaging modalities, namely MSCT and MRI, EUS still holds a leading role in the investigation of pancreaticobiliary disorders. EUS remains the most accurate method for the detection of small (< 3 cm) pancreatic lesions, including NETs and ampullary neoplasms, and the best test to define vascular invasion in pancreatic and periampullary tumors. The ability of tissue acquisition by EUS-FNA is pivotal for clinical decision-making in patients with pancreatic cancer; it demonstrates excellent sensitivity and specificity and appears to be safe when performed by experienced endosonographers. The adjunct of molecular analysis of aspirates, particularly K-ras point mutation analysis, could guide the differential diagnosis of solid pancreatic lesions, once incorporated to routine clinical practice. Furthermore, early diagnosis of asymptomatic tumors in screening programs for familial pancreatic cancer and MEN1 syndrome seems feasible and reliable, but data regarding the management of these patients are still inconclusive. Novel imaging techniques such as CH-EUS and EUS-elastography are promising for the detection of pancreatic cancer, which exhibits a characteristic imaging pattern; quantitative analysis enables an objective evaluation of lesions and will potentially increase intra- and inter-observer agreement, but prospective comparative studies are still pending.

EUS provides detailed imaging of pancreatic cysts and helps their differentiation according to subtle structural features. Although no single criterion has been established to predict malignancy, the size of lesions, the presence and size of mural nodules and the cyst growth rate seem useful in estimating the risk of malignancy. This has been well documented in the case of branch-duct type IPMNs, where long term EUS follow-up has

provided a deep insight to their natural history. The malignant potential can be further evaluated on the basis of biochemical (amylase, CEA) and molecular cyst fluid analysis (K-ras mutation). These markers also seem to compensate for the generally low yield of EUS-FNA based cytology, but refinement of the technique and the adjunct of EUS-FNA brushing may increase the cellularity of aspirates.

Early diagnosis of CP is challenging. EUS is equally sensitive, yet much safer than ERCP, and more sensitive than MRCP in detecting the subtle changes of mild disease. EUS-FNA seems to increase the specificity of the technique, but further improvement in tissue sampling and analysis is necessary to support the routine use of FNA for patients with CP. EUS could also add valuable information in cases of suspected AIP, by demonstrating characteristic morphologic features and a typical pattern on elastography and CH-EUS, compatible with the disease.

EUS is the most sensitive and specific modality for the diagnosis of small CBD stones (< 4 mm). This fact has a critical impact on the management of patients with biliary symptoms or AP; a strategy based on performing EUS as the primary diagnostic modality can prevent futile diagnostic ERCPs in more than two thirds of patients and select those who will benefit from a therapeutic ERCP with higher success and lower complication rates.

Probably the greatest challenge of EUS evolution is its expanding therapeutic potential. EUS-CPN for patients with pancreatic cancer and EUS-guided pseudocyst drainage are now established alternatives to more invasive and risky surgical or radiologic interventions, which are routinely performed by experienced endosonographers. EUS-guided pancreaticobiliary drainage and targeted fine needle injection therapy for pancreatic cysts and solid tumors are novel therapeutic applications with encouraging preliminary outcomes that await to be confirmed by further studies.

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Gene expression profiling and endothelin in acute experimental pancreatitis

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Abstract

AIM: To analyze gene expression profiles in an experimental pancreatitis and provide functional reversal of hypersensitivity with candidate gene endothelin-1 antagonists.

METHODS: Dibutyltin dichloride (DBTC) is a chemical used as a polyvinyl carbonate stabilizer/catalyzer, biocide in agriculture, antifouling agent in paint and

fabric. DBTC induces an acute pancreatitis flare through generation of reactive oxygen species. Lewis-inbred rats received a single i.v. injection with either DBTC or vehicle. Spinal cord and dorsal root ganglia (DRG) were taken at the peak of inflammation and processed for transcriptional profiling with a cDNA microarray biased for rat brain-specific genes. In a second study, groups of animals with DBTC-induced pancreatitis were treated with endothelin (ET) receptor antagonists [ET-A (BQ123) and ET-B BQ788]. Spontaneous pain related mechanical and thermal hypersensitivity were measured. Immunohistochemical analysis was performed using anti-ET-A and ET-B antibodies on sections from pancreatic tissues and DRG of the T10-12 spinal segments.

RESULTS: Animals developed acute pancreatic inflammation persisting 7-10 d as confirmed by pathological studies (edema in parenchyma, loss of pancreatic architecture and islets, infiltration of inflammatory cells, neutrophil and mononuclear cells, degeneration, vacuolization and necrosis of acinar cells) and the pain-related behaviors (cutaneous secondary mechanical and thermal hypersensitivity). Gene expression profile was different in the spinal cord from animals with pancreatitis compared to the vehicle control group. Over 260 up-regulated and 60 down-regulated unique genes could be classified into 8 functional gene families: circulatory/acute phase/immunomodulatory; extracellular matrix; structural; channel/receptor/transporter; signaling transduction; transcription/translation-related; anti-oxidants/chaperones/heat shock; pancreatic and other enzymes. ET-1 was among the 52 candidate genes up-regulated greater than 2-fold in animals with pancreatic inflammation and visceral pain-related behavior. Treatments with the ET-A (BQ123) and ET-B (BQ-788) antagonists revealed significant protection against inflammatory pain related mechanical and thermal hypersensitivity behaviors in animals with pancreatitis ($P < 0.05$). Open field spontaneous behavioral activity (at baseline, day 6 and 30 min after drug treatments

(BQ123, BQ788) showed overall stable activity levels indicating that the drugs produced no undesirable effects on normal exploratory behaviors, except for a trend toward reduction of the active time and increase in resting time at the highest dose (300 $\mu\text{mol/L}$). Immunocytochemical localization revealed that expression of ET-A and ET-B receptors increased in DRG from animals with pancreatitis. Endothelin receptor localization was combined in dual staining with neuronal marker NeuN, and glia marker, glial fibrillary acidic protein. ET-A was expressed in the cell bodies and occasional nuclei of DRG neurons in naïve animals. However, phenotypic expression of ET-A receptor was greatly increased in neurons of all sizes in animals with pancreatitis. Similarly, ET-B receptor was localized in neurons and in the satellite glia, as well as in the Schwann cell glial myelin sheaths surrounding the axons passing through the DRG.

CONCLUSION: Endothelin-receptor antagonists protect against inflammatory pain responses without interfering with normal exploratory behaviors. Candidate genes can serve as future biomarkers for diagnosis and/or targeted gene therapy.

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Key words: Gene expression; Endothelin receptors; Pancreatitis; Pain; Dibutyltin dichloride; Hypersensitivity; Hyperalgesia

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INTRODUCTION

Abdominal pain ranging from mild to severe pain is the chief symptom of patients with pancreatic disorders. Neural innervation of the pancreas is important in the initiation and maintenance of inflammation. Activation of pancreatic sensory neurons causes release of neurotransmitters in the spinal cord and neurogenic activation signals in the pancreas itself producing plasma extravasation and neutrophil infiltration. Abundant evidence has suggested that endothelins (ETs) may play a role in the transmission of nociceptive information in animals^[1-5] and in humans^[1,6,7]. ET1 has been shown to induce abdominal constrictions in mice, incapacitation in dogs, and intradermal injection into humans caused wheal and flare and itching responses^[1]. Additionally, duc-

tal fibrosis induced constriction is enhanced through endothelin autocrine loops caused by stellate cell activation. Therefore, endothelin cascade is implicated as a major contributing factor in pancreatic pain in both pancreatitis and pancreatic cancer^[8-11].

Multifunctional ETs^[1-3] comprise a family of peptides of 21 amino acids which interact with their specific receptor subtypes that are involved in regulation of blood flow, cell proliferation, muscle contraction or relaxation, secretion and ion transport^[12]. The ETs are expressed by a variety of cell types including endothelial cells, macrophages, astrocytes and neurons. Previous data also indicate that ETs can have direct effects on the peripheral sensory nervous system (including neurons and glial cells and may directly be involved in signaling nociceptive events in peripheral tissues)^[3]. In inflammatory states the levels of ETs are increased^[1]. In mammals, ETs produce their biological effects *via* activation of two receptor subtypes, the endothelin-A (ET-A) receptor and the endothelin-B (ET-B) receptor^[3]. ET-A and ET-B receptors are expressed in different cell types in peripheral nerve and sensory ganglia and are involved in pain transmission^[3]. ET receptor blockade in severe acute pancreatitis leads to systemic enhancement of microcirculation, stabilization of capillary permeability, and improved survival in rats^[13,14]. ET-A receptors are mainly localized in dorsal root ganglia (DRG), enteric motor neurons^[15], and brain blood vessels. In peripheral nerves, small unmyelinated fibers, are reported to express both ET-A and ET-B receptors. ET-B receptors are expressed primarily in the glia, epithelia, ependymal, in addition to neuronal cells. ET-A receptor action produces vasoconstriction and they are involved in hypoxia mediated neuropathic pain, while action of ET-B receptors results in vasodilatation that has been implicated in inflammatory pain and nociception^[16]. ET-1 is a potent vasoconstrictor peptide increased in inflammatory states and known to induce pain in animals through its actions on endothelin receptors^[3]. In addition, ET-1 increases capillary permeability changes and plays a role in aggravating the development of acute hemorrhagic pancreatitis through its action on the pancreatic microcirculation^[17]. ET-1 is recognized as the key player in the immune-mediated hypernociception and inflammatory diseases as depicted in autoimmune pancreatitis^[18]. Indeed, ET-1 is considered as the main cause of pancreas microcirculation disturbance during acute pancreatitis. ET-1 increases capillary permeability changes. Nitric oxide (NO) is the mediator of the cascade of inflammatory responses^[19]. Normally, ET-1 and NO together are in a dynamic balance regulating the elasticity of blood vessels, and maintaining the peripheral resistance of vessels and local vasomotor function. Once this balance is disrupted, it leads to vasomotor dysfunction and microcirculation disturbances^[20].

Objectives of the present study were to analyze the gene expression profile in the thoracic spinal cord and DRG to elucidate whether pancreatitis can induce gene regulation in these tissues. One of the genes upregulated

by dibutyltin dichloride (DBTC)-induced pancreatitis was endothelin-1. As a test of phenotypic expression and functional significance, the present study examined localization of endothelin-A and B receptors in the pancreas and the DRG, as well as determined the effects of pharmacological agents known to be ET-A or ET-B receptor antagonists. Thus, we used the chemically-induced pancreatitis model by a single *iv* injection of the polyvinyl carbonate stabilizer/catalyzer, biocide in agriculture, anti-fouling agent in paint and fabric of DBTC that results in acute pancreatitis through reactive oxygen species. Pancreatitis induced by DBTC persists through seven days allowing a more clinically relevant study of the ongoing processes evoked by activation of viscer-nociceptive pathways that are likely maintaining the central sensitization state characteristic of experimental and clinical pancreatitis^[21,22]. We hypothesized that the pancreatitis-induced hypersensitivity is mediated by the endothelin imbalance and its action on related receptors. Furthermore was tested the notion that pain related responses generated by endothelins could be pharmacologically reduced using specific endothelin receptor antagonists. To reduce the activation in viscer-nociceptive pathways, we assessed the ability of ET-A (BQ123) and ET-B (BQ788) receptor antagonists to reverse the behavioral syndrome induced by the experimental pancreatitis as described previously^[21,22]. This behavioral syndrome is a measurable end point indicative of the central sensitization state ongoing in the spinal cord and peripheral nerves.

MATERIALS AND METHODS

Animal study

All animal procedures were approved by the Institutional Animal Care and Use Committee. Male Lewis-inbred rats (125-150 g) were purchased from Harlan Laboratories (Indianapolis, IN). Rats were housed 2 per cages with a 12-h/12-h light/dark cycle and allowed access to food and water *ad libitum* except during behavioral testing. Rats were monitored daily for continued weight gain and general health. Health Status and Procedures were documented daily on the post-operative evaluation form. After one week acclimatization/quarantine, they were assigned to experimental groups. Baseline behavioral measures were assessed prior to induction of pancreatitis with DBTC and one week later, before and after treatment with ET-A antagonist BQ123 or ET-B antagonist BQ-788. A flowchart for the experimental design is provided in (Figure 1).

Animals were divided into groups: Naïve control, Vehicle/phosphate buffer (PBS) and DBTC/PBS. The study was repeated for each of the endothelin antagonists (Table 1).

Induction of persistent acute pancreatitis: Acute persistent pancreatitis was induced in Lewis rats by a single tail vein injection with DBTC (Sigma-Aldrich, St Louis, MO). DBTC was dissolved in 95% ethanol (two parts)

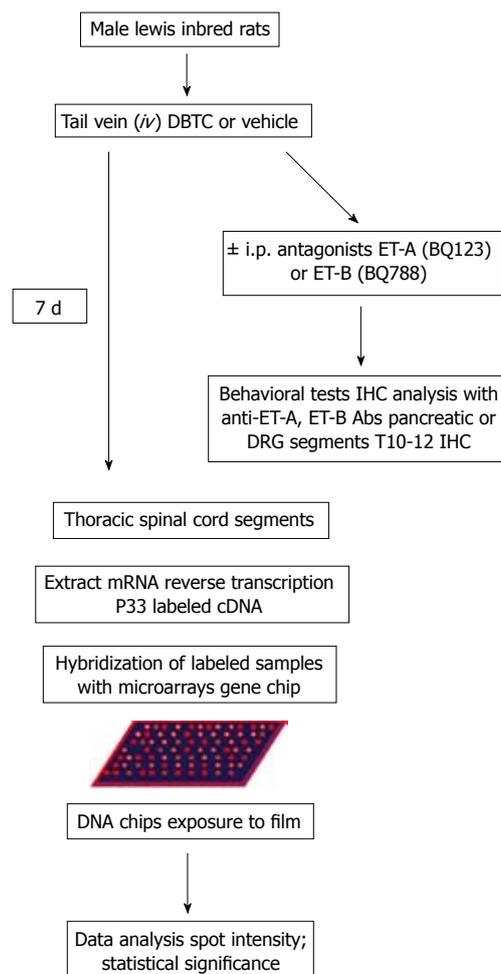


Figure 1 Schematic drawing of the study design. DBTC: Dibutyltin dichloride; DRG: Dorsal root ganglia. ET: Endothelin; IHC: Immunohistochemistry.

Table 1 Experimental design

No.	Groups (<i>n</i> = 5 per group)	<i>iv</i> injection	Drug administration (<i>ip</i>)
1	Naïve	Non	Non
2	Vehicle/PBS	Vehicle	0.1 mol/L PBS
3	Vehicle/BQ-123 or BQ-788	Vehicle	300 µmol/L
4	DBTC/PBS	DBTC	0.1 mol/L PBS
5	DBTC/BQ-123 or BQ-788	DBTC	33 µmol/L
6	DBTC/BQ-123 or BQ-788	DBTC	100 µmol/L
7	DBTC/BQ-123 or BQ-788	DBTC	300 µmol/L

Rats were injected with dibutyltin dichloride (DBTC) or vehicle (ethanol + glycerol) *via* tail vein to induce pancreatitis. On day 7 animals were treated with sham or endothelin receptor A (BQ-123) or endothelin receptor B (BQ-788) antagonists and tested for the behavioral hypersensitivity. Each group was constituted of at least *n* = 5. Experiment was repeated at least once. PBS: Phosphate buffer.

and then mixed with glycerol (three parts). In rats anesthetized with isoflurane inhalation, a maximum volume of 200 µL of DBTC (8 mg/kg body weight) was injected into the tail vein at a rate of 25 µL/min over 10 min using a syringe pump (Harvard Apparatus 22). Sham control rats received the vehicle (95% ethanol + glycerol, 2:3) also *via* tail vein. All animals (DBTC and sham controls)

were fed Teklad 8626 chow and given 10% alcohol + 5% apple juice in their drinking water as a maintenance diet to support persistence pancreatitis in the model.

Tissue acquisition, RNA extraction, and gene chip analysis: Rat DRGs and segments of T10-T12 spinal cord were taken 7 d after injection at the peak of pancreatic inflammation and processed for transcriptional profiling using the rat brain-biased cDNA nylon array generated by Millennium Pharmaceuticals Inc. (Cambridge, MA) for gene discovery (both normal and subtractive). The cDNA libraries, array construction, hybridization, sequencing, process technology, informatics, TRACE for library construction, expressed sequence tag sequencing, data acquisition, and software have been detailed previously^[23]. Therefore, rats were sacrificed and tissues were rapidly dissected from three animals per each group, frozen in dry ice, and stored at -80 °C before RNA isolation. Frozen tissues were homogenized in 1 mL of the TRIZOL reagent (Invitrogen, Carlsbad, CA) per 50-100 mg tissue and total RNA was extracted following the manufacturer's protocol. Briefly, total RNA from each sample was column purified, and oligonucleotide primers flanking the cloning site was used to amplify the cDNA insert by polymerase chain reaction. After purification over CHROMA SPIN TE+30 columns, the labeled cDNA was annealed at 65 °C for 1 h with 10 µg poly (dA) > 200 (Amersham Pharmacia). At 2×10^6 cpm/mL, the annealed cDNA mixture was added to array filters in preannealing solution containing 100 mg/mL sheared salmon sperm DNA in 7% sodium dodecyl sulphate, 0.25 mol/L sodium phosphate, 1 mmol/L ethylene diamine tetraacetic acid, and 10% formamide. Following overnight hybridization the radioactive signals captured by a Fuji BAS 2500 phosphorimager (Fuji Medical Systems, Stamford, CT) and were quantified by using ARRAY VISION software (Imaging Research, St. Catherine's, ON, Canada). Array hybridizations were performed in triplicate. Expression profiling data analysis and data clustering algorithm were done according to the published methodology^[23]. Regulated genes were selected based on the array spot intensity that was normalized to the average of housekeeping genes and normal controls across the array. Total 3 animals/each group was used for the microarray analysis and the study repeated at least $\times 2$. The average greater than or equal 2 fold changes in gene expressions were included in the study. Animal study design is shown in the Figure 1.

Pain-related behavioral assessments: Experimental procedures: Day 0: Baseline testing of abdominal nociceptive responses to mechanical and heat stimuli was applied to the upper left abdominal quadrant skin of rats as previously described^[21,22,24]. Pain-related behavior was assessed throughout the study by an observer blinded to group assignment. (1) Assessment of Secondary Mechanical Hyperalgesia/Allodynia by Testing Abdominal Withdrawal Threshold. Mechanical hypersensitivity in the abdominal area was quantified by measuring the number

of withdrawal events (either abdominal withdraw from the von Frey filament or consequent licking of the abdominal area, or whole body withdrawal) in response to normally innocuous or sub-threshold mechanical stimuli. The stimuli, applied to the upper left abdominal skin area, were von Frey filaments with bending forces of 3.52 mN, 10.78 mN and 47.24 mN (considered sub-threshold stimuli), and with bending forces of 215.6 mN (used as a supra-threshold stimulus). Reflex testing for "referred" secondary mechanical hyperalgesia/allodynia with von Frey fibers was developed by Max von Frey, who in 1896 identified "pain spots" on human skin, and has remained the standard in the field of pain assessment in humans and in animals. Mechanical nociceptive assay scores were expressed as an average percent response to ten applications of the von Frey filaments or the total number of withdrawal events to ten applications of three individual filaments as described previously^[21,22,24]. Before testing, all animals were shaved on the rostral abdominal area and placed into clear plastic enclosures (7 cm \times 4 cm \times 4 cm) on the metal meshed (3 cm \times 3 cm) platform (36 cm \times 29 cm \times 21.5 cm) and acclimated for 30 min. The von Frey filaments were applied from underneath through the mesh floor to the surface of the rostral abdominal area at different points. A single trial consisted of 10 applications of every von Frey filament. Each application was held for 1-2 s with a 5 s rest interval between applications to allow the animal to cease any response and return to a relatively inactive position. Totally, there were 3 trials through the graded series with a 10 min interval between trials. The withdrawal events for the three trials were averaged to get a single value per rat per time point. A percentage response was calculated (average of withdraw events/10 \times 100) for the study. A positive response was defined as an abrupt withdrawal (flick response) of the abdomen during stimulation or immediately after the removal of stimulus. The abdominal withdrawal tests were performed at baseline; before DBTC insult (day 6); and on day 7 after endothelin receptor antagonist' injection and von Frey filaments mechanic stimulation at 0 min, 20 min, 40 min, 75 min after BQ123 or BQ788 i.p. injection. Comparisons were made to baseline response; (2) Assessment of Secondary Thermal Hyperalgesia. In this assay, rats were placed on a regulated hotplate (52 °C) in a clear plastic enclosure (14 inches \times 14 inches). The latency to responses (foot flick, jumping, licking, *etc.*) was determined on day 7 after DBTC insult and before (0) and 20 min, 40 min and 75 min after injection of endothelin receptor antagonists; and (3) Open Field Box Spontaneous Exploratory Behavioral Measures. On day 6, spontaneous exploratory behavioral activities were collected using open field 16 \times 16 Photobeam Activity System (PAS) with FLEXFIELD software coupled to a PC (San Diego Instrument, Inc. CA). The PAS allows acquisition of movements in an x, y and z axis oriented grid system within an activity chamber (40 cm \times 40 cm \times 40 cm) by recording the number of times photobeams are obstructed. Data was collected

in 5 min intervals for 30 min. Six different behavioral measures of spontaneous activity were examined: rearing events, rearing time (s), active time (s), rest time (s), distance traveled (inch), and total counts (number of beams broken). Resting time was defined as a period when the animal remained in place for 1 s or longer. Active and rest time are important to determine effect of the treatment on the total amount of time spent for both exploratory and stationary movements. Changes in each parameter were evaluated individually.

Endothelin receptor antagonist administration: ET-A receptor antagonist (BQ-123) and ET-B receptor antagonist (BQ-788) from American Peptide, CA, were tested in this model to assess their effect on pain related behavioral modification. The drugs were dissolved in PBS with final concentration in 1 mL and administrated (i.p.). Rats were treated with ET-A and ET-B receptor antagonists (0 min, 20 min, 40 min and 75 min) day 7 after induction of pancreatitis. The concentrations used are based on the respective K_i for the drugs. The K_i for BQ-123 has been reported to be 10 nmol/L^[25]. The K_i for BQ-788 has been reported to be 100 nmol/L^[26]. The 33 μ mol/L, 100 μ mol/L and 300 μ mol/L treatments were used for generating the BQ-123 dose response curve by examining the effects of post-treatment on behavioral testing in animals with pancreatitis ($n = 5$ per each dose). Then, the maximal effective dose (300 μ mol/L) was selected for the rest of study including for the drug, ET-B receptor antagonist (BQ-788, American Peptide, CA). Control rats received PBS injections (i.p.).

Therefore, seven different experimental groups were designed: BQ-123 or BQ-788 treated rats with or without pancreatitis; PBS treated rats with or without pancreatitis as well as naïve rats. Five rats in each group were included in this study (Table 1).

Morphine administration: Morphine was injected in cumulative doses of 1, 5 and 10 mg/kg body weight or sham saline given i.p. to rats ($n = 6$ /group). Systemic administration of morphine is used as a gold standard analgesic in different pain related behavioral modification studies.

Necropsy and sample collection

Perfusion and tissue collection: At the end of the experiment, rats were given an overdose of pentobarbital (75 mg/kg). Fresh pancreatic tissues were collected. Dorsal root ganglia (DRG) and spinal cord from 10th-12th thoracic segments were dissected as they receive the sensory information from the neuronal fibers that innervate the pancreas. Samples were flash frozen in liquid nitrogen and kept at -80°C and processed for transcriptional profiling microarray analysis biased for rat brain-specific genes as mentioned above and for immunohistochemistry. The study design is shown in the Figure 1.

Immunohistochemical study

Spinal cord, DRG and pancreatic tissues collected

for immunohistochemical analysis: At the end of the experiment, rats were given an overdose of pentobarbital and transcardially perfused with 4% buffered paraformaldehyde. Spinal cord segments T7-T12 and DRGs were taken 7 d after injection at the end of the experiment. Tissue were removed and stored overnight at 4°C followed by immersing in 30% sucrose. The frozen sections were cut as 30 μ m thickness. The fresh pancreas was directly fixed in 4% paraformaldehyde for 24 h followed by storing in 70% alcohol. The tissue was processed for paraffin sections (4 μ m) which were mounted onto the chrome-gelatin pre-coated glass slides. After deparaffinization in xylene for 5 min ($\times 2$), the sections were hydrated gradually with 100%, 95%, 80% and 70% alcohol followed by washes with distilled water. Polyclonal rabbit anti- ET-A and ET-B receptor (Alomone Labs, Jerusalem, Israel) were used for staining frozen sections of the DRG. In addition, paraffin sections of pancreas were prepared from rats with different treatments. Then, immunohistochemical staining procedures for both frozen and paraffin section were performed. After rinsing in 0.1 mol/L PBS, the tissues were blocked with 5% normal goat serum in 0.1 mol/L PBS plus 0.05% Triton X-100 and 0.3% bovine serum (NGSTB) for 40 min. Then, the tissues were double stained with specific antibodies produced in two different species in the following combinations: (1) Endothelin receptor A and neuronal marker (NeuN); and (2) Endothelin receptor B and glial fibrillary acidic protein marker for all glia (GFAP) (satellite and Schwann cells). This was accomplished by incubating the cut tissue sections with rabbit anti-ET-AR (1:400) and mouse anti-NeuN (1:5000) or ET-BR (1:200) and mouse anti-GFAP (1:500) diluted in 1% NGSTB overnight at room temperature. After rinsing with 0.1 mol/L PBS, the tissues were incubated for 1 h in a fluorescent tagged secondary antibody, red Alexa fluor 568 goat anti-rabbit IgG (1:1000) and green Alexa fluor 488 goat anti-mouse IgG (1:1000) diluted in 1% NGSTB. Finally, the tissues were rinsed with 0.1 mol/L PBS, cover-slipped with mounting media hard set with hard set mounting media with blue 4',6-diamidino-2-phenylindole counterstain (Vector Labs, Burlingame, CA). Pictures were taken using the Act-1 program with a Nikon E1000 microscope.

Statistical analysis

All data are presented as mean \pm SD unless otherwise stated. Statistical analysis was performed by the ANOVA-test (two way analysis of variance). If significant differences were found Bonferroni post *hoc* and when appropriate student *t* test statistical analysis were performed to assess changes over time for each group separately to compare (1) baseline and 7 d after induction of pancreatitis; (2) before and after drug treatment; and (3) drug treated group compared to controls without drug treatment. Statistical significance was set at $P \leq 0.05$.

RESULTS

Animals that received sham vehicle significantly gained

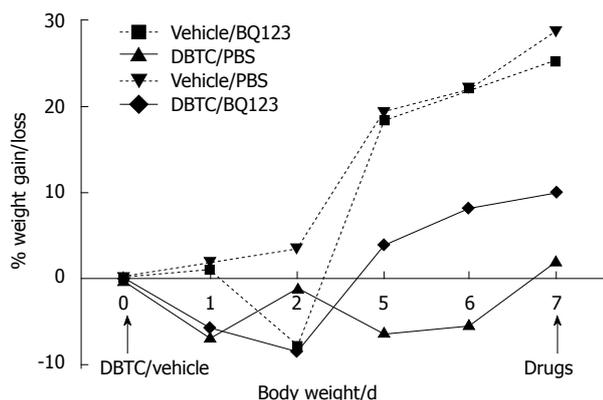


Figure 2 Timeline demonstrates % body weight gain/loss in rats during the study comparing naive rats and rats with pancreatitis (n = 5). DBTC: Dibutyltin dichloride; PBS: Phosphate buffered saline.

weight (27%) on the diet (vehicle: D1 *vs* D7, *P* < 0.01) compared the DBTC induced pancreatitis rats which differed (vehicle *vs* DBTC) by an average of 21% (Figure 2).

Gene chip analysis

Rat DRG segments of T10-12 spinal cord were taken at the peak of inflammation and processed for gene expression profiling with a microarray biased for rat brain-specific genes. An average of 3 animals/each group was used for the microarray analysis and the study repeated at least twice. The genes were normalized with sham controls and average greater than or equal 2 fold changes in gene expressions were demonstrated in the Table 2. Modified genes were classified into groups according to their functionality (Table 3). The identified genes included: circulatory/acute phase, extracellular matrix, signaling transduction, transcription/translation-related, antioxidants/chaperones/heat shock and pancreatic and other enzymes. Candidate genes are among those modified in comparison to normals and are involved in different signaling pathways in DBTC induced pancreatic inflammation as shown in the Table 2.

Acute pancreatitis regulates gene expression in spinal cord

Subtraction of overlapping normal genes revealed 321 unique genes that were classified into 9 different functional groups (Table 3). The candidate genes included 261 that were up-regulated and 60 downregulated genes in this model. Amongst these, fifty-two distinct genes that were upregulated greater than 2-fold were chosen as candidates for the pancreatitis, visceral pain and pancreatic cancer pathway specific and listed in Table 2. Additionally, 53 selected genes which were down regulated in this pancreatitis model are shown in Table 2.

Pain related behavioral activity

Secondary mechanical hyperalgesia: Animals developed persistent pancreatic inflammation during the study as detected in pathological samples and secondary pain-

Table 2 Up-regulated and down-regulated genes expressed¹

Group	Regulated genes	Abbreviation	Fold
Up-regulated genes¹			
1	B-lymphocyte surface antigen		7.3
1	Endothelin-1	ET-1	3.2
1	Eosinophil chemotactic protein	ECP	11
1	T-cell surface glycoprotein CD5		2.6
1	Tumor necrosis factor	TNF- α	4
2	Laminin α 5	LAMA5	2.5
2	Matrix gla protein	MGP	7.4
2	Integrin α		27
3	Epithelial-cadherin	E-CADHERIN	3.1
3	Transmembrane ER-resident protein, type I	IRE1b	23.5
4	ATP binding cassette	ABC-transporter	23
4	C-C chemokine receptor type 8	C-C CKR-8,	3.4
		GPR-CY6	
4	GABAA receptor	GABA _A R	14.3
4	Glutamate receptor ionotropic, AMPA 1	GluR-1	9.1
4	Putative G protein-coupled receptor	GPR68 (OGR-1)	3.1
5	Calcium binding protein	CHP	2.6
5	Caveolin-1	CAV1	3.8
5	Iron response regulator	IRR	2.8
5	LDL receptor-related protein 6	LDL-R6	3.6
5	Insulin-like growth factor binding complex acid label chain precursor	ALS	2.7
5	Proto-oncogene tyrosine-protein kinase FES/FPS	C-FES	8
5	RAS GTPase-activating-like protein	RAN	3.9
5	Retinoic acid-inducible gene-1	RAIG1	6.4
5	Transforming growth factor-b	TGFb	2.8
5	Guanine nucleotide binding protein	G (Y) α -11	4.7
6	Nuclear factor of activated T cells	NF-ATc	3.5
6	Signal transducer and activator of transcription 5B	STAT5b	3
6	Transcription factor	TF	11.8
7	Glutathione peroxidase gastrointestinal	GSHPX_GI	2.5
7	Extracellular superoxide dismutase	EC-SOD	2.9
7	Heat shock related 70 kD proteins2		2.3
7	NADH dehydrogenase (ubiquinone)		2.3
7	Serum amine oxidase	SAO	2.2
8	ACE-related carboxypeptidase	ACE2	3.6
8	Aminopeptidase N	APN	2.3
8	ATP-dependent RNA helicase P54	DDX6	2
8	a enolase		2
8	a-Tryptase	TRYPTASE 1	2.6
8	cAMP cAMP-inhibited cGMP 3' 5'-cyclic phosphodiesterase		9.4
8	Cholecystokinin type A receptor	CCK-AR	2.8
8	Dopamine β -hydroxylase	DBH	5.4
8	GAMMA-glutamyltransferase 1	Gamma-GT	5.6
8	(GDP-D-MANNOSE dehydratase	GMD	3.5
8	Glycolipid transfer protein	GLTP	2.6
8	Lipase		3.8
8	Lysophosphatidic acid acyltransferase-b	LPAAT- b	3.4
8	Malonyl-CoA decarboxylase	MLYCD	4.8
8	NTPDase 3; CD39L 3	ENTPD3	7.2
8	Pancreatic isozyme	Glucokinase	3.4
8	Phosphate regulating neutral endopeptidase	NEP	11
8	Putative NAD (P)-dependent cholesterol dehydrogenase	MDH	3
8	Red cell phosphatase1, isozyme F	ACP1	2.3
Down-regulated genes¹			
1	Insulin precursor		2.8

1	IG GAMMA-3 chain C region	HDC	2
1	Interleukin 1 receptor 1 precursor	IL-1R, IL-1R α	3.8
1	Growth regulated protein (neutrophil-activating protein 3)	NAP-3	2.3
1	Natural killer cells antigen CD94	KP43	4.2
1	Monocyte chemotactic protein 1		4.3
1	T-cell surface glycoprotein CD3 ϵ chain precursor		5.2
1	Heparin-binding growth factor 1		7.3
2	EGF-containing fibulin-like extracellular matrix protein 1	FIBL-3	2.1
2	Homocontig12		2.3
2	MT4-MMP		2
3	Glycosyl structure		2
3	Microtubule-associated protein 2	MAP2C	2.1
3	Mucosal addressin cell adhesion molecule-1		2.1
3	Peripheral myelin protein 22	PMP-22	3.8
4	Growth factor regulated channel 5	GRC5	2.1
4	Growth hormone secretagogue receptor type 1		2
4	a platelet-derived growth factor receptor (CD104A antigen)	PDGFR α	2.1
4	Glucose transporter type 5, small intestine (fructose transporter)		2.2
4	Dopamine receptor		2.3
4	C-C chemokine receptor type 9	CCR-9	2.4
4	Monocarboxylate-transporter 3	MCT 3	2.9
4	Orphan receptor	GRF	3.6
4	G protein-coupled receptor	GRP1	3.6
4	Neuropeptide Y Receptor Type 4	NPY4-R	10.6
4	β 3 Adrenergic Receptor		2.5
5	G25K GTP-binding protein, placental isoform (GP)		2
5	MAD 2		2
5	Ribosomal S6 protein kinase		2
5	Metalloproteinase Inhibitor 3	TIMP-3	2.2
5	GTPase RhoD	RhoD	2.4
5	Ankyrins		2.7
5	Putative RHO/RAC effector protein		4.2
5	Protein farnesyltransferase β subunit (RAS Proteins prenyltransferase)	FTASE- β	2.4
6	GTP-binding protein	RHEB	2.2
6	GTP-binding protein 1	GP-1	2.3
6	hLHX 6 1 α		2
6	AMP-binding protein		2.7
7	Thyroid peroxidase	TPO	2.1
8	Ubiquitin-conjugating enzyme E2 (ubiquitin-protein ligase)	UBE2	2
8	Steryl-sulfatase (steroid sulfatase) (steryl-sulfate sulfhydrolase)	ASC	2
8	Histone deacetylase 2	HD2	2.1
8	Corticosteroid 11- β -dehydrogenase, isozyme 2		4.3
8	Hydroxymethylglutaryl-CoA synthase		4.4
8	ATP synthase oligomycin sensitivity conferral protein	OSCP	4.9
8	Indoleamine 2,3-dioxygenase	IDO	2.1
8	Lysozyme		2.2
8	ubiquitin C-terminal hydrolase	UCH	2.3
8	Tyrosine aminotransferase, L-tyrosine: 2-oxoglutarate Aminotransferase	TAT	2.7
8	Corticosteroid 11- β -dehydrogenase, isozyme 2		4.3
8	Hydroxymethylglutaryl-CoA synthase		4.4
8	ATP synthase oligomycin sensitivity conferral protein	OSCP	4.9

8	Glycogen phosphorylase	8.5
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¹52 selected genes ($n = 3/\text{group}$, study repeated $\times 2$). Dibutyltin dichloride induced pancreatitis regulated genes (up or down-regulated) expression normalized with sham controls. Group 1: Circulatory/acute phase/immunomodulatory; Group 2: Extracellular Matrix; Group 3: Structural; Group 4: Channel/receptor/transporter; Group 5: Signaling transduction; Group 6: Transcription/translation-related; Group 7: Antioxidants/chaperones/heat shock; Group 8: Pancreatic and other enzymes; Group 9: Unidentified genes. Total genes = 321; up-regulated = 261, down-regulated = 60.

related cutaneous mechanical hypersensitivity. Abdominal mechanical testing with von Frey microfilament demonstrated statistically significant increases in withdrawal events on day 6 post DBTC injection in pancreatitis animals and are presented as % number of events ($n = 5$) (Figure 3). In contrast, no changes were detected in the sham control rats. Data obtained were consistent for 2 different microfilaments except more intense responses were detected with the larger microfilament (3.52 mN *vs* 10.78 mN) as expected, demonstrating consistency for abdominal response with the von Frey mechanical test (Figure 3A and B).

On day 7 after induction of pancreatitis, groups of animals were simultaneously treated (i.p. injection) with 3 different doses of ET-A antagonist (BQ123, 33, 100 and 300 $\mu\text{mol/L}$). Animals with pancreatitis had dose dependent responses in contrast to control animals that were not affected by the antagonists. While 33 $\mu\text{mol/L}$ BQ123 did not provide a statistically significant effect (Figure 4), 300 $\mu\text{mol/L}$ provided significant protection against inflammatory pain related behaviors in pancreatic animals tested at 75 min after drug administration. Therefore, studies continued only with the 300 $\mu\text{mol/L}$ dose (Figure 4).

Next we compared efficacy of BQ123 (ET-A receptor antagonist) to the BQ788, an ET-B antagonist (Figure 5). Both drugs were effective in normalizing mechanical hypersensitivity response. However, ET-A receptor antagonist had a longer lasting effect compared to ET-B receptor antagonist, with a more robust response. The ET-A receptor antagonist (BQ123) and the ET-B receptor antagonist (BQ788) abolished abdominal mechanical hypersensitivity as early as 20 min (Figure 5A). The reduced events using BQ123 demonstrated more vigorous response persisting through 75 min post treatment ($n = 5$), while the number of withdrawal events increased again at 75 min for the ET-B receptor antagonist demonstrating less efficacy and persistence of effect for this agent than for the ET-A receptor antagonist in management of pain related behavioral modifications in this model.

Hotplate whole-body thermal responses: Rats with pancreatitis reacted to the hotplate with reduced response times compared to naïve rats indicating increased thermal hypersensitivity (Figure 5B). Both ET-A receptor antagonist (BQ123) and ET-B receptor antagonist (BQ788) significantly increased response latencies, rein-

Table 3 Modified genes were classified into 9 different groups according to their functions, while some genes' functionality may overlap¹

Groups	G1	G2	G3	G4	G5	G6	G7	G8	G9	%	Total
Up	17	17	8	30	46	23	9	67	44	81.3	261
Down	7	3	5	11	12	4	1	14	3	18.7	60
Sum	24	20	13	41	58	27	10	81	47		321
Total%	7.5	6.2	4.1	12.8	18.1	8.4	3.1	25.2	14.6	100	
Sum	8.7	4.62	5.86	13.98	16.56	6.24	1.86	20.52	7.26	118.7	334

¹52 selected genes ($n = 3/\text{group}$, study repeated $\times 2$).

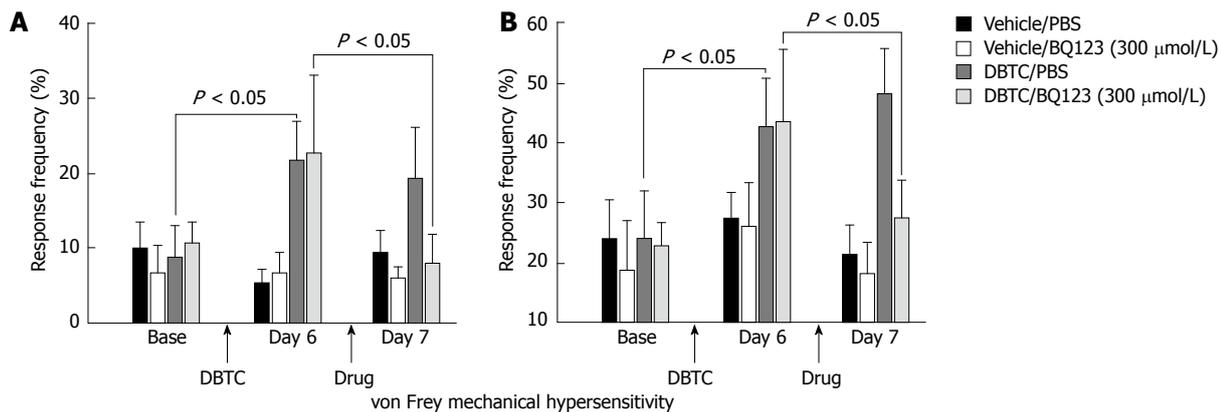


Figure 3 Behavioral responses to mechanical stimuli using 2 von Frey microfilaments of different strengths (g-Force). A: 3.52 mN; B: 10.78 mN, at baseline and on day 6 (dibutyltin dichloride-induced pancreatitis) and day 7 (drug treatment). BQ123 (300 $\mu\text{mol/L}$) abolishes abdominal hypersensitivity 75 min post treatment with no effect in naïve animals ($n = 5$). PBS: Phosphate buffered saline; DBTC: Dibutyltin dichloride.

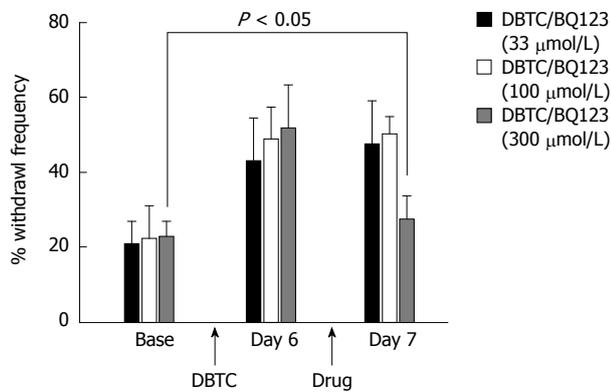


Figure 4 Response to von Frey monofilaments for rats at baseline, after induction of pancreatitis with dibutyltin dichloride which promotes mechanical hypersensitivity (day 6), and responses 75 min (day 7) after the three doses of BQ123 ($n = 5$). DBTC: Dibutyltin dichloride.

stating response times back to the levels of the vehicle control animals.

Additionally as expected abdominal hypersensitivity to von Frey mechanical stimulation and hyperalgesia (data not shown) were attenuated by the i.p. administration of the morphine in a cumulative, dose dependent manner in rats on day 7 ($n = 6$). As systemic administration of morphine is currently a gold standard therapy in different experimental pain models, data for the endothelin receptor antagonists used in this study correlated well to those obtained with morphine administration (correlation

not shown).

Open field exploration: Spontaneous behavioral activity was conducted before induction of pancreatitis (baseline), 6 d after induction of pancreatitis, as well as 30 min after drug treatments (BQ123, BQ788) for comparison to sham control animals. The overall stable activity levels indicate that the drugs produced no undesirable effects on normal exploratory behaviors (data not shown). No changes were noted in the six behavioral measures except a trend toward reduction of the active time and an increase in resting time measured at the highest dose (300 $\mu\text{mol/L}$). These data indicate that the agents do not interfere with normal exploratory behaviors.

Pancreatic pathology

Pancreatic sections stained with HE from naïve animals receiving sham treatment showed normal histological architecture and islets. In contrast DBTC treated rats develop persistent pancreatitis demonstrated as edema in parenchyma, loss of pancreatic architecture, infiltration of inflammatory cells including neutrophil and mononuclear cells, degeneration vacuolization and necrosis of acinar cells (not shown).

Endothelin-A and B receptor expression in pancreas

Endothelin-A and B receptor expression in naïve or pancreatitis DBTC rats was observed in the vascular endothelia (Figure 6). ET-A receptor appeared to be more

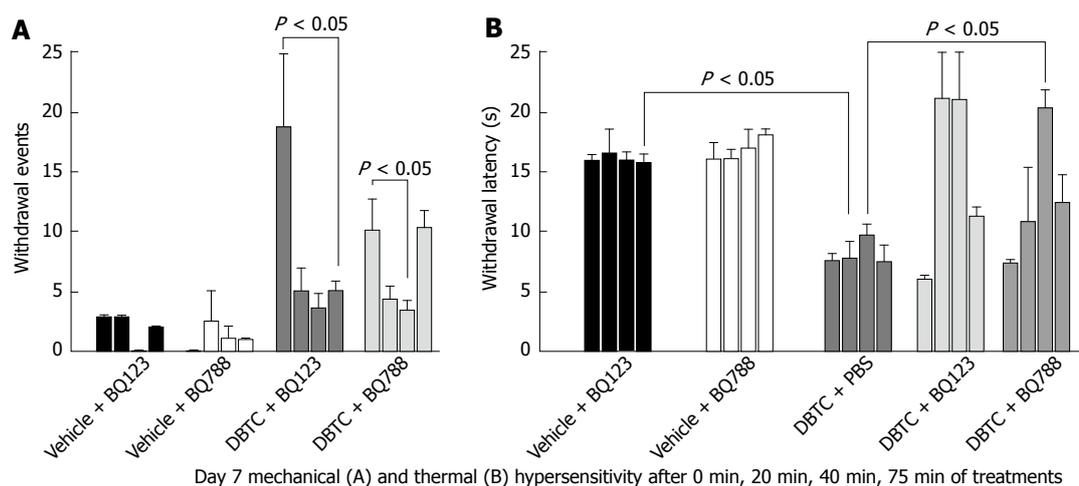


Figure 5 Mechanical and thermal hypersensitivity. A: Rats with pancreatitis demonstrate fewer withdrawal events in response to mechanical stimuli at time points after treatment with BQ123 [endothelin (ET)-A receptor antagonist, 300 $\mu\text{mol/L}$] and BQ788 (ET-B receptor antagonist, 300 $\mu\text{mol/L}$). The response to BQ788 persisted for a shortened amount of time compared to the longer lasting to BQ123. Data is presented as actual number of events ($n = 5$); B: Endothelin-A receptor antagonist (BQ123) normalized secondary thermal hypersensitivity that was shortened after induction of pancreatitis. The effect persisted longer for BQ123 compared to shorter response to BQ788 antagonist ($n = 5$). PBS: Phosphate buffered saline; DBTC: Dibutyltin dichloride.

condensed around constricted vessels in pancreatic compared to naïve control animals. However, no quantitation was done since overall, minor detectable differences were evident in ET-A and ET-B receptors expression in the pancreas in comparisons between naïve and pancreatitis animals.

Endothelin A and B in DRG

Based on immunocytochemical localization, ET-A and ET-B receptors were increased in DRG (T10-12) from pancreatitis compared to naïve rats (Figure 7). ET-A receptor expression was observed in all sizes of primary sensory neurons of the DRG. In contrast, ET-B receptors were primarily localized on the Schwann cells (myelin sheaths) surrounding the axons that were passing through the DRG from naïve or pancreatitis rats.

Endothelin receptor localization was combined in dual NeuN and GFAP (Figure 8). ET-A was expressed in the cell bodies and occasional nuclei of neurons of different sizes in the DRG in naïve animals. However, phenotypic expression of ET-A receptor was greatly increased in neurons of all sizes in rats with DBTC induced pancreatitis. Similarly, ET-B receptor was also localized in neurons of all sizes, in the satellite glia, as well as in the Schwann cell glial myelin sheaths surrounding the axons passing through the DRG and satellite glia.

DISCUSSION

In this investigation, DBTC-treated animals developed persistent pancreatic inflammation detected in pathological analysis and secondary pain-related hypersensitivity. The DRG and spinal cord from 10th-12th thoracic segments were selected for gene microarray analysis, as they portray the major source of sensory neuronal fibers in the pancreas. Amongst the 52 candidate genes upregulated in three animals in each experimental group (run

in duplicate) was ET-1. ET-1 was upregulated greater than 2-fold in the animals with pancreatic inflammation and visceral pain-related behavior. This finding illustrates the benefits of gene array analysis in identifying relevant genes with possible direct roles in mediating pain in visceral inflammatory states. Visceral mechanical testing with von Frey microfilaments demonstrated functional significance showing increases in abdominal withdrawals on day 6-7 post DBTC insult in pancreatitis animals. In contrast, no changes were detected in normal rats receiving sham vehicle alone. Similarly, spontaneous pain related behaviors were unchanged in naïve rats treated with endothelin antagonists indicating that the agent had no effect on normal exploratory behaviors. Single highest dose treatment with ET-A antagonist (BQ123) provided significant protection against inflammatory pain related behaviors in animals with pancreatitis, while the ET-B antagonist (BQ788) had a short lasting effect.

In the current study, ET-A and ET-B receptors were both detected in DRG. ET-A receptors trigger vascular constriction and ET-B receptors act as vasodilators, but their role in neurons is not defined.

Although, ET-A and ET-B receptors are both present in the thoracic spinal segment, and DRG, ET-B receptors are primarily expressed in DRG satellite cells and ensheathing Schwann cells^[27] where they can stimulate the synthesis and release of prostaglandin E₂, an active compound in inflammatory pain^[16]. In addition, ET-B receptors present on keratinocytes mediate the release of β -endorphin from these cells with a local analgesic effect^[18]. ET-1 has a mitogenic effect, promoting cancer cell growth in colon and pancreas in which there is also upregulation of ET-A receptors and moderate down-regulation of ET-B receptors^[28].

In our hands, ET-A receptors appeared to be more localized around constricted vasculature in pancreatic tissues of animals with DBTC-induced pancreatitis. While,

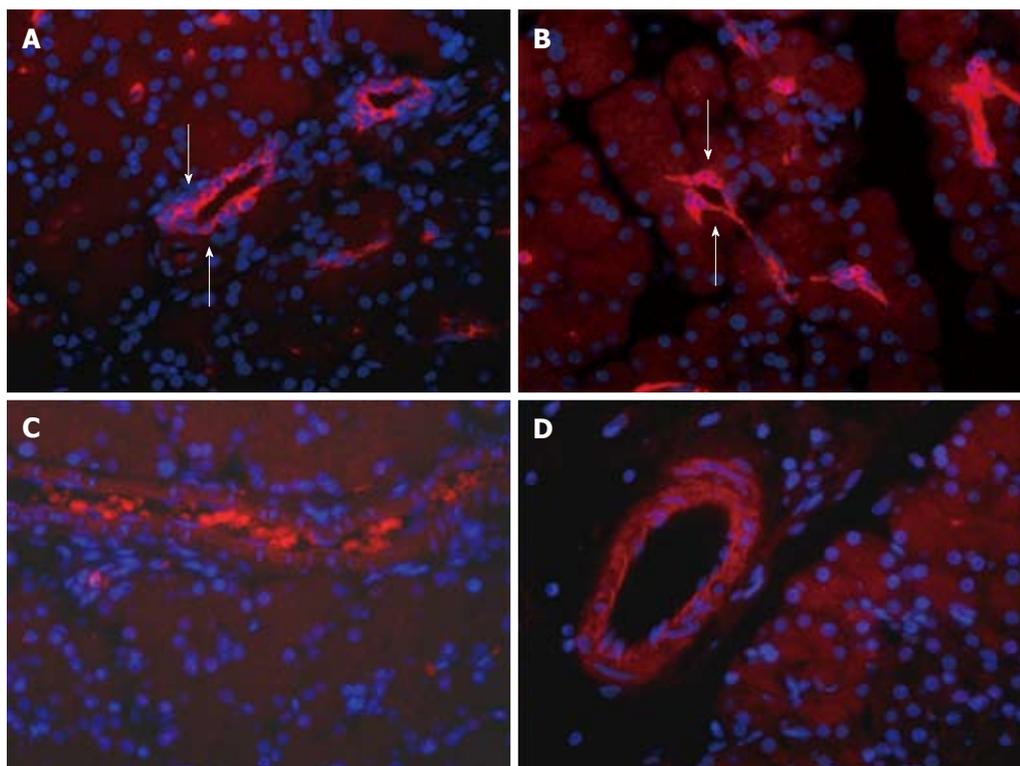


Figure 6 Pancreatic tissues from naïve and pancreatitis animals. A: Naïve endothelin (ET)-AR; B: Dibutyltin dichloride (DBTC) ET-A receptor; C: Naïve ET-B receptor; D: DBTC ET-B receptor. ET-A receptors are expressed in ducts (arrows) which appear more constricted in pancreatitis animals (B: DBTC ET-A receptor). ET-B receptors were expressed on the vasculature (4',6-diamidino-2-phenylindole, nuclear blue counterstaining) 40× magnification ($n = 5$).

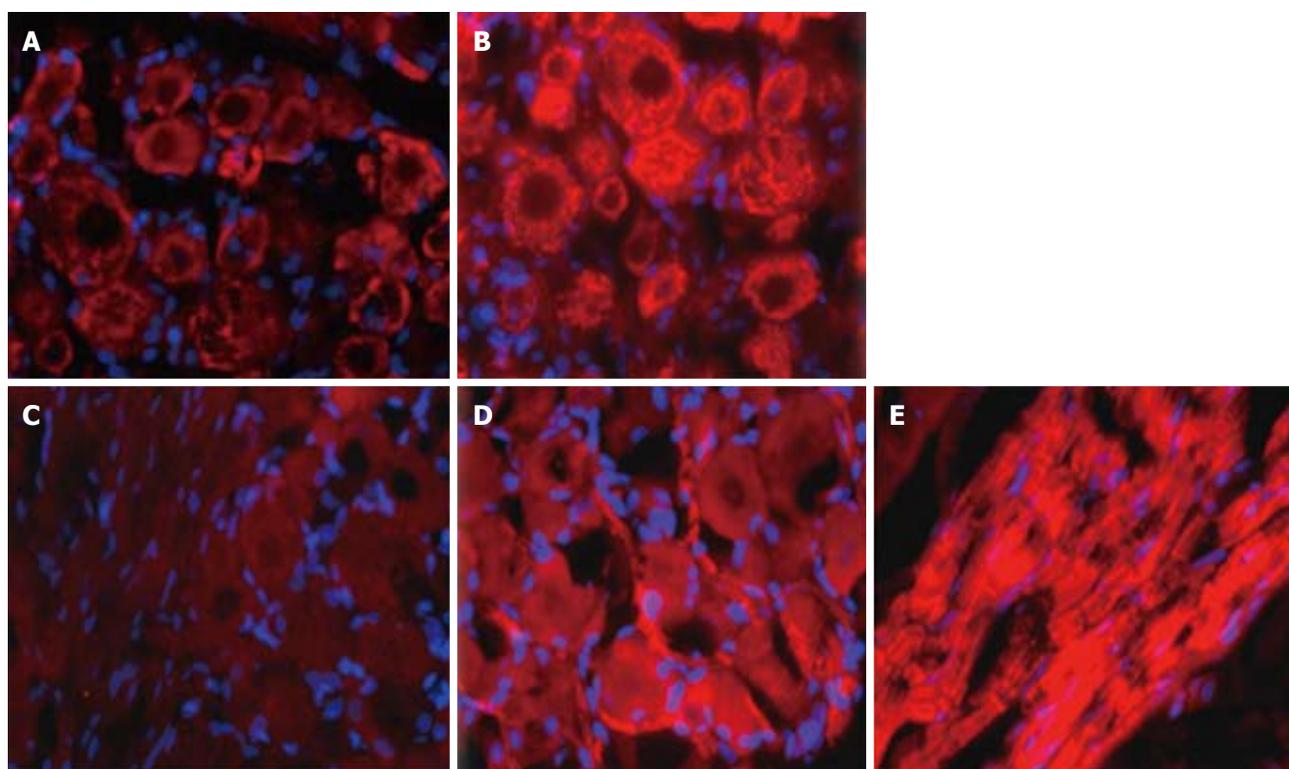


Figure 7 Immunohistochemical analysis of the localization of endothelin-A and endothelin-B receptors in dorsal root ganglia from naïve or pancreatitis rats. A: Naïve endothelin (ET)-A; B: Dibutyltin dichloride (DBTC) ET-A; C: Naïve ET-B; D, E: DBTC ET-B ($n = 5$). ET-A receptor expression is shown in primary sensory neurons (A, B). In contrast, ET-B receptors (B-E) are primarily localized on the Schwann cells (myelin sheaths) surrounding the axons passing through the dorsal root ganglia from naïve or pancreatitis rats (4',6-diamidino-2-phenylindole nuclear blue staining and Alexa Fluor 568, red).

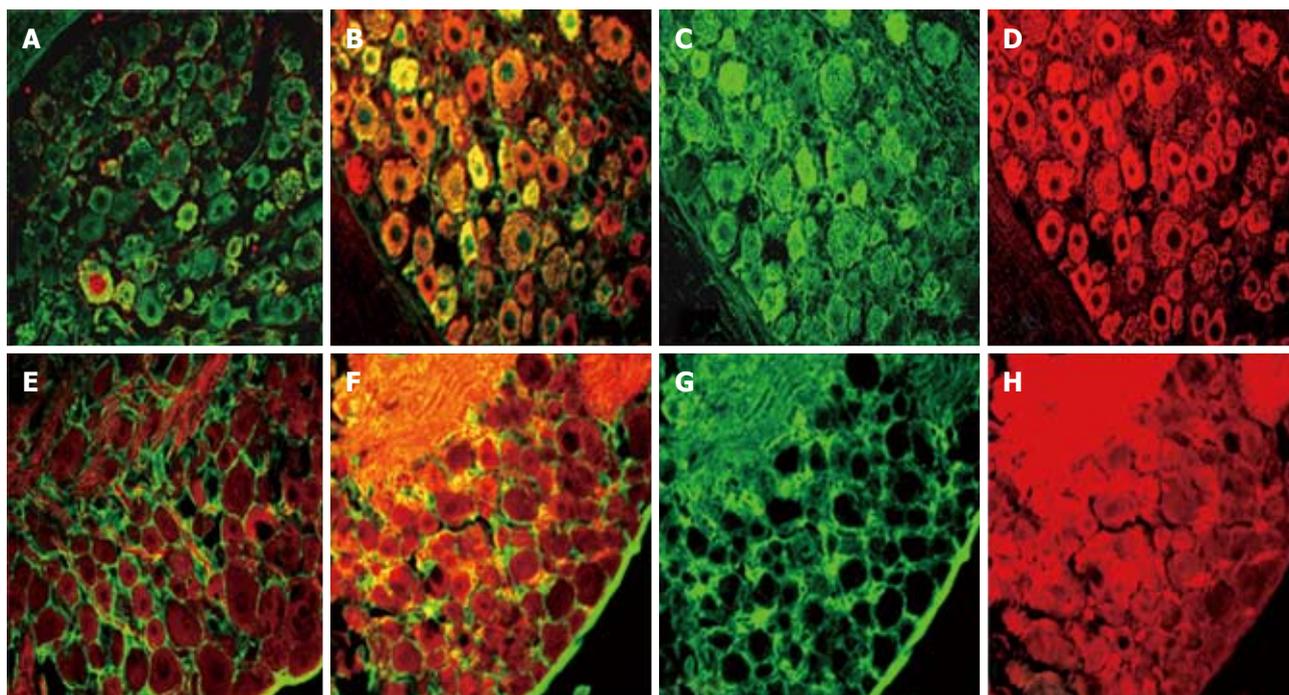


Figure 8 Endothelin receptor A and B in dorsal root ganglia (T10-12). A: Double staining of Endothelin receptor A with dual staining for neuronal (NeuN) and glial fibrillary acidic (GFAP) markers from naïve animal; B: Dual staining for Endothelin A and NeuN in a pancreatic animal; C: NeuN in a pancreatic animal; D: Endothelin receptor A in a pancreatic animal. Significant increases in the endothelin (ET)-A are noted in dorsal root ganglia (DRG) neurons of all sizes on day 7 after induction of pancreatitis; E: Dual staining for Endothelin B and GFAP in a naïve animal; F: Dual staining for Endothelin B and GFAP in a pancreatic animal; G: GFAP in a pancreatic animal; H: Endothelin receptor B in a pancreatic animal; Significant increases in the ET-B receptor are noted localized in satellite glia and Schwann cell myelin in thoracic DRG on day 7 after induction of pancreatitis. Alexa Fluor 568, red; Alexa Fluor 488, green ($n = 5$).

modest detectable differences were evident in the ET-B expression of the pancreatic tissues in comparisons of animals with pancreatitis to naïve controls.

Animals with pancreatitis demonstrated trend toward a reduction in active time and an increase in resting time. Immunohistological analysis revealed increased ET-A in DRG neurons of all sizes and ET-B in myelin sheath and satellite glia. The changes in spontaneous behaviors induced by pancreatitis were normalized by application of the ET-A antagonist (BQ123), suggesting that this receptor has a role in pancreatitis-induced behavioral changes.

Injection of ET-1 is reported to cause severe pain *via* activation of ET-A receptors, in the sciatic nerve chronic constriction injury model^[29]. In the present study, both the endothelin-A (BQ-123) and B (BQ-788) receptor antagonists significantly reversed the thermal hypersensitivity assessed using the hot plate method at 20 min, 40 min after injection. However, the ET-B antagonist was ineffective at the 75 min time point while the efficacy for the endothelin-A receptor antagonist persisted. Neither agents affected vehicle injected animals. Additionally, we demonstrated that the thermal and mechanical hyperalgesia induced in pancreatitis animals were equally well normalized by the administration of morphine (10 mg/kg) with a dose dependent response, whereas a lower dose of BQ-123 and BQ-788 were required to reverse the nocifensive responses in the pancreatic rats. Similarly, administration of the ET-A receptor antagonist reversed the attenuation of spontaneous exploratory behaviors

observed in pancreatic animals.

These data reveal that nocifensive responses invoked by the persistent pancreatitis in animals can be ameliorated by systemic post-treatment with endothelin-A receptor and to a lesser degree endothelin-B antagonist similar to conventional morphine administration. As has been suggested for neuropathic pain^[29], ET-A receptor antagonists deserve future study as potential novel therapy including against inflammatory pain.

Previous studies indicate that treatment with non-selective ET-A and ET-B (LU 302872) and selective ET-A (LU 302146) antagonists had no effect on the pancreatic pathological (edema and inflammatory infiltration) nor on trypsinogen activation 4h after caerulein-induced acute pancreatitis model. A slight increase in the pathological necrosis and vacuolization suggested the possible undesired effects of these compounds in the model^[30]. In contrast ET-1 at the high dose was found to be beneficial on morphological changes and trypsinogen activation in that model^[31]. In our hands we did not detect any undesirable pathological effects or any histological improvement in treated animals after single dose i.p. administration of endothelin-1 receptor antagonists (BQ123, BQ788).

In severe acute pancreatitis, microcirculatory disorders and increased capillary permeability contribute to multiple organ dysfunction syndrome, while, ET-A receptors stabilize capillary leakage and improved organ function^[32]. Of interest, upregulated genes reported here substantiate this finding. For example, multifunction pro-inflamma-

tory cytokine IL-6 levels were significantly upregulated in DRG of rats with pancreatitis on day 6 after DBTC injection^[20]. Relative quantification of target cDNA levels in primary stellate cultures using real-time polymerase chain reaction revealed a dose-dependent reduction of endothelin-1 after treatment with inhibitors of histone deacetylases^[33]. This is relevant to anti-cancer activities and, therefore, of growing clinical interest^[34-36]. It is well established that diabetes can occur in acute pancreatitis as well as chronic pancreatitis. Insufficient pancreatic enzyme activity and dosing is treated in pancreatic steatorrhea with administration of lipase with meals in patients^[37]. In this model we detected modulation of genes for the inflammatory markers of pancreatitis, including upregulated lipase, α enolase and α trypsinase, while insulin precursor, glucose transporter type 5, and glycogen phosphorylase were down regulated. In accordance with these findings the increased levels of the serum amylase and lipase were reported on day 3 and peaked on day 7 in this DBTC induced pancreatitis model, consistent with pancreatitis in patients^[22]. Finally, amongst those genes found to be downregulated were peripheral myelin protein 22, α platelet-derived growth factor receptor, dopamine receptor, and neuropeptide Y receptor. Further studies are warranted to investigate the role of these candidate genes as novel targeted therapeutic modalities in patients. ET-A receptor antagonists deserve future study as a potential novel therapy against inflammatory and neuropathic pain.

In conclusion, we report 8 different groups of genes modified in this model of pancreatitis. These candidate genes may be useful as future biomarkers for diagnostic and/or targeted gene therapy. As an example, endothelin-1 gene was upregulated and subsequently, ET-A and ET-B receptor antagonists were found to reverse inflammatory pain responses. These results demonstrate the potential utility of the gene microarray analysis to identify candidate genes for analgesic development.

COMMENTS

Background

Abdominal pain ranging from mild to severe pain is the chief symptom of patients with pancreatic disorders. Neural innervation of the pancreas is important in the initiation and maintenance of inflammation. Activation of pancreatic sensory neurons causes release of neurotransmitters in the spinal cord and neurogenic activation signals in the pancreas itself producing plasma extravasation and neutrophil infiltration. Endothelins (ET) cascade is implicated as a major contributing factor in pancreatic pain in both pancreatitis and pancreatic cancer.

Research frontiers

Multifunctional ETs comprise a family of peptides which interact with their receptors that are involved in regulation of blood flow, cell proliferation, muscle contraction or relaxation, secretion and ion transport. The ETs are expressed by endothelial cells, macrophages, astrocytes and neurons. ETs activate the peripheral sensory nervous system and may directly be involved in signaling nociceptive events in peripheral tissues. ETs are increased in inflammatory conditions. ETs produce their biological effects via activation of the ET-A receptor and the ET-B receptor.

Innovations and breakthroughs

The ET-1 was amongst the 52 candidate genes upregulated (average of 3 animals/group and study repeated X2) greater than 2-fold in an experimental pancreatitis model. ET receptor antagonists were safe and effective in ameliorating mechanical and thermal hypersensitivity in doses lower than the gold standard

morphine with exception of no detectable side effects.

Applications

The candidate genes reported in this investigation may be useful as future diagnostic tool and targeted gene therapies. ET-A and to a lesser extent ET-B receptor antagonists can reverse pancreatic inflammatory pain responses. The results demonstrate the possible utility of the gene array analysis to identify candidate genes for analgesic development.

Terminology

The pain systems are comprised of peripheral neurons and their receptors, the nociceptors, central neuronal transmitting pathways and neurons with excitatory or inhibitory effects on nociceptive information. Nociceptors are specialized receptors on nerve endings that respond to noxious to mechanical and thermal stimuli interpreted as pain. Dorsal root ganglia are the cell bodies of peripheral nerves and are located adjacent to the spinal cord in the vertebral column. ET interacts with its receptors and regulates blood flow, cell proliferation, muscle contraction or relaxation, secretion and ion transport.

Peer review

This paper reports a potentially interesting approach to an important problem, pain in pancreatitis. The paper is an original report focusing on the gene profiling to provide rationale for potential casual mechanisms and pharmacological block of functional pain related behaviors with specific antagonists. The agent dose response data present important information for potential future human translational studies.

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Colometer: A real-time quality feedback system for screening colonoscopy

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Abstract

AIM: To investigate the performance of a new software-based colonoscopy quality assessment system.

METHODS: The software-based system employs a novel image processing algorithm which detects the levels of image clarity, withdrawal velocity, and level of the bowel preparation in a real-time fashion from live video signal. Threshold levels of image blurriness and the withdrawal velocity below which the visualization could be considered adequate have initially been determined arbitrarily by review of sample colonoscopy videos by two experienced endoscopists. Subsequently, an overall colonoscopy quality rating was computed based on the percentage of the withdrawal time with adequate

visualization (scored 1-5; 1, when the percentage was 1%-20%; 2, when the percentage was 21%-40%, *etc.*). In order to test the proposed velocity and blurriness thresholds, screening colonoscopy withdrawal videos from a specialized ambulatory colon cancer screening center were collected, automatically processed and rated. Quality ratings on the withdrawal were compared to the insertion in the same patients. Then, 3 experienced endoscopists reviewed the collected videos in a blinded fashion and rated the overall quality of each withdrawal (scored 1-5; 1, poor; 3, average; 5, excellent) based on 3 major aspects: image quality, colon preparation, and withdrawal velocity. The automated quality ratings were compared to the averaged endoscopist quality ratings using Spearman correlation coefficient.

RESULTS: Fourteen screening colonoscopies were assessed. Adenomatous polyps were detected in 4/14 (29%) of the collected colonoscopy video samples. As a proof of concept, the Colometer software rated colonoscopy withdrawal as having better visualization than the insertion in the 10 videos which did not have any polyps (average percent time with adequate visualization: 79% \pm 5% for withdrawal and 50% \pm 14% for insertion, $P < 0.01$). Withdrawal times during which no polyps were removed ranged from 4-12 min. The median quality rating from the automated system and the reviewers was 3.45 [interquartile range (IQR), 3.1-3.68] and 3.00 (IQR, 2.33-3.67) respectively for all colonoscopy video samples. The automated rating revealed a strong correlation with the reviewer's rating (ρ coefficient= 0.65, $P = 0.01$). There was good correlation of the automated overall quality rating and the mean endoscopist withdrawal speed rating (Spearman r coefficient= 0.59, $P = 0.03$). There was no correlation of automated overall quality rating with mean endoscopists image quality rating (Spearman r coefficient= 0.41, $P = 0.15$).

CONCLUSION: The results from a novel automated real-time colonoscopy quality feedback system strongly

agreed with the endoscopists' quality assessments. Further study is required to validate this approach.

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Key words: Colonoscopy; Quality assurance; Quality improvement; Withdrawal time; Colon cancer; Bowel preparation

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INTRODUCTION

Colorectal cancer (CRC) is a major cause of cancer mortality worldwide, with more than one million new cases diagnosed annually^[1]. CRC screening reduces CRC incidence and mortality^[2-4]. Currently, colonoscopy is widely accepted as the most accurate method of screening the colon for CRC^[5-7]. Although there is good evidence for its positive impact in the reduction of CRC, it is also recognized that its effectiveness is dependent on the quality of the procedure^[8] which, may be highly variable in clinical practice. Several studies have highlighted important limitations in the accuracy of colonoscopy. Ineffective bowel preparation, an inability to consistently intubate the caecum, and rapid withdrawal times are significant contributors to missed lesions during colonoscopy^[9-11].

It has been suggested that a high-quality examination that ensures the detection and removal of all neoplastic lesions is key to screening efficacy^[12]. In response, professional societies have proposed the use of various quality-assessment indicators. Crucial quality indicators for colonoscopy were published in 2002 by the American Society for Gastrointestinal Endoscopy/American College of Gastroenterology^[13,14]. The assurance that colonoscopy procedures are completed with adherence to these quality standards has become important^[15,16] in the quest for high quality and effective CRC screening. Current quality indicators for colonoscopy such as mean withdrawal times and bowel preparation are endoscopist-based^[17,18]. Therefore, the quality of a colonoscopy for an individual patient may vary even when performed by an experienced endoscopist^[19,20]. The effectiveness of colonoscopy depends on adequate visualization of the entire colon and diligence during examination of the mucosa. Many colon segments can have low diagnostic yield caused by transiently rapid withdrawal, for example, when passing acute angulations of the organ^[21]. A quality indicator that

is only based on the total withdrawal time cannot recover information about colon segments which have been poorly visualized due to rapid withdrawal. In addition, the effectiveness of any colorectal cancer screening program is critically dependent on adequate bowel preparation^[22]. Poor bowel preparation contributes to inadequate visualization of the colonic mucosa. A universal, automated, real-time, feedback-based, and non-operator dependent method which could provide information about the dynamics and the level of the bowel preparation of the procedure would be particularly useful in this regard.

We propose a colometer system^[23]; a software-based, automated image analysis tool to improve the quality of the screening colonoscopy thus providing three major outputs: (1) real-time visual feedback indication of image changing velocity and image blurriness to the endoscopist; (2) automated summative statistics report provided immediately following the colonoscopy, including withdrawal time, % time of adequate visualization, and a novel graph of dynamics over time; and (3) automated stool coverage analysis for the documentation of bowel preparation. All of these outputs can be obtained automatically. Hence, this method could allow future colonoscopy quality control in the day-to-day medical practice setting.

The goal of our study was to comparatively validate the performance of the real-time feedback system for screening colonoscopy against expert opinion in clinical practice.

MATERIALS AND METHODS

System overview

The conceptual block diagram of the entire colometer system is shown in Figure 1. Initially, a high-speed analog video acquisition device (VCE-Express, Imperx, Boca Raton, FL, United States) was connected to an EPX-1 colonoscope (Pentax Canada, Mississauga, Ontario) through its S-video port before a standard colonoscopic procedure to enable a real-time video acquisition. A high-performance laptop workstation (Thinkpad W520, Lenovo Canada, North York, Ontario) equipped with Matlab 7.14 (The Mathworks, Inc., MA, United States) was employed to interface with the video acquisition unit and to allow a real-time video processing of the colonoscopy video stream at sampling frequency of 30 frames per second.

A real-time visual feedback of the colonoscopy image changing velocity and image blurriness was embedded within this video utilizing Matlab (Image Processing Toolbox™). A custom-design user control interface was developed on the workstation using Matlab (MathWorks, Natick, MA, United States) to allow the user to control the real-time feedback indicator and to provide an offline summative report.

Real-time visual feedback system

In this study, a total of 14 screening colonoscopy videos from a single endoscopy unit (Foothills Medical Campus, Calgary Zone, Alberta Health Services), Calgary, Canada) were collected, processed, and rated in a real-

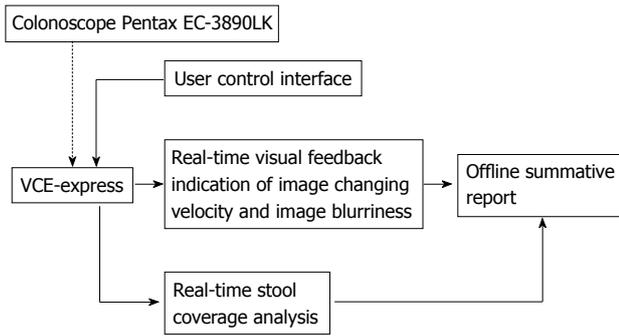


Figure 1 Conceptual block diagram of the proposed colometer system.

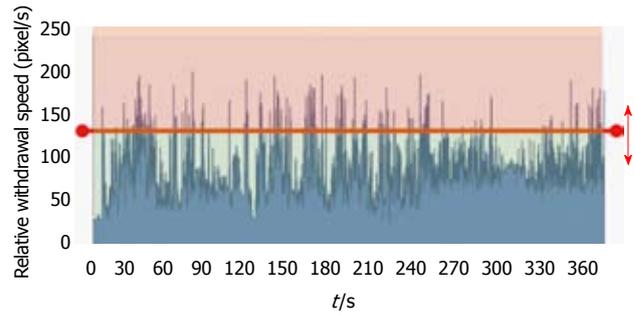


Figure 2 Relative withdrawal speed over time. Red line is the speed threshold configured by the endoscopists.

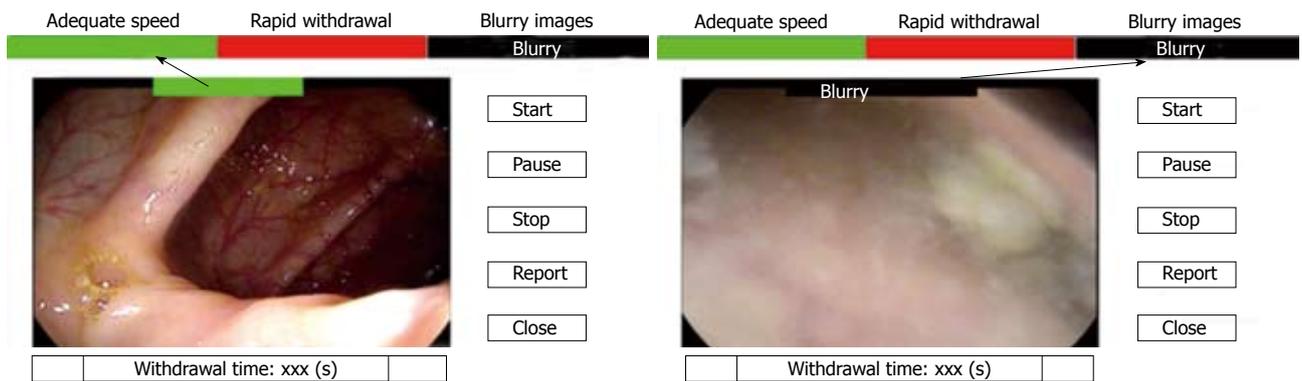


Figure 3 A sample frame with focused (left) and poor image clarity. Text indicators “adequate speed”, “rapid withdrawal”, “blurry”, were embedded into the video in a real-time to provide a visual feedback to the endoscopist.

time fashion. The colonoscopy videos were completely anonymized videos without any patient or endoscopist information on the images, filenames, or in the file meta-data. The videos were acquired for a quality assurance exercise (practice audit), for maintenance of certification purposes (for Andrews CN) and was thus exempt from consent requirements. Three other experienced gastroenterologists reviewed these videos in a blinded fashion and rated the withdrawal velocity, image quality, and colon preparation of each (scored 1-5; 1, poor; 3, average; 5, excellent) as well as overall quality on the same scale. The automated quality ratings were compared to the averaged endoscopist quality ratings.

Image changing velocity measurement: In general, the dynamics of moving objects in a video sequence can be assessed by frame differencing technique. If the temporal changes of pixel intensity have changed in a successive sequence of frames, it had to be due to the changing dynamics of objects within the image^[24]. Most frame differencing methods offer low computational complexity, which was the main constraint when designing the system to operate in real-time^[25]. Since the frame differencing technique is contrast-based and the colonoscopic image contrast can be manually adjusted according to endoscopist’s preference, a normalized approach was chosen to eliminate this problem.

Initially, an overall minimum and maximum values of velocity changes for all 14 videos were determined in real

time with 30 ms time delay. Subsequently, two different threshold ranges between these values were selected corresponding to an adequate and rapid withdrawal speeds. These ranges were further optimized to achieve agreement with the average rating from the gastroenterologists (Figure 2).

Image blurriness measurement: Colonoscopy videos also contain many blurry (out-of-focus) frames due to frequent shifts of camera positions while moving along the colon. Current endoscopes are equipped with a single, wide-angle lens that cannot be focused^[26]. Sharpness, brightness and contrast of the image are optimized using the endoscopist’s skills. In addition, the tip of the colonoscope during the procedure can be temporally buried in mucosa or closely face the colonic wall, which also results in blurry images. It is estimated that the average number of blurry frames in a colonoscopy video is 37%. However, that number can range as high as 60%, depending on endoscopists, patients and colon preparation^[27]. Numerous methods have been proposed to assess blurriness in colonoscopy videos. However, a real-time algorithm for blurriness measurement for the colonoscopy video has not yet been implemented. A variance metric calculated as the variance of the whole image was utilized to measure the blurriness of the video frames in our real-time application^[28]. During blurry video sequence, information about the image changing velocity is not important and thus was not calculated. Figure 3 shows comparison

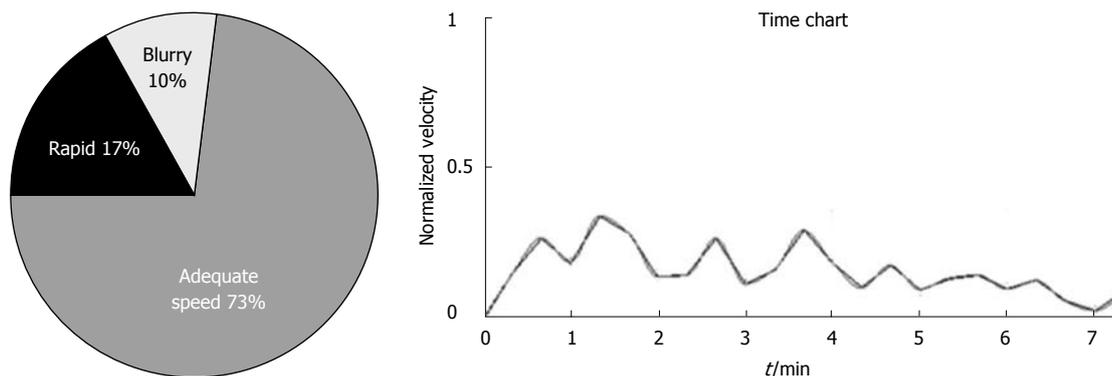


Figure 4 An automatically generated chart providing % of time of adequate speed in a colonoscopy procedure (left) and chart showing filtered velocity over time (right).

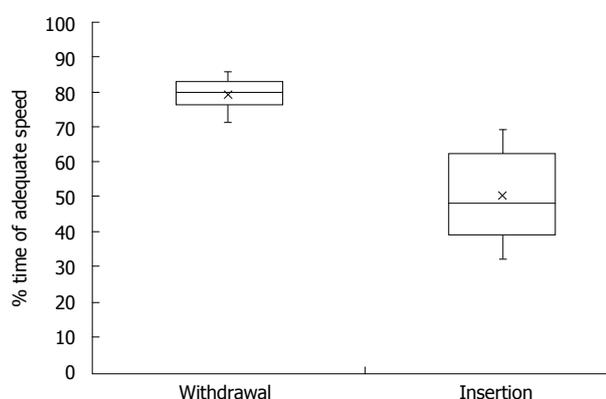


Figure 5 Comparative results (withdrawal vs insertion) for 10 colonoscopy procedures. The output mean values are marked with a cross.

between focused and blurred snapshots from a colonoscopy withdrawal video.

Stool coverage analysis: The core factor to be detected in the stool coverage analysis is the level of cleanliness in the colon. A previously reported color recognition algorithm was used for measuring this feature based on recorded colonoscopy videos^[23]. In this study, the stool coverage analysis was successfully integrated within the withdrawal and blurriness algorithm in real-time with reasonable correlation to Ottawa stool scores^[29,30]; these results are reported elsewhere^[31].

Statistical analysis

Based on these thresholds, a summative statistics report following each colonoscopy could be provided, including withdrawal time, % time with adequate visualization, and a novel graph of image changing dynamics over time. This graph may allow endoscopists to see through which portion of the colon the withdrawal was too fast. An example of a summative colometer report is given as an example in Figure 4. Moving average filter was used to smooth the velocity data for every 20 s. The peaks in the image changing dynamics graph indicate the period with frequent abrupt image changes during scoping. The slope of the line on this dynamics-time graph reveals

useful information about the acceleration of the scoping sequences. Using this information missed colonic segments could be estimated. Comparisons of continuous variables used Student's *t* test and correlations of ordinal data used Spearman coefficient. Free-margin multirater Kappa statistics were calculated for inter-rater variability^[32]. A level of *P* < 0.05 was used to determine statistical significance.

RESULTS

Proof of concept

For a colonoscopy procedure, it is widely known that the visualization quality during withdrawal is higher than the visualization quality during insertion process. Based on this assumption, a comparative study was performed to validate the functionality of the proposed algorithm by comparing the system outputs (% of time of adequate speed in a colonoscopy) between the insertion and withdrawal for 10 colonoscopy procedures. The 10 videos, which were used for validation of the functionality of the proposed algorithm, were different from the videos used to validate the automated scores against expert endoscopist. The average percentage for the colonoscope withdrawals was 79.3% ± 4.96% and it was 50.3% ± 13.95% for the insertion procedures. In a total of 10 collected videos, there was a significant difference of 29.1% ± 11% (*P* < 0.01) between both procedures. Comparative results are shown in Figure 5.

Correlation between the system output and the subjective endoscopist evaluation

Subsequently, an overall colonoscopy quality rating was computed based on the percentage of the withdrawal time with adequate visualization (scored 1-5; 1, when the percentage was 1%-20%; 2, when the percentage was 21%-40%, *etc.*). Adenomatous polyps were detected in 4/14 (29%) of the collected colonoscopy video samples. There were large differences in the withdrawal times during which no polyp was removed (range: 4-12 min). The percentage time with adequate visualization in the videos (*i.e.*, not blurry and not over the velocity

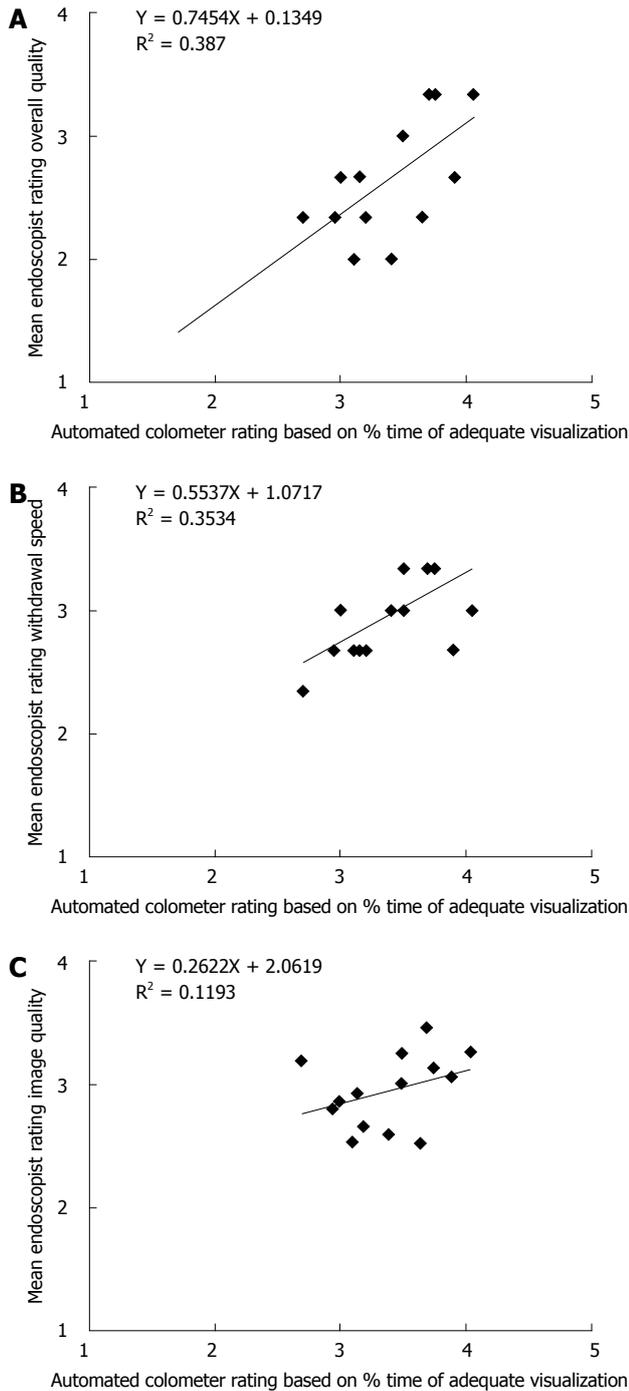


Figure 6 Correlation between the overall quality ratings from the Colometer system. A: Mean endoscopist overall quality rating ($\rho = 0.65, P < 0.01$); B: Mean endoscopist withdrawal speed rating ($\rho = 0.59, P < 0.01$); C: Mean endoscopist image quality rating ($\rho = 0.41, P = 0.15$).

threshold) ranged from 54% to 81% (mean $68\% \pm 2\%$). The median quality rating from the automated system and the reviewers was 3.45 [interquartile range (IQR), 3.10-3.68] and 2.67 (IQR, 2.33-3.00), respectively, for all colonoscopy video samples. However, there was significant variability in the endoscopist ratings (free-margin kappa statistic = 0.20).

The automated overall quality rating revealed a strong correlation with the reviewers overall quality rating

(Spearman r coefficient = 0.65, $P = 0.01$) as can be seen in Figure 6A. Similarly, there was good correlation of the automated overall quality rating and the mean endoscopist withdrawal speed rating (Spearman r coefficient = 0.59, $P = 0.03$) (Figure 6B). There was no correlation of automated overall quality rating with mean endoscopists image quality rating (Spearman r coefficient = 0.41, $P = 0.15$) (Figure 6C). There was also no correlation of the automated blurriness or excessive velocity metrics and mean endoscopists ratings (data not shown).

Comparative analysis between the withdrawal standard and the proposed algorithm

The mean withdrawal time of the videos was 5.8 min (± 0.4 min SE, range 4-10 min). Out of the 14 colonoscopy withdrawal videos, 2 videos with 6 min withdrawal time were rated as poor quality with low diagnostic yield by 3 endoscopists and confirmed with the colometer. There was no correlation of automated overall quality rating and withdrawal time (Spearman r coefficient = 0.11, $P = 0.70$).

DISCUSSION

Colonoscopy is widely used for CRC screening^[33-35] and its miss rate for advanced adenomas, neoplastic lesions or adenomas remains a concern^[36,37]. Obviously, the rate of adenoma detection is closely related to the quality of the colonoscopy^[38]. Quality assurance initiatives have been adopted by most national gastroenterology societies, with the mean withdrawal time for colonoscopy strongly correlating with adenoma detection rate. It has been accepted that endoscopists who use withdrawal times longer than 6 min detected significantly more adenomas. In this study, we present a novel, real-time colonoscopy video quality indication system for evaluating the adequacy of image clarity, the withdrawal velocity, and the bowel preparation quality.

The colometer system is a user-friendly method for evaluating the quality of individual screening colonoscopies. This is in contrast to many endoscopist-based quality indicators, such as mean withdrawal time^[39]. For example if an endoscopist has a 6 min mean withdrawal rate, many of that endoscopist's colonoscopies will presumably have a withdrawal time below 6 min^[40] and may have lower adenoma detection rates. The individuals with the more rapid withdrawals will not likely be aware of this fact. Further, calculation of mean withdrawal rates is labor-intensive, and even if published, will not likely affect patient choice of endoscopist. Thus a simple and immediate objective quality summary that can be integrated into the patient's colonoscopy report would be ideal. This is especially so in areas where reimbursement may be tied to procedure quality, as has been proposed in the United States^[41].

In our study, widely recommended criteria for colonoscopy procedures were analyzed, including withdrawal time, quality of the bowel preparation and the video qual-

ity. Quality indicators for colonoscopy have been selected to establish competence in performing colonoscopy procedures and to help define areas for continuous quality improvement^[42,43]. The Colometer quality measurements appear to correlate well with these outcomes in this small study.

This new computer-based method is based on image processing analysis of the live video feed from the colonoscope processor, and just as easily can be run on digitized video files for retrospective review. The method uses widely available video processing technology and can be retrofitted onto any endoscope processor with digital video output. No remote processing of video is required, which will prevent any privacy breach risks. Output of summative metrics is immediate, and could be added to individual endoscopy reports, as well as database recording for simple collection of quality control data in the practice setting on a large scale.

This study was designed to show the proof of the Colometer concept and compare it to subjective quality assessments based on retrospective video recordings of colonoscopy. The software however is designed to provide real-time visual feedback to the endoscopist during the withdrawal itself. We did not evaluate the actual feedback to endoscopists during the colonoscopy to determine whether this type of output (like a speedometer for the scope driver) affects withdrawal speed or other quality measures, but studies in this regard are underway. Further uses could be used in the education and (re)credentialing fields.

This study has some limitations. First, it was a small pilot-study looking at mostly good quality colonoscopies. The performance characteristics of Colometer may change with a broader spectrum of colonoscopy withdrawals. Second, overall quality of a screening colonoscopy is a multi-dimensional concept, which may not be completely captured by a small number of quality metrics. However, the established metrics are certainly not perfect (as shown by the fact that 2 of the videos with a 6 min withdrawal time were in fact of poor quality). The advantage of an automated system however removes the variability and subjectivity of current quality metrics. Third, the small numbers in this study may have led to underpowering of the correlations. Additionally, the inter-rater variability was high. This is due to a number of factors, including the fact that the rating scale used was unvalidated, despite its simplicity and face validity. Moreover, the raters were not trained prior to the study to “anchor” their responses, which we admit is a limitation of this study. Interestingly, the rater with the most experience in colonoscopy quality assessments had outstanding correlation with colometer (data not shown), and thus inter-rater correlation will likely be tighter in future anticipated studies. Finally, some endoscopists may resist the concept of real-time constant scrutiny of their screening colonoscopies. However, it is clear that high-quality screening colonoscopy requires skill and attention, in addition to expensive infrastructure and has measurable

(although small) patient risk. We therefore should strive for continuous quality improvement of this procedure, and if heightened, non-punitive, scrutiny improves it (i.e., the Hawthorne effect) this is likely a positive direction for patients.

In conclusion, a new real-time feedback system for screening colonoscopy procedures was proposed. The results from this system based on the withdrawal dynamics and image quality strongly agreed with the endoscopists’ quality ratings. The colometer system could facilitate a real-time colonoscopy quality feedback for clinical practice, with easily accessible, in depth colonoscopy quality assessment for individual patients. Such a device could also be a potential training tool for new endoscopists. Further study is required in order to better define the optimal quality thresholds and to validate this approach in a larger clinical trial.

COMMENTS

Background

Colonoscopy is widely accepted as the most accurate method of screening the colon for colorectal cancer (CRC) however its effectiveness is dependent on the quality of the procedure which may be highly variable in clinical practice. Image quality and withdrawal time or velocity are important determinants of quality. A novel automated computer-based assessment tool was described and tested.

Research frontiers

It has become clear that colonoscopy quality is a major determinant of the detection of polyps in CRC screening, and there has been significant recent interest in the creation and evaluation of quality metrics for colonoscopy.

Innovations and breakthroughs

To date, there has been limited research on computerized colonoscopy video quality assessment in the published literature. The Colometer software represents an advance by using simple algorithms to characterize a complex procedure, with the added advantages of offering a real-time feedback tool to the endoscopist, immediately available patient-level quality metrics, and with minimal hardware or software requirements.

Applications

The colometer could be potentially applied to clinical screening colonoscopy practice to assess and ultimately improve colonoscopy quality.

Terminology

Colonoscopy quality: A multi-dimensional concept that affects the ability to identify and remove pre-cancerous polyps from the colon in screening colonoscopy for CRC. It incorporates many factors, including the effectiveness of the bowel preparation, the withdrawal speed of the scope, and the skill and care taken to thoroughly assess all surfaces of the colon.

Peer review

The manuscript is well written and has useful information that would be of interest to the readers.

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Use of butyrate or glutamine in enema solution reduces inflammation and fibrosis in experimental diversion colitis

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Abstract

AIM: To investigate whether butyrate or glutamine enemas could diminish inflammation in experimental diversion colitis.

METHODS: Wistar specific pathogen-free rats were submitted to a Hartmann's end colostomy and treated with enemas containing glutamine, butyrate, or saline. Enemas were administered twice a week in the excluded segment of the colon from 4 to 12 wk after the surgical procedure. Follow-up colonoscopy was performed every 4 wk for 12 wk. The effect of treatment was evaluated using video-endoscopic and histologic scores and measuring interleukin-1 β , tumor necrosis factor-alpha, and transforming growth factor beta production in organ cultures by enzyme linked immunosorbent assay.

RESULTS: Colonoscopies of the diverted segment showed mucosa with hyperemia, increased number of vessels, bleeding and mucus discharge. Treatment with either glutamine or butyrate induced significant reductions in both colonoscopic ($P < 0.02$) and histological scores ($P < 0.01$) and restored the densities of collagen fibers in tissue ($P = 0.015$; $P = 0.001$), the number of goblet cells ($P = 0.021$; $P = 0.029$), and the rate of apoptosis within the epithelium ($P = 0.043$; $P = 0.011$) to normal values. The high levels of cytokines in colon explants from rats with diversion colitis significantly decreased to normal values after treatment with butyrate or glutamine.

CONCLUSION: The improvement of experimental diversion colitis following glutamine or butyrate enemas highlights the importance of specific luminal nutrients in the homeostasis of the colonic mucosa and supports their utilization for the treatment of human diversion colitis.

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Key words: Diversion colitis; Butyrate; Glutamine; Short-chain fatty acids; Cytokines

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INTRODUCTION

Diversion colitis is a complex, nonspecific inflammatory disease that occurs in an excluded colonic segment in almost all patients submitted to a fecal diversion, such as loop colostomy or Hartmann's procedure (end colostomy with closure of the distal colon's segment)^[1,2]. Clinical manifestations typically include tenesmus with abundant rectal discharge of mucus or blood and abdominal pain. The inflammatory process and the severity of symptoms are highly variable, and occasionally, areas of erosions and ulcerations spread throughout the segment and may cause severe colonic hemorrhage^[3,4]. In some cases, it may be difficult to distinguish diversion colitis from other diseases that mimic not only the clinical manifestations but also the colonoscopic appearance. It is essential that diversion colitis not be mistaken for other diseases, especially ulcerative colitis or Crohn's disease, for this may delay the initiation of appropriate treatment. Reestablishment of the fecal stream is the ideal therapy for diversion colitis, but the best outcomes are directly related to the correct diagnosis^[5]. However, many patients need to remain with the colostomy for long periods, and some will never attain the reconstruction of intestinal continuity. As a consequence, it is expected that diversion colitis will impair the quality of life in a significant number of patients.

The diagnosis and follow-up of diversion colitis usually require endoscopic and histologic analyses, but none of these are specific^[6]. The endoscopic appearance ranges from absent to severe inflammatory features, including mucus discharge, luminal narrowing, friability, erythema, ulceration and a distorted mucosal vascular pattern^[7,8]. Mucosal biopsy of the diverted segment is an important auxiliary tool for the clinical or surgical management. Histological findings usually include mucosal edema, inflammatory infiltration with follicular lymphoid hyperplasia, vascular congestion, decreased number and depth of crypts, expansion of cellular elements and edema of the lamina propria^[9,10].

Bacterial proliferation^[11,12], overproduction of free

oxygen radicals^[13], impairment of butyrate oxidation^[14,15], defective transport of short-chain fatty acids (SCFAs)^[16], and immunological factors^[17] are some of the pathogenic mechanisms proposed for explaining the intestinal inflammation after a surgical fecal diversion. Nevertheless, the exact etiopathogenesis of diversion colitis remains unclear. Although an imbalance in the mucosal production of proinflammatory and immunoregulatory cytokines has been implicated in the etiopathogenesis of inflammatory bowel disease (IBD)^[18,19], this mechanism has not been well characterized in diversion colitis, and its potential pathogenic role is yet to be determined.

Some new data suggest that the lack of luminal nutrients may be crucial in the development of diversion colitis. SCFAs, in particular butyrate, are considered the major energy source for colonocytes and have been associated with a mucosal trophic effect^[16,20,21]. Therefore, it seems that dietetic supplementation with SCFAs or glutamine increases the function and repair of the intestinal mucosa under several conditions, such as radiotherapy, chemotherapy, inflammation, trauma and sepsis^[22-24]. In animal models, butyrate and glutamine administered through the oral route or enemas reduce inflammatory processes of the intestinal colonic mucosa^[25-29]. However, the available data concerning the treatment of human diversion colitis are still not clear.

Hence, the aim of this study was to investigate whether administering essential nutrients for colonocytes, such as butyrate and glutamine, could alleviate mucosal inflammation and minimize scarring lesions in a model of diversion colitis.

MATERIALS AND METHODS

Animals

The present study was carried out at the Center of Experimental Surgery of the School of Medicine of the Federal University of Rio de Janeiro (UFRJ). The care and use of animals and the procedures reported in this study were approved by the Ethical Committee for Laboratory Animals of the UFRJ and were in accordance with the guidelines of the International Care and Use Committee of the National Institutes of Health and the Guide for the Care and Use of Laboratory Animals.

Sixty adults Wistar rats (*Rattus norvegicus*) of both sexes, specific pathogen-free and five months old, with mean body weight 250 g, were maintained on a 12 h/12 h light/dark cycle in a temperature-controlled room (24 °C). Animals were fed standard rat chow and submitted to fasting for 24 h prior to the surgical procedure. Water was offered *ad libitum* during the whole experiment.

Experimental groups

Animals were randomly assigned to four groups of 12 animals each: the control group consisted of rats without any intervention (no surgery) but subjected to a colonoscopy and to the extraction of the distal segment of the colon. The treatment groups were submitted to a

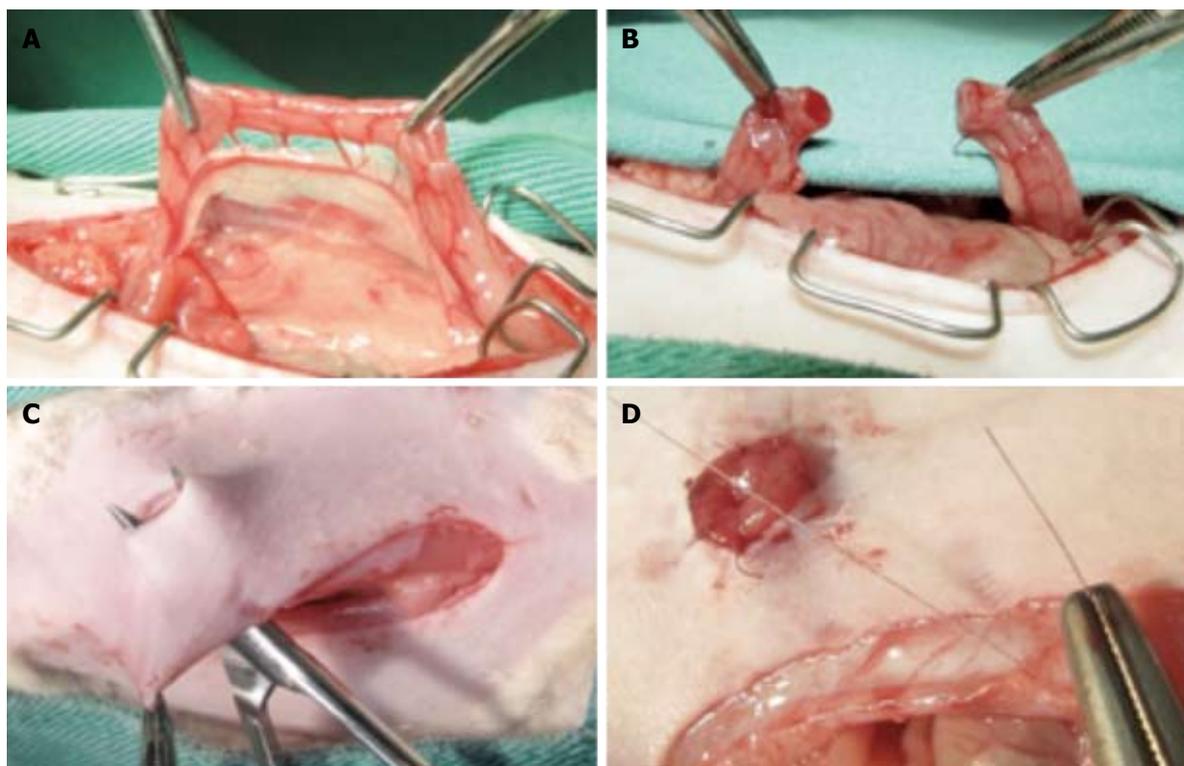


Figure 1 Steps of the surgical procedure. A: Isolation of the distal colon exposing the vascular arcade; B: Section of distal colon; C: Confection of colostomy; D: Completion of colostomy.

Hartmann's colostomy (standardized surgical procedure) followed by treatment with enemas containing saline, butyrate, or glutamine.

Surgical procedure

The anesthetic procedure consisted of the intraperitoneal administration of ketamine (1 mg/kg) associated with xylazine (0.1 mg/kg). Immediately after anesthetic administration, animals were immobilized in the dorsal decubitus position, and the skin was cleaned with an antiseptic surgical scrub solution after shaving the surgical site.

After a midline abdominal incision, animals were subjected to a Hartmann's colostomy. With the exception of the control group, all animals had the left colon transected transversally and the proximal segment brought out as an end colostomy, while the distal segment was sewn over and left within the abdomen as a blind rectal pouch. After the surgical manipulation, the laparotomy was closed by two layers of continuous suture (Figure 1). In the first two postoperative days, animals received analgesia with dipyrone (1 g/L) in the drinking water. A caloric supplement with glucose was also provided because they had been deprived of solid food for the 24 h before surgery.

Treatment protocols

A standard volume of 1 mL containing saline (placebo), butyrate (40 mmol) or glutamine (50 g/L) was instilled into the distal excluded segment of the colon through a rubber cannula (4 cm long), under light anesthesia. Ani-

mals were maintained in the Trendelenburg position for 2 min after the procedure. Enemas were administered twice a week beginning at week 4 after surgery and going through week 12.

Colonoscopies

Colonoscopies were performed under light anesthesia before the surgical procedure and thereafter every 4 wk, starting at 4 wk and going through the total follow-up of 12 wk. In addition to the real-time evaluation of mucosal appearance, video-endoscopic recorded movies were also used to confirm the results before and after the different treatments. Criteria for the endoscopic analysis were based on four parameters: hyperemia, number and size of vessels, bleeding, and mucous secretion, as previously described^[7]. Each parameter was assigned 0 to 4 points, and the sum of all parameters was used to generate a final endoscopic score.

After the 8th-week colonoscopic procedure, half of the animals from each group ($n = 6$) were euthanized by anesthetic overdose, without any pain or suffering. Three samples were excised from the colon for histological assessment and for organ cultures. The other animals from all groups ($n = 6$) were euthanized after the last colonoscopic procedure.

Histologic examination

Specimens were fixed in 40 g/L formaldehyde saline, embedded in paraffin, cut into serial sections of 5 μ m, and submitted to the different staining procedures. His-

tomorphological analysis were performed under light microscopy by two independent observers who were unaware of the experimental data and who examined all tissue sections and captured images (Leica Microsystems Ltd, Switzerland).

The following histological parameters involving inflammatory and trophic alterations were studied in hematoxylin-eosin-stained slides: mucosal edema, lymphoplasmacytic inflammatory infiltrate, lymphoid follicular hyperplasia, vascular congestion, number and depth of crypts, and the density of cellular elements in the lamina propria. Each parameter was assigned 0 to 2 points, and a total score was obtained by the sum of all parameters, as previously described^[30].

Assessment of collagen deposition in the colon wall

The phosphomolybdic acid-picro-sirius red dye was used to stain collagen fibers in the tissue of serial paraffin sections obtained as described above. At least 10 different areas per tissue section were analyzed under light microscopy at $\times 100$ magnification. The density of collagen fibers was calculated as the area positively stained for collagen in relation to total intestinal tissue using an imaging analysis system (Leica QWin Plus V 3.5.1, Leica Microsystems Ltd, Switzerland).

Assessment of goblet cells in the colonic mucosa

The periodic acid of Schiff (PAS) was used to stain goblet cells within the intestinal epithelium. The density of goblet cells was defined as the percentage of PAS-positive cells within at least 500 epithelial cells in the crypts and in the surface epithelium of longitudinally sectioned colonic pits.

Assessment of apoptosis in the colonic mucosa

To analyze apoptosis within the colon, fragmented DNA was stained by the terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay, with the Apoptag[®] Plus Peroxidase In Situ Apoptosis Kit (Millipore, Billerica, MA, United States). Paraffin sections were first deparaffinized, incubated with proteinase K solution for 15 min at room temperature, and then immersed in hydrogen peroxide to block endogenous peroxidase activity. After rinsing with phosphate buffered saline (PBS), slides were incubated with equilibration buffer for at least 1 min, followed by incubation with a solution containing TdT enzyme. Sections were incubated at 37 °C for 1 h. A second section from each sample, incubated without TdT enzyme, constituted the negative controls. Positive controls were prepared by treating samples with DNase I (Sigma, Deisenhofen, Germany). The reaction was terminated by washing the slides with pre-warmed stop/wash buffer solution for 10 min. Specimens were subsequently incubated with non-immune horse serum for 20 min and subsequently with an anti-digoxigenin peroxidase conjugate for 30 min at room temperature. After being rinsed in PBS, all sections were developed with a solution containing hydrogen peroxide

and diaminobenzidine. Preparations were lightly counterstained in Harris's hematoxylin, dehydrated, and mounted in Permout (Fisher Scientific, Pittsburgh, PA, United States). Morphologically preserved TUNEL-positive cells and apoptotic bodies were referred to as apoptotic cells and identified by using pre-defined measurements in the computer-assisted image analyzer in conjunction with careful evaluation of morphologic criteria. Percentages of apoptotic cells were defined by the number of immunoreactive cells in relation to total cells (immunoreactive and nonimmunoreactive cells; $\times 400$ magnification), counted among at least 500 epithelial cells in the crypts and in the surface epithelium of longitudinally sectioned colonic crypts. Two independent observers who were unaware of the experimental data examined all tissue sections and captured images.

Organ culture and cytokine measurements

Colonic mucosal explants were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Life Technologies, United States), 10 mmol HEPES (Pro-mega, United States), penicillin (100 kU/L) and streptomycin (100 mg/L) (Sigma-Aldrich, St. Louis, MO, United States) for 24 h at 37 °C in a 50 mL/L CO₂ humidified incubator. After incubation for 24 h, the supernatant was collected and stocked at -20 °C. Samples were centrifuged and the supernatants used to measure the concentrations of the cytokines tumor necrosis factor-alpha (TNF- α), interleukin (IL)-1 β and transforming growth factor beta (TGF- β) by enzyme-linked immunosorbent assay (ELISA) (R and D Systems, MN, United States). The total protein content of the biopsy specimens was estimated by the bicinchoninic acid method, and values were used to normalize ELISA results. The minimum detectable concentrations of rat TNF- α , IL-1 β , and TGF- β were typically less than 5.0 ng/L.

Statistical analysis

Statistical analyses were performed using the statistical software SPSS for Windows (version 10.0.1, SPSS Inc., 1989-1999, United States). Significant differences among the experimental groups were evaluated by one-way ANOVA followed by pairwise multiple comparisons using Dunnett's T3 test. Colonoscopic score changes before and after treatment were compared by the Wilcoxon matched-pair signed-rank test. Values are expressed as medians (1st quartile, 3rd quartile). The level of significance was set at $P < 0.05$.

RESULTS

An inflammatory process similar to human diversion colitis was well reproduced in the experimental model, and the peak inflammation was observed at 8 wk after induction.

Endoscopic changes in the colon

Diversion colitis manifested predominantly as patchy inflammatory lesions in the excluded distal colon. Colo-

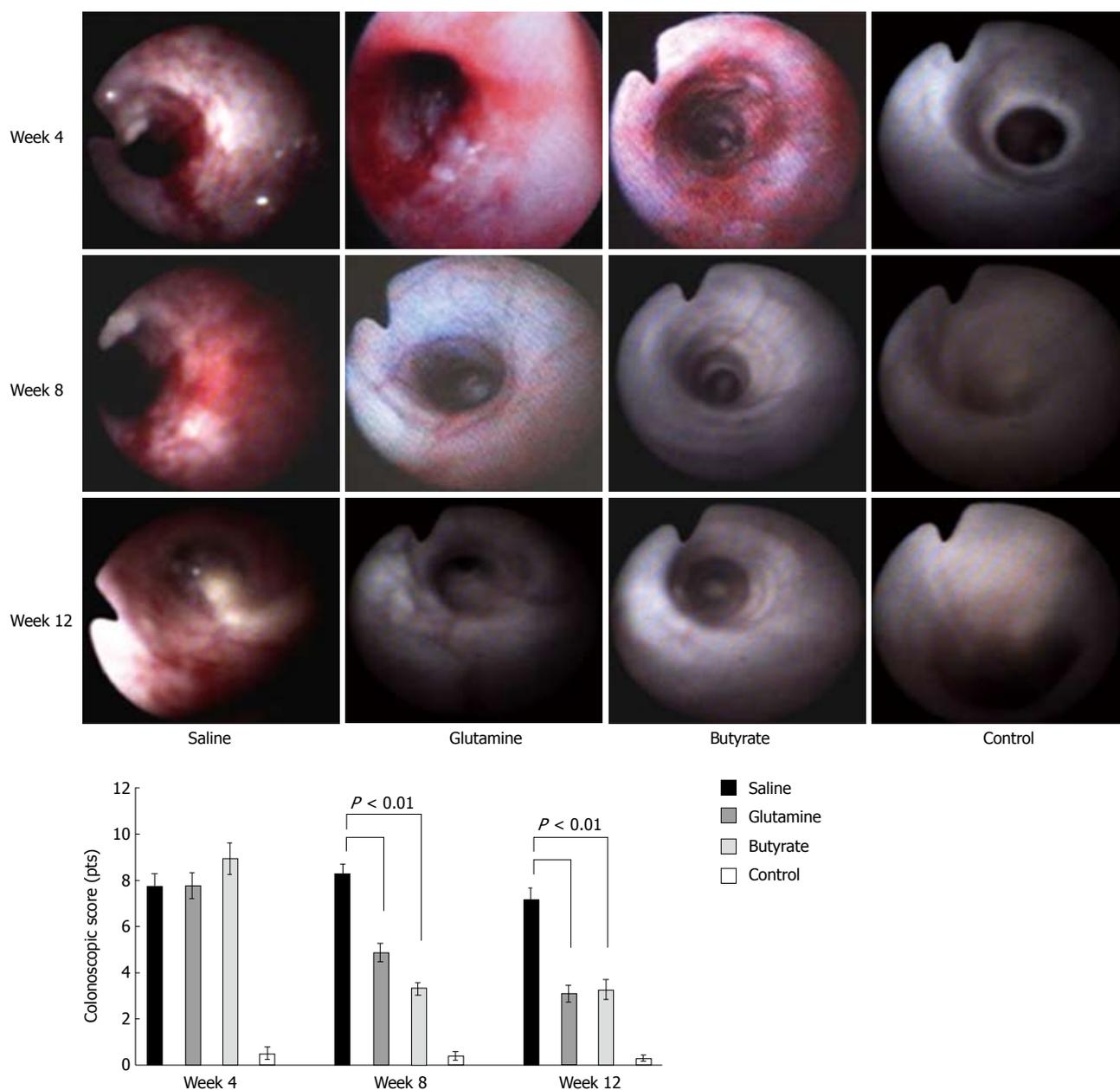


Figure 2 Endoscopic images of the diverted segments of the colon treated with different protocols at 4 wk, before treatment, and at 8 wk, the peak of inflammation in this experimental model. Diversion colitis is characterized by the presence of a fragile mucosa, hyperemia, spontaneous bleeding, and increased number of vessels and mucous secretion (at 8 wk). Colonoscopic images were semi-quantitatively analyzed with an endoscopic score. Animals treated with either glutamine or butyrate showed significantly reduced colonoscopic scores compared to animals without any treatment at 8 and 12 wk. Significant differences are noted ($n = 6$ in each group).

noscopy images of the diverted segment with colitis displayed a fragile mucosa, with hyperemia, increased number of vessels, spontaneous bleeding, and increased mucous secretion (Figure 2). Animals treated with either butyrate or glutamine showed a significant reduction in the endoscopic scores compared to saline-treated animals at both 8 and 12 wk (Figure 2).

Histological changes in the colon

Four weeks after surgery, the histological evaluation of the involved areas of the colon showed mucosal edema, inflammatory infiltration with follicular lymphoid hyperplasia, vascular congestion, decreased number and depth

of crypts, expansion of lamina propria cellular elements, and evidence of transmural inflammation (Figure 3A-F). Significantly higher histologic inflammatory scores were found in the intestinal mucosa of all animals submitted to colitis induction through colostomy compared to the normal mucosa of controls. Butyrate- or glutamine-treated animals presented significantly reduced histologic scores of the colon compared to those of the saline-treated group at 8 and 12 wk (Figure 3G).

Number of goblet cells in the colonic lamina propria

The number of goblet cells in the colonic mucosa was significantly lower in saline-treated rats, compared with

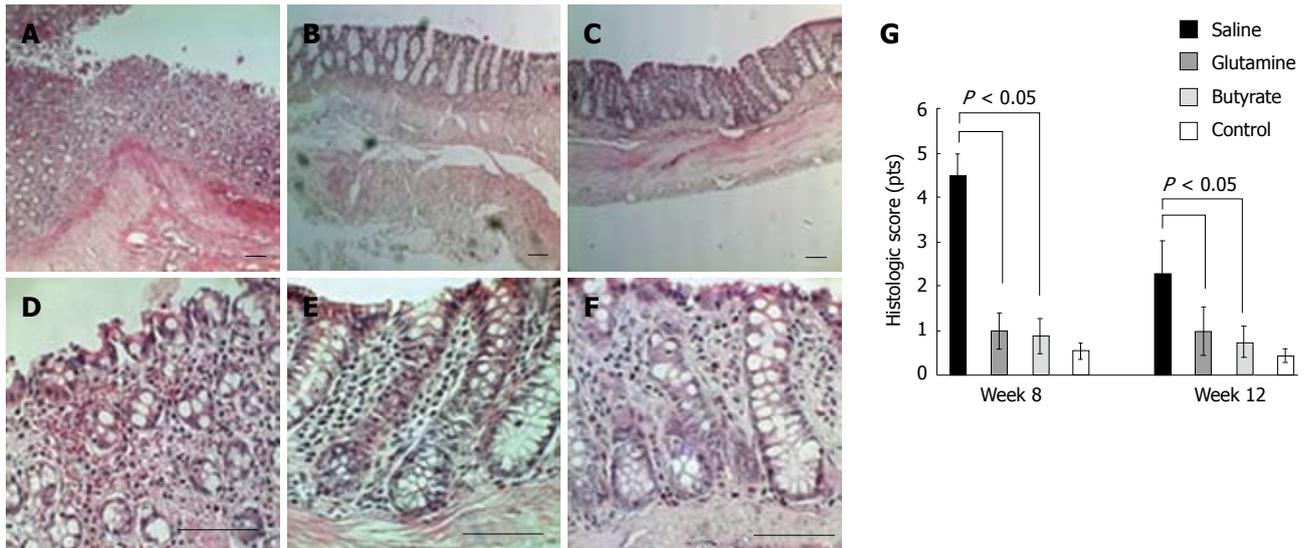


Figure 3 Hematoxylin-eosin-stained slides of diverted colon samples obtained at 8 wk. A, B: Images were captured at $\times 100$ and $\times 400$ magnifications, respectively. Diversion colitis is characterized by a predominantly mononuclear cell infiltration, hyperemia, vasodilatation, and associated atrophic changes; C, D: Glutamine-treated animals show a slightly inflamed colon; E, F: Butyrate-treated animals show an almost normal colon ($n = 6$ in each group). Bars represent $50 \mu\text{m}$.

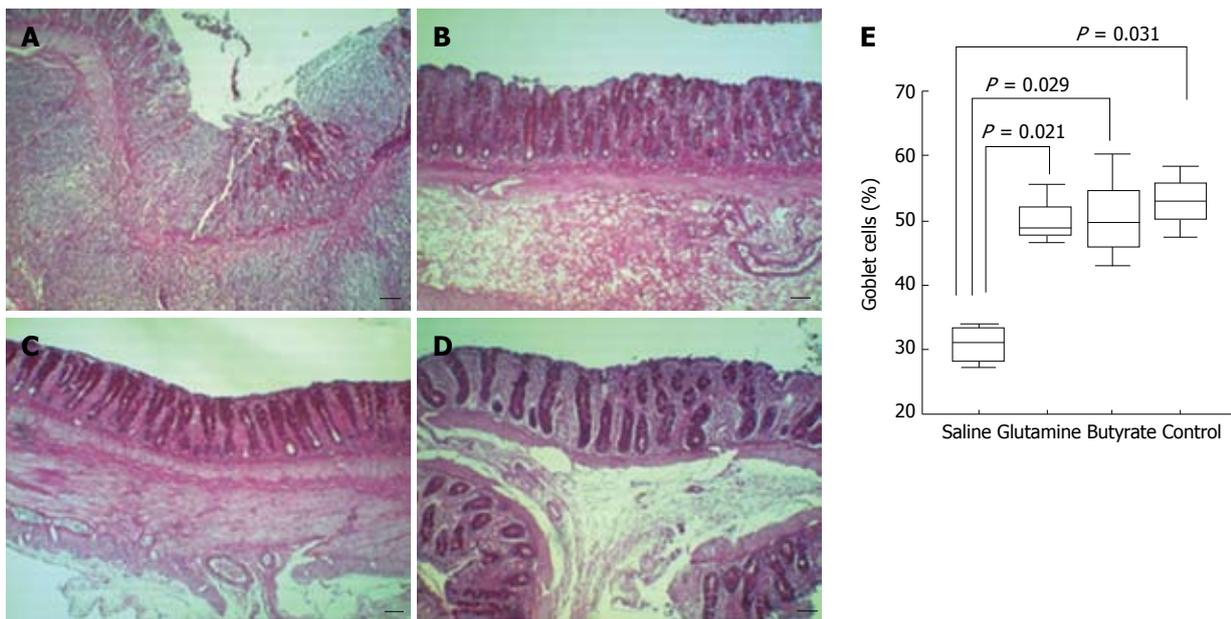


Figure 4 Quantitative analysis of goblet cells using periodic acid-schiff-stained slides of diverted colon samples obtained at 8 wk. Images show a significant reduction in the number of goblet cells in slides from animals with placebo-treated (A) colitis compared to (B) glutamine-treated, butyrate-treated (C) and control animals (D). Images were captured at $\times 100$ magnification. In the slides, bars represent $50 \mu\text{m}$. In the graph (E), horizontal bars represent medians, boxes represent the 25th and 75th percentiles, and vertical bars represent ranges. Significant differences are noted ($n = 6$ in each group).

the higher numbers found in the therapeutic glutamine or butyrate groups, which were similar to those of the normal control group (Figure 4).

Deposition of collagen fibers in the colon wall

Increased densities of collagen fibers were found in saline-treated animals, with a diffuse distribution throughout the colon wall. In contrast, rats treated with glutamine or butyrate enemas showed a significant reduction in collagen deposition, with levels similar to those observed in control animals (Figure 5).

Apoptotic rates in the colonic mucosa

In this diversion colitis model, significantly reduced rates of apoptotic TUNEL-positive cells were observed in the epithelium of glutamine- ($P = 0.042$) and butyrate-treated colitic animals ($P = 0.011$) compared to saline-treated animals. No significant difference was detected between glutamine- and butyrate-treated animals (Figure 6).

Cytokine production by the colonic mucosa

TNF- α , IL-1 β , and TGF- β production by the colonic mucosa of the diverted segment obtained at 8 wk was

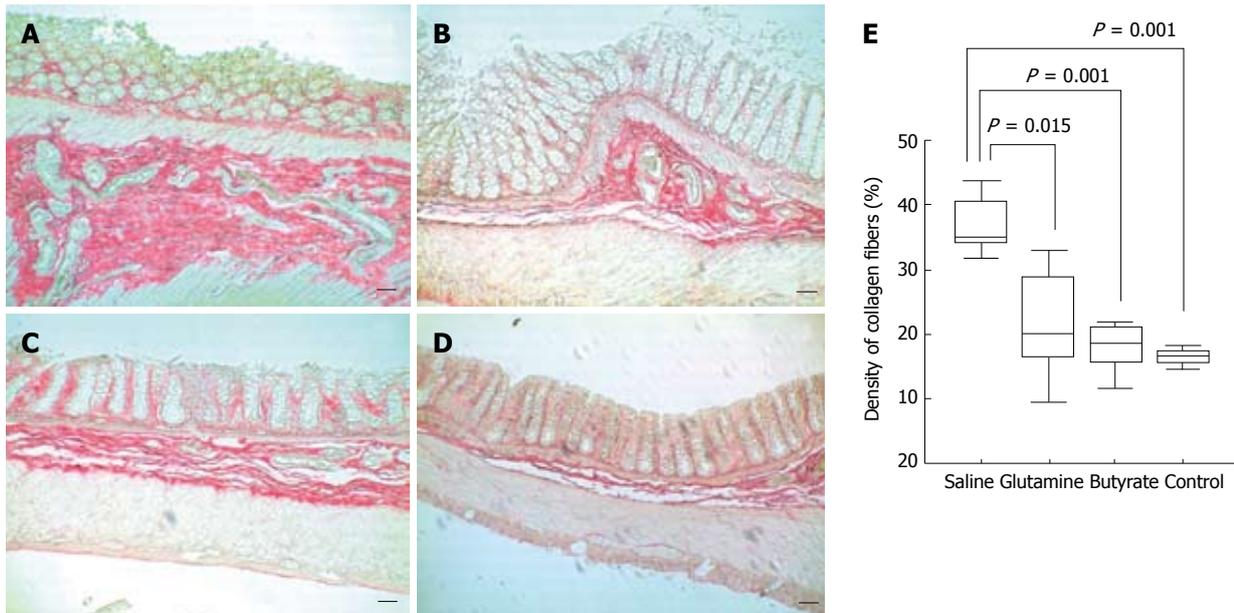


Figure 5 Quantitative analysis of collagen fiber deposition in picric acid-stained slides and apoptotic terminal deoxynucleotidyl transferase dUTP nick end labeling-positive cells of diverted colon samples obtained at 8 wk. Images show a significant increase of collagen density in slides from animals with placebo-treated (A) colitis compared to (B) glutamine-treated, butyrate-treated (C) and control animals (D). Images were captured at $\times 100$ magnification. In the slides, bars represent 50 μm . In the graph (E), horizontal bars represent medians, boxes represent the 25th and 75th percentiles, and vertical bars represent ranges. Significant differences are noted ($n = 6$ in each group).

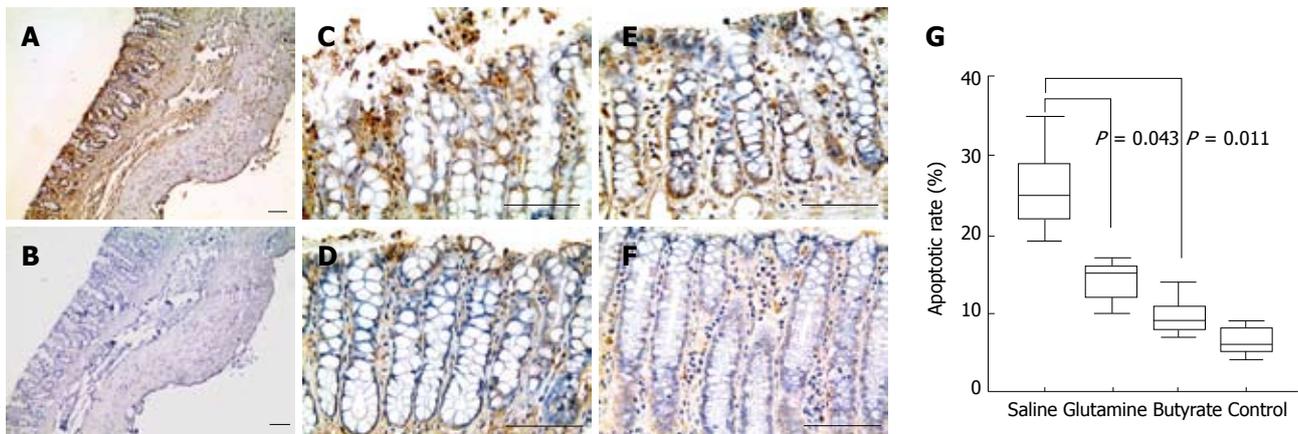


Figure 6 Quantitative analysis of apoptotic terminal deoxynucleotidyl transferase dUTP nick end labeling-positive cells of diverted colon samples obtained at 8 wk. Images show a significant reduction in the number of apoptotic cells in slides from animals with placebo-treated (A) colitis compared to (B) glutamine-treated, butyrate-treated (C) and control animals (D). Images were captured at $\times 100$ magnification. In the slides, bars represent 50 μm . In the graph (G), horizontal bars represent medians, boxes represent the 25th and 75th percentiles, and vertical bars represent ranges. Significant differences are noted ($n = 6$ in each group).

evaluated by ELISA in the supernatants of 24 h organ cultures. High levels of TNF- α (Figure 7A), IL-1 β (Figure 7B), and TGF- β (Figure 7C) were detected in the supernatants of saline-treated colitis, and significantly lowered to normal values following treatment with glutamine (Figure 7A) or butyrate (Figure 7A-C).

DISCUSSION

The experimental model presented in this study demonstrates that the simple deviation of the fecal stream through a colostomy is capable of inducing inflammation similar to human diversion colitis, even in specific

pathogen-free animals. Both glutamine and butyrate treatments significantly attenuated the inflammatory process, supporting a crucial role for specific luminal nutrients in intestinal homeostasis. We show that the inflammatory process is characterized by inflammatory cell infiltration, collagen fiber deposition, and atrophic changes in the colon, with loss of terminally differentiated goblet cells and increased local apoptotic rates, resulting in colonic remodeling. In addition, we show that a Th1-type of immune response is likely to be responsible for the inflammatory process and that the simple feeding of glutamine or butyrate can downregulate pro-inflammatory cytokines, reducing colonic inflammation.

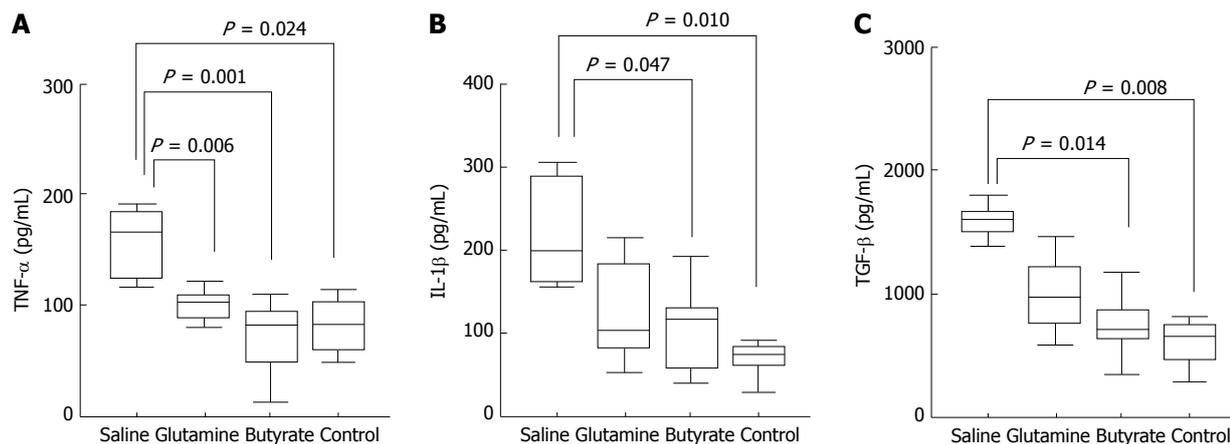


Figure 7 Cytokine production by the colonic mucosa of the diverted segment, obtained at 8 wk. Cytokines in 24 h organ cultures were measured by enzyme-linked immunosorbent assay and presented in pg/mL of culture supernatant, normalized to the protein content of tissues. A: High levels of tumor necrosis factor- α (TNF- α) measured in saline-treated colitis were restored to normal values following treatment with glutamine or butyrate; B, C: The high levels of interleukin (IL)-1 β and of transforming growth factor beta (TGF- β) detected in supernatants of saline-treated colitis decreased significantly after treatment with butyrate. Horizontal bars represent medians, boxes represent the 25th and 75th percentiles, and vertical bars represent ranges. Significant differences are noted ($n = 6$ in each group).

Diversion colitis constitutes an inflammatory process that develops within a colon segment following surgical deviation of the bowel traffic, and it usually includes the whole excluded segment, with findings common to other inflammatory conditions of the colon^[1,2]. Hence, it would be expected that the human disease could be reproducible in animal models^[30,31]. In accordance with our results, another study in rats demonstrated that inflammatory abnormalities can be detected after the fourth week following colostomy, reaching their peak between week 8 and 12^[32]. In the present study, we aimed to establish an inflammatory curve for diversion colitis. To accomplish this, we performed serial colonoscopies every two weeks from week 4 to week 20 in a pilot study, testing loop colostomy *vs* Hartmann's surgery. We confirmed that the inflammatory process appears after week four, peaking at week 8, and that the best results were obtained with Hartmann's colostomy. In addition, we showed that at sixteen week, most animals displayed a spontaneous attenuation of the inflammation. Because of these findings, we determined the best period of time for the study of inflammation and treatment outcomes in the experimental model.

In humans, diversion colitis typically occurs between three and thirty-six months after surgery, and once developed, the pathologic process and clinical manifestations can persist for decades^[10,33]. Nevertheless, it seems important to note that the biologic cycle of rats is faster compared with humans, and as a consequence, the timing of the establishment of colitis and the response to treatment are also expected to be different. Regarding the severity of diversion colitis, there appears to be considerable variation among individuals submitted to colostomy^[7,10]. This fact can be explained, at least in part, by the type of surgical procedure. For example, the loop colostomy technique allows the passage of some fecal residues to the excluded segment, sufficient to nourish colonocytes and most likely preserving their viability and function.

Although the exact mechanism underlying the pathogenesis of diversion colitis is yet to be determined, the deficiency of SCFAs is thought to play an important role in the inflammatory process^[34,35]. Because SCFAs constitute the major source of energy for human colonocytes^[36,37], SCFA depletion in the defunctionalized colon segment would most likely result in a homeostatic imbalance of a rapid-turnover tissue. Energy deprivation can limit cell restoration or induce colonocyte apoptosis, consequently disrupting the epithelial barrier. In this work, the reduction of goblet cells together with increased apoptotic rates within the colonic mucosa reflects the characteristic atrophic changes of the inflammatory process. Once the mucosal barrier is disrupted, the lamina propria is overexposed to luminal antigens, thus exacerbating the inflammatory response. Of note, some studies suggest that SCFA deprivation may constitute an additional factor for the development or the exacerbation of IBD^[35,38,39]. In addition, enemas containing SCFAs have been utilized with relative success in subsets of patients with IBD^[40,41].

Among the SCFAs, butyrate is the preferential nutrient for colonocytes. *In vitro* in both rat and human colonocytes, most oxygen consumption is due to butyrate oxidation^[14,15]. Therefore, in this work we chose to work with butyrate in its isolated form, devoid of other SCFAs, in contrast to previous works^[35,42,43].

Although the etiopathogenesis of IBD is complex and multifactorial, the chronic inflammatory process involves the excessive production of proinflammatory cytokines by the intestinal mucosa^[44]. In particular, in Crohn's disease, the disruption of the intestinal epithelial barrier and increased apoptosis are paralleled by a predominant local Th1/Th17-type of immune response^[18,45,46]. In our model, we observed a remarkable mononuclear cell accumulation within the lamina propria and atrophic changes in the epithelial layer, together with overproduction of IL-1 β and TNF- α . Most interesting, butyrate and to a lesser

extent glutamine significantly reduced the production of these Th1-type pro-inflammatory cytokines. In addition, the high levels of cytokines were correlated with the other macroscopic and histologic parameters used to analyze diversion colitis.

The highest levels of TGF- β were also detected in samples from animals with diversion colitis, in conjunction with the increased densities of collagen fibers. TGF- β is a multifunctional cytokine with potentially high anti-inflammatory activity, but it is also critical in extra-cellular remodeling in chronic inflammatory bowel diseases^[47,48]. Here, we showed that treatment with topical butyrate significantly reduced the levels of TGF- β and collagen deposition in the colon. Although the mechanism responsible for the reduction of TGF- β is not clear, it is likely that the immunomodulatory actions of butyrate, including suppression of pro-inflammatory cytokines, could indirectly downregulate TGF- β ^[49,50]. Independently of the exact mechanism responsible for the downregulation of TGF- β , it is relevant to highlight the anti-fibrotic effect of butyrate in this model. This finding seems to indicate a potential influence of butyrate counteracting fibrogenesis, an end-point consequence of many different diseases in which chronic inflammation is the dominant pathological process.

In conclusion, the successful treatment of this experimental model with either glutamine or butyrate supports the hypothesis of luminal nutrient deprivation as a major etiopathogenic mechanism underlying diversion colitis. In particular, butyrate, with its remarkable anti-inflammatory and regenerative effects, appears as a potential new alternative to topical therapy for human diversion colitis.

COMMENTS

Background

The lack of luminal nutrients has been suggested to play a crucial role in the pathogenesis of diversion colitis. The aim of this study was to test whether butyrate or glutamine enemas could diminish inflammation in a model of diversion colitis.

Research frontiers

Investigating the effects of topical administration of specific nutrients in an experimental model of diversion colitis may be relevant to mechanisms of chronicity and new therapeutic approaches in human diversion colitis.

Innovations and breakthroughs

The novelty of the work is not exclusively related to the use of topical butyrate or glutamine to treat experimental diversion colitis or other inflammatory conditions but rather to the new mechanistic observation of the anti-inflammatory action and intestinal mucosal repair of specific luminal nutrients. Furthermore, authors clearly show that the therapeutic agents proposed, in particular butyrate, actively regenerate the mucosa and influence colonic remodeling, resulting in colitis amelioration. Another fundamental and unique point of this work is the successful real-time demonstration of the anti-inflammatory effect of butyrate or glutamine in diversion colitis by a video-colonoscopy imaging system. To the knowledge, this *in vivo* observation of the benefit of topical therapy has never been presented in the field of intestinal inflammatory disorders.

Applications

The findings in experimental diversion colitis appear to be shared with other inflammatory disorders and offer a conceptually new approach to the treatment of chronic inflammatory diseases. In particular, butyrate, with its remarkable anti-inflammatory and regenerative effects, appears as a potential new alternative to

topical therapy for human diversion colitis.

Peer review

This experimental study shows the improvement of experimental diversion colitis following glutamine or butyrate enemas. The results obtained in this study clearly highlight the importance of specific luminal nutrients in the homeostasis of the colonic mucosa and support the utilization of these nutrients for the treatment of human diversion colitis.

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Adeno-associated virus mediated delivery of Tregitope 167 ameliorates experimental colitis

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adeno-associated virus-mediated delivery of Tregitope 167 in an experimental colitis model.

METHODS: The trinitrobenzene sulfonate (TNBS) model of induced colitis was used in Balb/c mice. Subsequently after intravenous adeno-associated virus-mediated regulatory T-cell epitopes (Tregitope) delivery, acute colitis was initiated by intra-rectal administration of 1.5 mg TNBS in 40% ethanol followed by a second treatment with TNBS (0.75 mg in 20% ethanol) 8 d later. Control groups included mice not treated with TNBS (healthy control group) and mice treated by TNBS only (diseased group). At the time of sacrifice colon weight, the disease activity index and histology damage score were determined. Immunohistochemical staining of the colonic tissues was performed to assess the cellular infiltrate and the presence of transcription factor forkhead Box-P3 (Foxp3). Thymus, mesenteric lymph nodes, liver and spleen tissue were collected and the corresponding lymphocyte populations were further assessed by flow cytometry analysis for the expression of CD4+ T cell and regulatory T cell associated markers.

RESULTS: The Tregitope 167 treated mice gained an average of 4% over their initial body weight at the time of sacrifice. In contrast, the mice treated with TNBS alone (no Tregitope) developed colitis, and lost 4% of their initial body weight at the time of sacrifice ($P < 0.01$). The body weight increase that had been observed in the mice pre-treated with Tregitope 167 was substantiated by a lower disease activity index and a decreased colon weight as compared to the diseased control group ($P < 0.01$ and $P < 0.001$, respectively). Immunohistochemical staining of the colonic tissues for CD4+ showed that inflammatory cell infiltrates were present in TNBS treated mice with or without administration with tregitope 167 and that these cellular infiltrates consisted mainly of CD4+ cells. For both TNBS treated groups CD4+ T cell infiltrates were observed in the sub-epithelial layer and the lamina

Abstract

AIM: To explore the anti-inflammatory potential of

propria. CD4+ T cell infiltrates were also present in the muscularis mucosa layer of the diseased control mice, but were absent in the Tregitope 167 treated group. Numerous Foxp3 positive cells were detected in the lamina propria and sub-epithelium of the colon sections from mice treated with Tregitope 167. Furthermore, the Foxp3 and glycoprotein A repetitions predominant markers were significantly increased in the CD4+ T lymphocyte population in the thymus of the mice pre-treated with adeno-associated virus serotype 5 (cytomegalovirus promoter-Tregitope 167), as cytomegalovirus promoter compared to lymphocyte populations in the thymus of diseased and the healthy control mice ($P < 0.05$ and $P < 0.001$, respectively).

CONCLUSION: This study identifies adeno-associated virus-mediated delivery of regulatory T-cell epitope 167 as a novel anti-inflammatory approach with the capacity to decrease intestinal inflammation and induce long-term remission in inflammatory bowel disease.

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Key words: Adeno-associated virus; Regulatory T cell epitope; Inflammatory bowel diseases; Adeno-associated virus

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INTRODUCTION

Inflammatory bowel diseases (IBD) are inflammatory diseases that affect mostly young adults^[1,2]. Although the precise pathogenesis has not been identified, it is generally accepted that IBD result from inappropriate mucosal immune system responses against intestinal flora and other luminal antigens^[3-5]. IBD are associated with a reduction in quality of life^[6-8] and no curative treatments are available.

Despite the fact that novel treatment strategies, including tumor necrosis factor (TNF)-neutralizing antibodies, have greatly expanded the therapeutic armamentarium, these therapeutics do not prevent complications in IBD and many patients still have to undergo surgery^[9]. New treatment strategies that would prevent the initiation of inflammation and enable long-term remission would improve the lives of millions of individuals who are af-

ected by IBD world-wide^[10,11].

Recently, biological therapies that target immune pathways have emerged as a new therapeutic approach for the treatment of immune-mediated diseases. They include administration of monoclonal antibodies against inflammatory cytokines^[12] and those that influence immune responses such as certain small molecules, Helminths and stem cells^[10,13,14]. Since IBD are immune-mediated diseases, these biological therapies are highly promising treatment approaches and have the potential to achieve mucosal tolerance and long-term remission in IBD^[10,12-14]. Here, we introduce regulatory T-cell epitopes (Tregitopes)^[15,16] as novel biological agents that could create new possibilities for the regulation of inflammation and postulate that Tregitopes, delivered by adeno-associated virus (AAV), could be developed as a new therapeutic modality for the treatment of IBD.

Tregitopes are a set of putative regulatory T cell epitopes present in the immunoglobulin G molecule, which have been identified by using computational epitope mapping^[15,16]. Tregitope sequence 167 (Tregitope 167) and an additional sequence (Tregitope 289) were synthesized and shown to bind to multiple Major Histocompatibility complex (MHC) class II molecules and to suppress immune response when co-administered with an antigen. Tregitopes 167 and 289 were also shown to expand natural occurring regulatory T (nTreg) cells and to induce a regulatory phenotype and function in peripheral T (iTreg) cells^[15,16]. Harnessing the potential of Treg cells activated or induced by Tregitopes to regulate pathological immune responses in IBD may reduce the requirement for systemic immunosuppressive therapies. However, the use of immunomodulatory peptides in clinical applications for IBD so far have shown that the *in vivo* delivery of these peptides for therapeutic purposes is hindered by difficulties in obtaining sufficient and stable peptide concentrations^[17-19]. Therefore, novel means for stable delivery of regulatory peptides have to be explored. AAV present a good safety profile and have been shown to be effective as gene delivery vectors in the clinic for the treatment of a broad range of diseases^[20-22]. Therefore, AAV-mediated delivery represents an attractive approach to deliver the immuno-modulatory Tregitope peptides.

In the present study, the potential of AAV-mediated gene therapy for the therapeutic delivery of Tregitope 167 was explored. Systemic AAV-mediated administration of Tregitope 167 was shown to ameliorate the clinical and histo-pathological severity of trinitrobenzene sulfonate (TNBS) induced inflammatory colitis in mice. Hence, AAV-mediated delivery of regulatory T-cell epitopes appears to be a promising novel therapeutic approach for the treatment of IBD and could represent an alternative or adjunct to the use of immunosuppressive drugs.

MATERIALS AND METHODS

AAV vector production and characterization

Mouse Tregitope cDNA was synthesized (Integrated

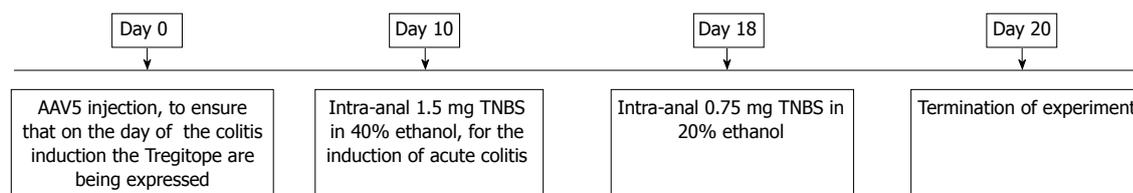


Figure 1 Schematic overview of the trinitrobenzene sulfonate induced colitis model. Mice were injected intravenously with either phosphate-buffered saline (PBS) or adeno associated virus (AAV) 5 [cytomegalovirus (CMV) promoter T-cell epitopes (Tregitope) 167]. Ten days after AAV-mediated Tregitope delivery acute colitis was initiated by intra-rectal administration of 1.5 mg trinitrobenzene sulfonate (TNBS) in 40% ethanol followed by a second TNBS treatment (0.75 mg in 20% ethanol) 8 d later. Control groups consisted of mice not treated by TNBS (healthy control group) and mice treated by TNBS only (diseased control group).

DNA Technologies, IDT, Inc) according to the published corresponding sequence^[15,16] and cloned into the plasmid pPSC10^[23] under the control of the cytomegalovirus (CMV) promoter. The Woodchuck hepatitis virus post-transcriptional enhancer was incorporated behind the Tregitope 167 cDNA to further optimize gene expression^[24]. The AAV vector, AAV5 (CMV-Tregitope 167) was produced according to a technology adapted from Negrete *et al.*^[23]. The AAV vector was purified with an anion column using the ÄKTA explorer system (GE-Healthcare). After purification, the concentration of AAV vector genomes copies (genome copies/mL) was determined at 9×10^{13} genome copies/mL by Taqman qPCR amplification. The biological infectivity of AAV5 (CMV-Tregitope 167) was demonstrated *in vitro* by PCR amplification of the “CMV-Tregitope 167” DNA fragment (product size 402 bp) on DNA isolated from HEK293T transduced with AAV5 (CMV-Tregitope 167). Primers designed and synthesized for Tregitope 167 and the CMV promoter were used.

Induction of colitis and study design

Balb/c mice (males, age 6–8 wk) were obtained from Harlan Laboratories, the Netherlands. The experimental protocol was approved by the ethical committee for animal welfare of the AMC (Academic Medical Center, Amsterdam, the Netherlands). Colitis was induced in mice by administration of TNBS (TNBS, Sigma-Aldrich), as described previously^[25]. The general procedure is summarized in Figure 1.

Mice were injected intravenously with either phosphate-buffered saline or AAV5 (CMV-Tregitope) 10 d before acute colitis was initiated by intra-rectal administration of 1.5 mg TNBS in 40% ethanol. Consecutively, a second TNBS treatment (0.75 mg in 20% ethanol) was done 8 d after the first TNBS treatment as described previously^[25]. Mice not treated with TNBS (healthy control group) and mice treated with TNBS only (diseased control group) were used as references to monitor colitis development. A concomitant sham AAV control vector was not used in this study as this control has been shown to be equivalent to saline control^[26,27]. Even though AAV-mediated gene transfer leads to the development of neutralizing antibodies against the vector capsid^[28], preventing vector re-administration, no inflammatory responses against the AAV capsid were documented in *in vivo* gene

transfer mice models using AAV vectors^[27,29].

Assessment of inflammation

The body weights of the mice were recorded daily, and wasting disease progression was expressed by the percentage of weight loss as compared to body weight at the day of initiation of TNBS treatment (day 10, Figure 1). Animals were withdrawn from the study when their weight loss was > 25% of their original body weight. At the time of sacrifice, colons were collected and presence of loose stool and visible fecal blood was assessed.

At the time of sacrifice, a composite score [disease activity index (DAI)] was established as described previously^[25]. Body weight loss was scored on a scale of 0–4 (0, < 1%; 1, 1%–5%; 2, 5%–10%; 3, 10%–15%; 4, > 15%). Loose stool was scored on a scale of 0–4 (0, normal; 1, loose droppings; 2, loose stools, colon filled with feces; 3, loose stool, feces only near cecum; 4, empty bowel). Visible fecal blood was scored on a scale of 0–4 (0, negative; 2, positive; 4, gross bleeding). The DAI consists of a combination of body weight loss, loose stool and visible fecal blood scores divided by 3 as described previously^[25].

Colon tissue weights were recorded and used as an indicator of disease-related intestinal wall thickening. Increased colon weight has been shown to correlate with increased colon inflammation^[25]. Colons were first divided longitudinally into two parts: one part was immediately frozen in liquid nitrogen for protein extraction and cytokine determination, while the second part was stored in formalin and embedded in paraffin for (immuno-) histological evaluation. Blood was collected by orbital puncture immediately following sacrifice and plasma was separated by centrifugation (5000 r/min for 5 min). Plasma samples were stored at -80 °C until analysis.

Histological analysis

Colonic segments were fixed in 10% formalin overnight and thereafter stored in 70% ethanol before embedding in paraffin. Tissues sections (7 µm thick) were stained with haematoxylin and eosin (HE) for histology scoring. The histology damage score was calculated using the following criteria: percentage of area involved, number of follicle aggregates, edema, fibrosis, erosion/ulceration, crypt loss, and infiltration of mononuclear and polymorphonuclear cells, as described previously^[30]. The percentage of area involved and crypt loss were scored on a scale

of 0-4 (0, normal; 1, < 10%; 2, 10%; 3, 10%-50%; and 4, > 50%). Erosions were defined as 0 if the epithelium was intact, 1 if the lamina propria was involved, 2 if ulcerations involved the submucosa, and 3 when ulcerations were transmural. The severity of the other parameters was scored on a scale of 0-3 (0, absent; 1, weak; 2, moderate; and 3, severe)^[30]. A certified pathologist scored all the tissue sections (blinded analysis).

Immunohistochemistry

Colon tissues sections of 7 μ m were acetone-fixed and stained with rat-anti-mouse (ram) CD4 (1:100, BD550278), ram CD8a (1:50, BD550281), ram CD19 (1:50, BD550284), ram Foxp3 (1:100, eBiosciences14-5773-82) and ram F4/80 (1:500). Prior to anti-rat biotin conjugated secondary antibody (1:50, BD51-7605kc) and Streptavidin-HRP (BD) incubations, endogenous peroxidases were blocked by incubation with 0.3% H₂O₂ for 20 min. After 5 min of diaminobenzidine staining (BD), the sections were counter-stained with Haematoxylin, dehydrated and mounted in Entellan.

Flow cytometry

Thymus, mesenteric lymphoid nodes, liver and spleen tissue were collected upon sacrifice. Cell suspensions obtained from each of the tissue samples were prepared using 40 μ m cell strainers (BD Biosciences) and stained for T cell surface markers CD4 (clone RM4-5, eBioscience), CD8 (Clone 53-6.7, Miltenyi) and Treg cell surface markers glycoprotein A repetitions predominant (GARP) and CD25 (clone YGIC86 and clone PC61.5 respectively, both eBioscience) as well as for the intracellular Treg cell marker Foxp3 (clone FJK16, eBioscience). The analysis was performed by flow cytometry (FACSCalibur, BD Biosciences).

Statistical analysis

The results are presented as mean \pm SD or SE, where appropriate. Statistical analyses were performed using Prism 5.0 (GraphPad). Data were analyzed using a 1 way analysis of variance, followed by Dunn's post hoc test for multiple comparisons.

RESULTS

We investigated the potential for AAV5-mediated delivery of regulatory T-cell epitopes to prevent the development of TNBS induced colitis. Mice treated intra-rectally with TNBS in ethanol developed a severe illness as reflected in the progressive body weight loss over time and an increase in disease activity index, histology damage score and mucosal inflammatory parameters at the time of sacrifice.

Tregitope 167 delivery protects against TNBS colitis development

Development of colitis in the TNBS mice model is strongly associated with wasting disease^[31]. Daily weight determination is therefore important to determine colitis

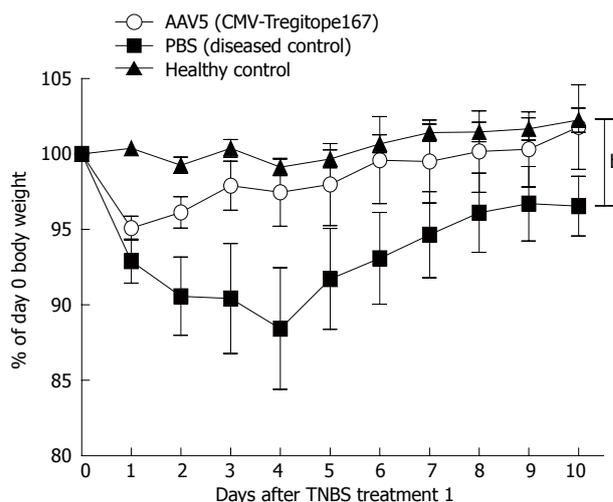


Figure 2 Adeno associated virus serotype 5 mediated delivery of regulatory T-cell epitope 167 ameliorates trinitrobenzene sulfonate induced colitis development over time. Mice were injected intravenously with either phosphate-buffered saline (PBS) or adeno associated virus (AAV) 5 [cytomegalovirus (CMV) promoter T-cell epitopes (Tregitope) 167]. 10 d after AAV-mediated Tregitope delivery, acute colitis was initiated by intra-rectal administration of 1.5 mg trinitrobenzene sulfonate (TNBS) in 40% ethanol followed by a second TNBS treatment (0.75 mg in 20% ethanol) 8 d later. Control groups consisted of mice not treated by TNBS (healthy control group) and mice treated by TNBS only (diseased control group). Disease progression was assessed by changes in daily body weight. Animals were withdrawn from the study when their weight loss was > 25% of their original body weight. Overall for the healthy controls, $n = 9$; adeno associated virus (AAV) 5 (CMV-Tregitope 167) treated, $n = 7$; diseased controls, $n = 6$, were included in the analysis. The data were analyzed using a 1 way analysis of variance, followed by Dunn's post hoc test for multiple comparisons. Data are presented as mean \pm SE of all the mice. ^b P value < 0.01 between PBS (diseased control group) and both AAV5 (CMV-Tregitope 167) treated and the healthy control group.

severity and is indicative of differences in colitis development between experimental groups^[31]. Animals were withdrawn from the study when their weight loss was > 25% of their original body weight.

The body weight of the mice was monitored daily after the first TNBS treatment as an indication of the severity in the colitis development between experimental groups (Figure 2). TNBS treated mice that were pre-administered with Tregitope 167, showed a body weight that increased over time and was comparable to the weight gain of untreated healthy control mice (Figure 2). The Tregitope 167 treated mice gained an average of 4% over their initial body weight at the time of sacrifice (Figure 3A). In contrast, the mice treated with TNBS alone (no Tregitope) developed colitis, and lost 4% of their initial body weight at the time of sacrifice (Figure 3A).

Increases in colon weight, as well as in the disease activity index are both indicative of colonic inflammation and were determined at the time of sacrifice (Figure 3). The body weight increase that had been observed in the mice pre-treated with Tregitope 167 was substantiated by a lower disease activity index (Figure 3B) and a decreased colon weight (Figure 3C) as compared to the diseased control group ($P < 0.01$ and $P < 0.001$ respectively).

The histology damage score was performed on HE

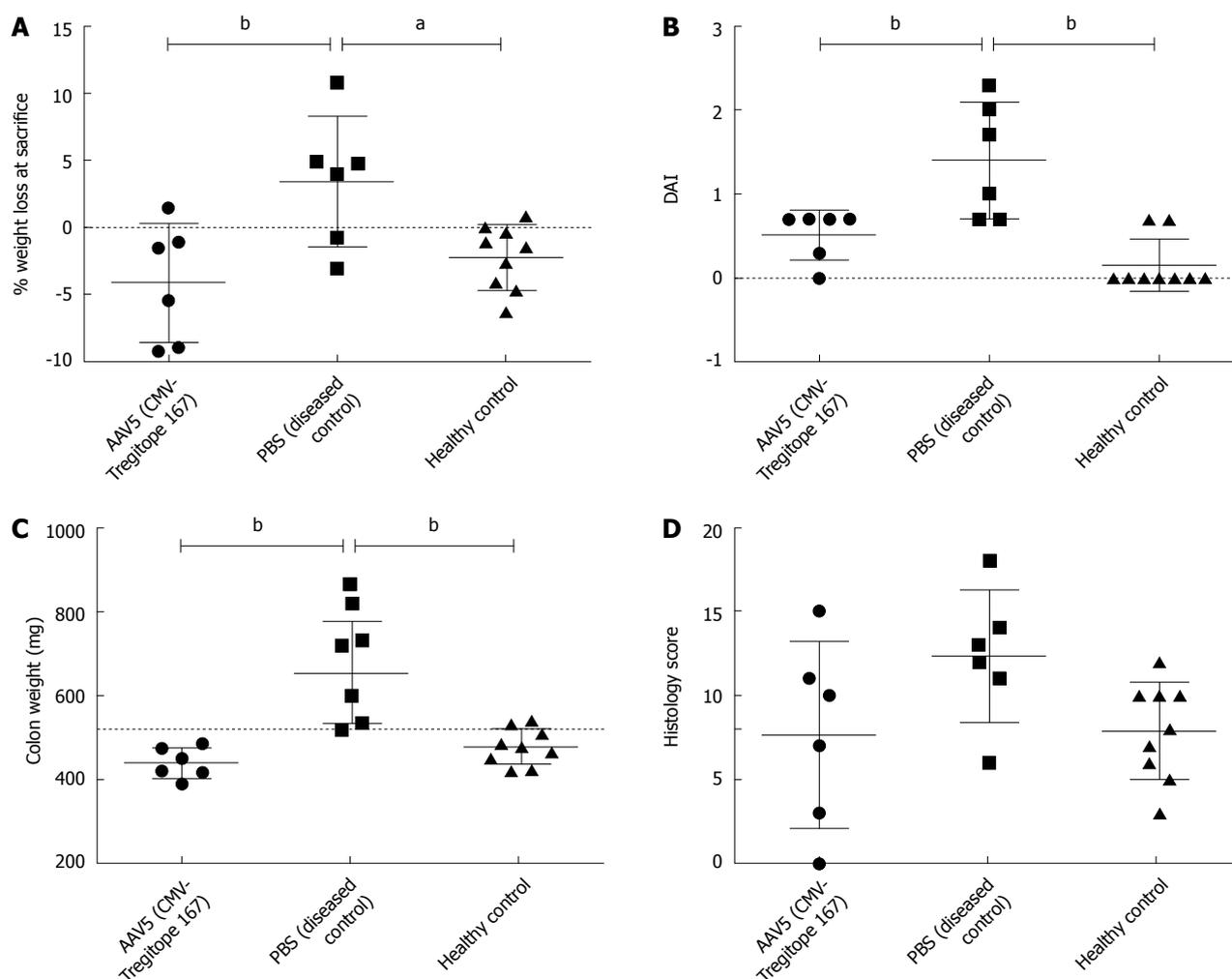


Figure 3 Adeno associated virus serotype 5 mediated delivery of regulatory T-cell epitope 167 alleviates colonic inflammation as determined at the day of sacrifice. Mice were injected intravenously with either phosphate-buffered saline (PBS) or adeno associated virus (AAV) 5 [cytomegalovirus (CMV) promoter T-cell epitopes (Tregitope) 167]. Ten days after AAV-mediated Tregitope delivery acute colitis was initiated by intra-rectal administration of 1.5 mg trinitrobenzene sulfonate (TNBS) in 40% ethanol followed by a second treatment with TNBS (0.75 mg in 20% ethanol) 8 d later. Control groups consisted of mice not treated by TNBS (healthy control group) and mice treated by TNBS only (diseased control group). Disease progression was assessed by changes in daily body weight as well as macroscopic and microscopic scores on the day of sacrifice. A: Animal body weight change on day 20 upon sacrifice. The values of body weight are expressed as a percentage of body weight on the day of the first TNBS treatment; B: Macroscopic disease score. The disease activity index consist of a combination of body weight loss, loose stool and visible fecal blood scores divided by 3 at the day of sacrifice; C: Assessment of colonic weight upon sacrifice as an index of disease-related intestinal wall thickening; D: Histological grading of colonic colitis scores. The histology damage score was calculated using the following criteria: percentage of area involved, number of follicle aggregates, edema, fibrosis, erosion/ulceration, crypt loss, and infiltration of mononuclear and polymorphonuclear cells. Individual mice are depicted; from the AAV5 (CMV-Tregitope 167) pre-treated group an outlier was removed. Animals were withdrawn from the study when their weight loss was > 25% of their original body weight. Overall for the healthy controls, $n = 9$; AAV5 (CMV-Tregitope 167) treated, $n = 6$; diseased controls, $n = 6$, were included in the analysis. The data were analyzed using a 1 way analysis of variance, followed by Dunn's post hoc test for multiple comparisons. Data are presented as mean \pm SD of all the mice. ^a $P < 0.05$, ^b $P < 0.01$ vs PBS (diseased control group).

stained tissue sections. The score was calculated using the following criteria: percentage of area involved, number of follicle aggregates, edema, fibrosis, erosion/ulceration, crypt loss, and infiltration of mononuclear and polymorphonuclear cells. The histological scoring showed that the AAV5 (CMV-Tregitope 167) pre-treated mice presented a decreased severity of colitis as compared to the diseased control group (Figure 3D) as a result of lower levels of inflammation, namely decreased cellular infiltrations, little crypt loss and the absence of erosions and ulceration (Figure 4).

The TNBS induced colitis model is characterized by

the local infiltration of CD4+ T cells in the intestinal mucosa^[32]. Immunohistochemical staining of the colonic tissues for CD4+ showed that, at the day of sacrifice, inflammatory cell infiltrates were present in TNBS treated mice with or without administration with Tregitope 167 and that these cellular infiltrates consisted mainly of CD4+ cells. For both TNBS treated groups CD4+ T cell infiltrates were observed in the sub-epithelial layer and the lamina propria. CD4+ T cell infiltrates were also present in the muscularis mucosa layer of the diseased control mice, but were absent in the AAV5 (CMV-Tregitope 167) pre-treated group (Figure 5).

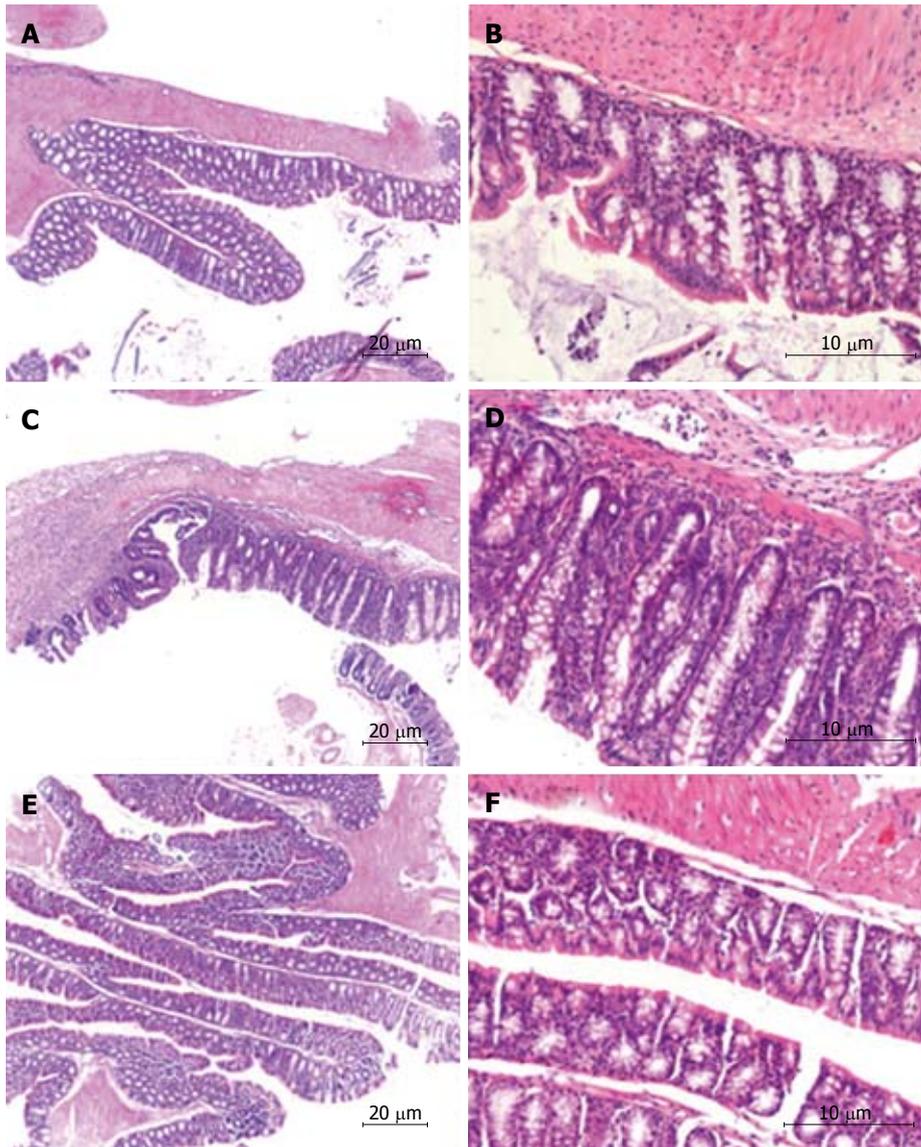


Figure 4 Hematoxylin and eosin-stained paraffin section from colon tissue. A, B: Healthy control mice; C, D: Diseased control mice; E, F: Adeno associated virus (AAV) 5 [cytomegalovirus (CMV) promoter T-cell epitopes (Tregitope) 167] pre-treated mice. Histological evidence that AAV5 (CMV-Tregitope 167) ameliorates trinitrobenzene sulfonate (TNBS)-induced pathology. Images depicted of an HE-stained paraffin section of a representative mouse colon from each group at the moment of sacrifice. The images of the diseased control (C, D) demonstrated acute inflammation: elongated villi, abundant transmurular cellular infiltrate, erosions and crypt loss as compared to both the AAV5 (CMV-Tregitope 167) pre-treated and healthy control mice.

Increase of regulatory markers expression in the intestinal mucosa and thymus of mice administered with Tregitope 167

The reported ability of Tregitopes to both activate and induce Treg cells led us to further assess the presence of Treg-cell associated markers in the colonic tissues.

Colon tissues were prepared and the presence of transcription factor Foxp3 was assessed by immunohistochemistry, so as to determine whether regulatory T cells were present in the peri-colonic infiltrates^[33]. Numerous Foxp3 positive cells were detected in the lamina propria and sub-epithelium of the colon sections from mice treated with Tregitope 167 (Figure 6). Foxp3 positive cells were absent or sporadic in the colon of healthy and diseased control mice (Figure 6).

Thymus, mesenteric lymph nodes, liver and spleen tissue were collected at the time of sacrifice and the corresponding lymphocyte populations were further assessed by flow cytometry analysis for the expression of the CD4 T cell surface marker and the Treg cell associated markers Foxp3^[34], CD25^[35] and GARP^[36,37] (Figure 7A-F). The Foxp3 and GARP markers were significantly increased in the CD4+ T lymphocyte population in the thymus of the mice pre-treated with AAV5 (CMV-Tregitope 167), as compared to lymphocyte populations in the thymus of diseased and the healthy control mice ($P < 0.05$ and $P < 0.001$ respectively, Figure 7G). CD4+ thymic lymphocyte population (mean \pm SD, 11% \pm 2%, $n = 6$) co-expressed Foxp3 and GARP in the thymus of AAV5 (CMV-Tregitope 167) pre-treated mice as compared to the

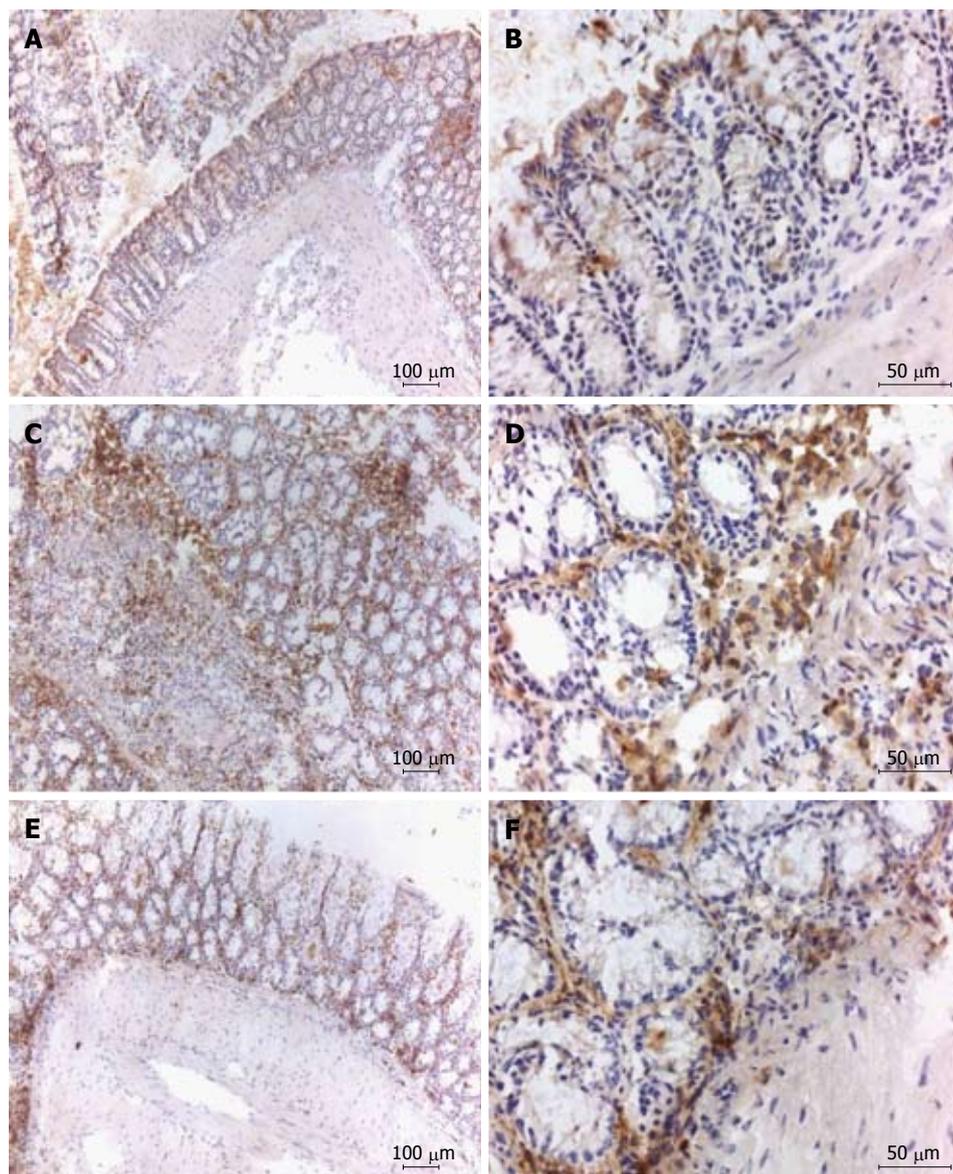


Figure 5 Immunohistochemistry pictures depicting CD4 staining colon tissue. A, B: Healthy control mice; C, D: Diseased control mice; E, F: Adeno associated virus (AAV) 5 [cytomegalovirus (CMV) promoter T-cell epitopes (Tregitope) 167] pre-treated mice. Specific immunohistochemical staining showed inflammatory cell infiltrates present in trinitrobenzene sulfonate (TNBS) treated mice with or without administration with Tregitope 167 consisted of CD4 positive cells, localized in the sub-epithelial layer, in the lamina propria (C-F) and for the diseased control also in the muscular layer (C,D). Depicted are representative data from a single mouse.

thymic lymphocyte population of diseased (mean \pm SD, 7% \pm 3%, $n = 6$) and the healthy control groups (mean \pm SD, 6% \pm 2%, $n = 9$), respectively (Figure 7G). Both the relative and absolute number of Foxp3 expressing T cells were expanded in the thymus after AAV5 (CMV-Tregitope 167) pre-treatment (Figure 7H). No significant differences in the expression of Foxp3 and GARP in the lymphocyte populations of the mesenteric lymph nodes, liver and spleen were identified.

DISCUSSION

Curative treatment approaches for Crohn's disease and ulcerative colitis represent a significant unmet medical need. Regulatory T (Treg) cells are key players in maintaining

peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammation^[33,38]. Therefore, novel strategies that aim for therapeutic tolerance induction and leverage Treg cells are currently being explored^[39]. In the present study, the potential for AAV-mediated delivery of an immunomodulatory peptide (Tregitope 167) was investigated.

In this study, we demonstrate that the systemic AAV-based delivery of Tregitope 167 has the potential to prevent the development of fulminant colitis in a TNBS-induced model of IBD. Tregitope 167 was used in our study as its binding affinity for the MHC molecule in Balb/c mice is superior to Tregitope 289 (De Groot, manuscript submitted for publication). The significant decrease of colonic inflammation in the Tregitope 167

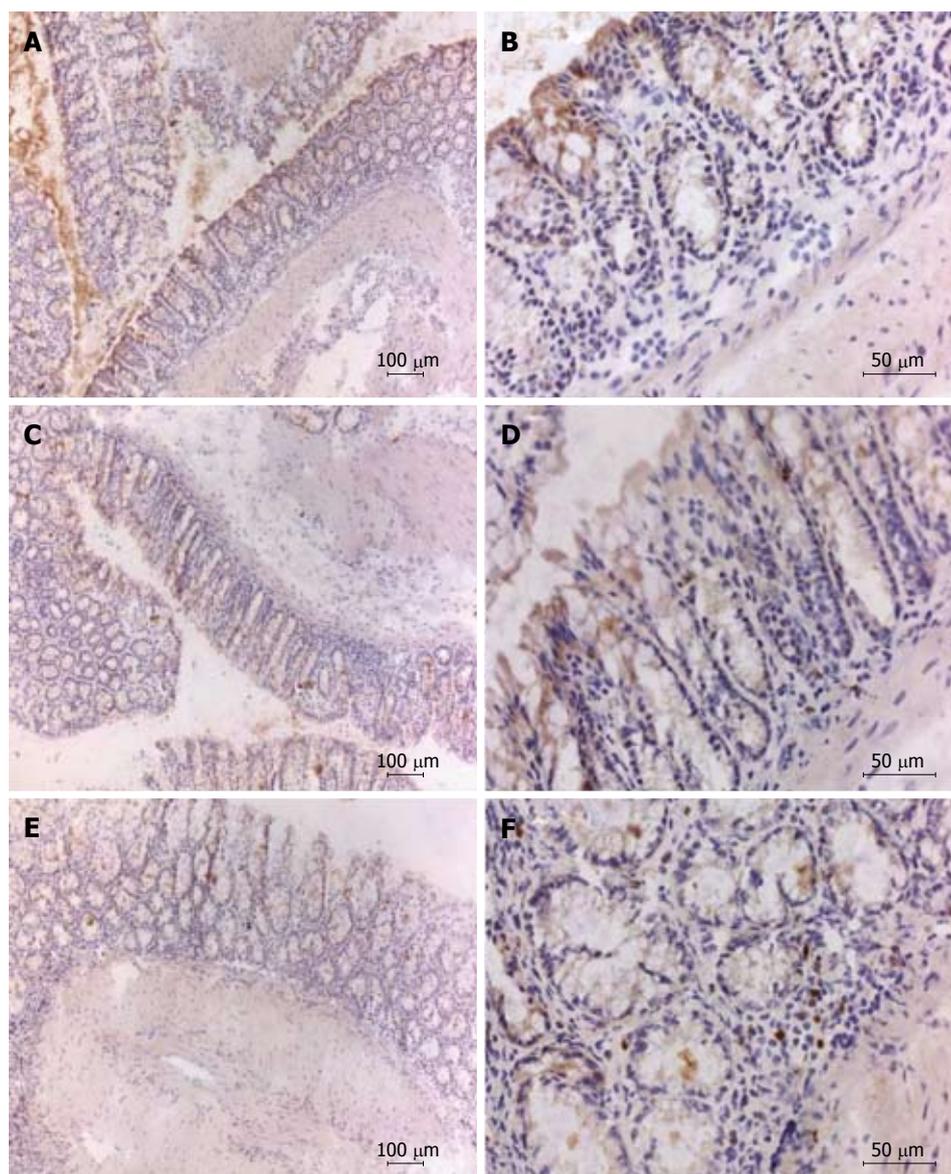


Figure 6 Immunohistochemistry pictures depicting forkhead Box-P3 staining colon tissue. A, B: Healthy control mice; C, D: Diseased control mice; E, F: Adeno associated virus (AAV) 5 [cytomegalovirus-regulatory (CMV) promoter T-cell epitopes (Tregitope) 167] pre-treated mice. Colon tissue were prepared and assessed by immunohistochemistry for the expression of the transcription factor forkhead Box-P3 (Foxp3) as a marker for regulatory T cells. Numerous Foxp3 positive cells were detected in the lamina propria and sub-epithelium of the colon sections from mice treated with Tregitope 167 (E, F). Foxp3 positive cells were rarely present in the colon of healthy and diseased control mice (A-D). Depicted are representative data from a single mouse.

pre-treated mice was reflected in an overall weight gain and substantiated by a decreased disease activity index, colon weight and histology damage score at the time of sacrifice. Additionally, there was less mucosal inflammation in the AAV (CMV-Tregitope 167) pre-treated mice. This therapeutic benefit corresponded with increases in the relative number of T cells expressing regulatory T cell markers in the colon tissues and among thymic lymphocytes of the Tregitope 167-treated mice.

IBD patients do not present defects in regulatory T cell function or phenotype^[40-42] and by consequence are more likely to benefit from therapies aiming at inducing and expanding Treg cells than patients affected by other autoimmune diseases. Tregitope 167 has the potential to both activate nTreg cells and induce iTreg cells^[15,16] and

may be suitable as a novel therapeutic agent for IBD. However, the use of immunomodulatory peptides in clinical applications for IBD has been hindered by difficulties associated with the systemic delivery of the therapeutic peptides in sufficient quantity and concentration to the target tissues^[17-19]. AAV has proven to be both effective and safe as a gene therapy delivery vector in the clinic^[20-22]. Therefore, AAV-mediated delivery of Tregitopes was explored in this study. The AAV serotype 5 (AAV5) was used since pre-existing immunity to AAV5 in humans has been shown to be low^[28,43]. Our data demonstrate that AAV5-mediated delivery may be an efficient approach for stable administration of Tregitopes *in vivo*. Further studies will need to be performed to determine the duration of the immunological tolerance that is evoked by induction

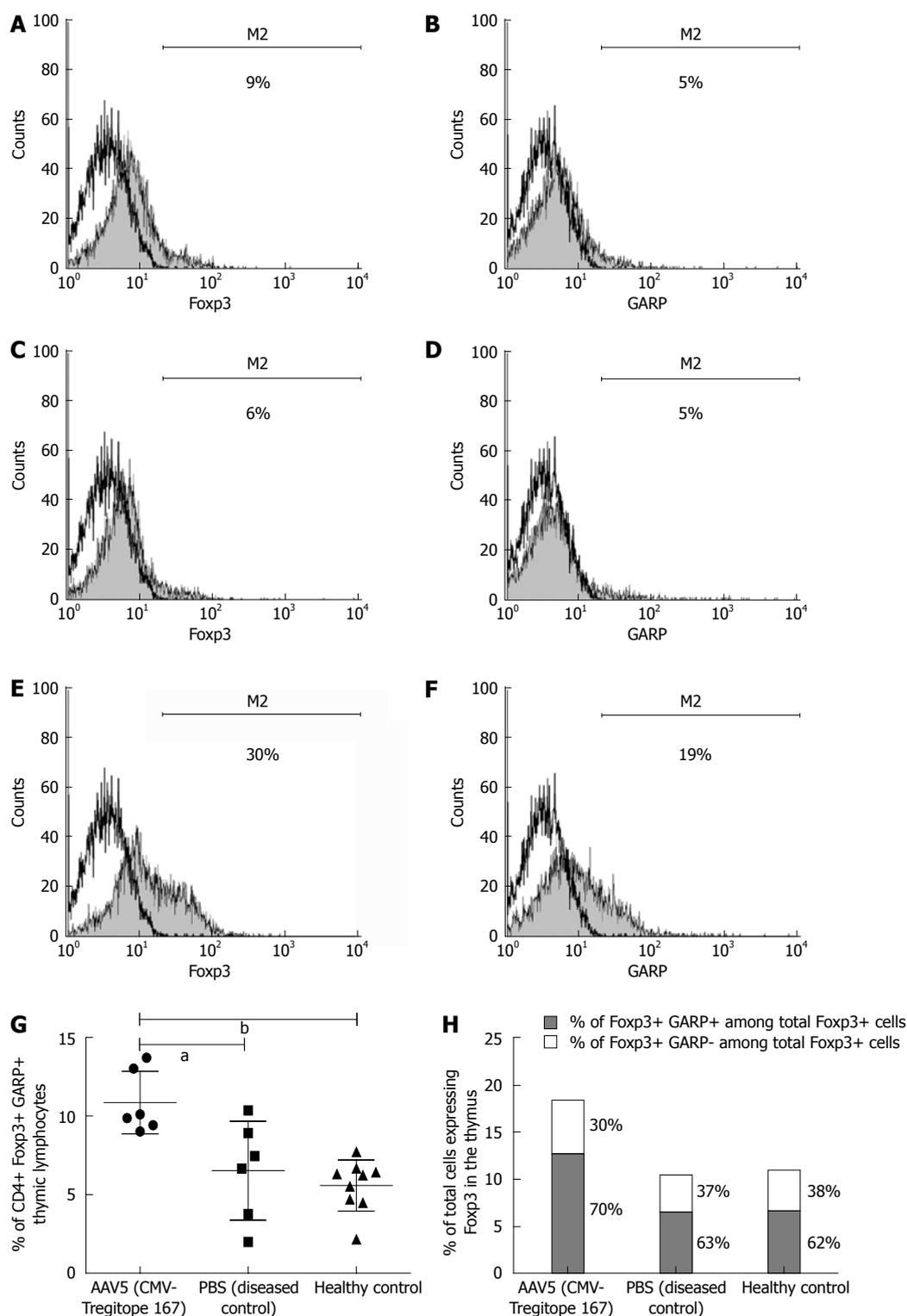


Figure 7 Adeno associated virus serotype 5 mediated delivery of regulatory T-cell epitope 167 induces forkhead Box-P3 and glycoprotein A repetitions predominant expression in the CD4 positive thymic lymphocyte population. Thymus tissue was collected upon sacrifice. Cells suspensions were prepared and stained for the following markers: CD4, glycoprotein A repetitions predominant (GARP) and forkhead Box-P3 (Foxp3) before analysis by flow cytometry (FACSCalibur, BD Biosciences). A: Histogram Foxp3 cell count: healthy control; B: Histogram GARP cell count: healthy control; C: Histogram Foxp3 cell count: diseased control; D: Histogram GARP cell count: diseased control; E: Histogram Foxp3 cell count: adeno associated virus (AAV) 5 [cytomegalovirus (CMV) promoter T-cell epitopes 167] pre-treated group (grey, filled in) vs unstained control (black continuous line). Gating was done on the CD4 positive thymic lymphocyte population. Depicted are representative data from a single mouse; F: Histogram GARP cell count: AAV5 (CMV-Tregitope 167) pre-treated group (grey, filled in) vs unstained control (black continuous line). Gating was done on the CD4 positive thymic lymphocyte population. Depicted are representative data from a single mouse; G: Depicted are percentages of CD4 positive, Foxp3 positive and GARP positive thymic lymphocytes. Individual mice are depicted; for the a AAV5 (CMV-Tregitope 167) pre-treated group one mouse did not have a thymus and therefore $n = 6$ mice were included in this analysis. The data were analyzed using a 1 way analysis of variance, followed by Dunn's post hoc test for multiple comparisons. Data are presented as mean \pm SD of all the mice. ^a $P < 0.05$, ^b $P < 0.01$ vs phosphate-buffered saline (PBS) (diseased control group) or healthy control; H: Both the relative and absolute number of Foxp3 expressing T cells were expanded in the thymus after AAV5 (CMV-Tregitope 167) pre-treatment. Mean % of lymphocytes expressing Foxp3 in the thymus of the three groups are depicted; for the AAV5 (CMV-Tregitope 167) pre-treated group one mouse did not have a thymus and therefore $n = 6$ mice were included in this analysis.

and activation of Treg cells.

Regulatory T (Treg) cells are considered to be essential in the counter-regulation of inflammatory reactions and Foxp3 is considered as a marker of the regulatory phenotype^[34,44,45]. Staining for Foxp3 in mice pre-treated with Tregitope 167 revealed the presence of Foxp3 positive cells in the lamina propria and sub-epithelium of the colon sections. Additionally, expression of both Foxp3 and GARP were increased in the thymic CD4+ T lymphocyte population in mice pre-treated with AAV5 (CMV-Tregitope 167), indicating an increase in activated regulatory T-cells^[34,36,37,44,45]. The presence of higher numbers of activated regulatory T cells corresponded with the prevention of fulminant intestinal inflammation *in vivo* in this TNBS model of IBD.

No increase in the Treg cell populations was observed in the mesenteric lymph nodes, liver and spleen in the current study. We hypothesize that this could be due to the duration of the experiment and the timing of the Treg cell evaluation. In other mouse models such as the model of spontaneous encephalomyelitis, the *de novo* generation of Treg cells was initiated intrathymically, and was followed by Foxp3 induction in peripheral tissue at later time points^[46].

Tregitopes are T cell epitopes naturally located in immunoglobulins that bind to multiple MHC class II alleles and induce Treg cell responses. We have demonstrated that antigen presenting cells (APCs) present Tregitopes to nTreg cells, engage feedback mechanisms promoting a tolerogenic APC phenotype, induce Treg cell expansion, and modulate antigen-specific effector T cell responses (Cousens and De Groot, manuscript submitted for publication). Proportions of APC expressing MHC II, CD80, and CD86 are suppressed, consistent with reported effects of intravenous immunoglobulin^[47] and of the immunoglobulin (Ig) G-derived peptide hCDR1^[48]. The basic mechanism of Tregitope tolerance induction is currently proposed to be as follows: (1) APC present Tregitopes to nTreg cells; (2) nTreg cells are activated to proliferate; (3) nTreg cells provide tolerogenic feedback signals to APC, modulating the APC phenotype; and (4) nTreg cells and tolerogenic APC together suppress antigen-specific T cell responses (Cousens and De Groot, manuscript submitted for publication).

A limitation of the colitis model used in this study was the acute necrotizing enterocolitis, occurring in the first 3 d after the first TNBS treatment, a presentation of disease which is unrelated to IBD^[25]. Therefore the surviving number of mice, included in the analysis was lower than anticipated, which, for some analysis, conflicted with statistical analysis of the data. As a consequence, a large variability in the colon mucosa cytokines levels was observed after TNBS induced experimental colitis and prevented an accurate analysis of those parameters. Therefore, further development of AAV mediated delivery of Tregitope 167 in different experimental models of inflammatory disease will be necessary to confirm the obtained results.

In summary, our data provide preliminary evidence supporting the potential use of AAV-based Tregitope delivery as a therapy for the treatment of IBD. Further investigations will permit to define the mechanism by which Tregitope exert their immune regulatory properties, the duration of the effect, the ability of Tregitopes to reduce disease that has already been established and their eventual impact on systemic immunity.

Overall, this study identifies AAV-mediated delivery of regulatory T-cell epitope 167 as a novel anti-inflammatory approach with the capacity to decrease intestinal inflammation and induce long-term remission in IBD.

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COMMENTS

Background

The focal point in the search for novel treatment options in inflammatory bowel diseases (IBD) is tolerance induction. In other words, exploring different mechanisms by which the immune system could maintain unresponsiveness to the antigens in the mucosal environment which cause destructive immune responses and thereby disease activity in IBD patients.

Research frontiers

The recently discovered regulatory T cell epitopes (Tregitopes) are highly promising peptides that were demonstrated to both activate and induce regulatory T cells *in vitro* and *in vivo*. Modulation of T cell responses with Tregitopes may contribute to the regulation of pathological inflammatory responses in IBD. However, the use of immunomodulatory peptides in clinical applications for IBD so far have shown that the *in vivo* delivery of these peptides for therapeutic purposes is hindered by difficulties in obtaining sufficient and stable peptide concentrations.

Innovations and breakthroughs

This study identifies adeno-associated virus (AAV)-mediated delivery of regulatory T-cell epitope as a novel anti-inflammatory approach with the capacity to decrease intestinal inflammation and induce long-term remission in IBD.

Applications

The significance of the achievements to IBD relate to possible novel therapeutic approaches, which could aim at long term tolerance induction. The described study identifies a novel anti-inflammatory strategy with the capacity to ameliorate the intestinal inflammatory response and restore mucosal immune tolerance.

Terminology

Tregitopes are T cell epitopes naturally located in immunoglobulins that bind to multiple major histocompatibility complex class II alleles and induce regulatory T cell responses. The non-pathogenic, replication-deficient AAV holds promise for gene therapy. The AAV vector has a good safety profile as it remains predominantly episomal. The therapeutic potential of AAV as a vector in gene therapy has also been demonstrated in a clinical setting in recent studies.

Peer review

This interesting paper is investigating the possibility of AAV mediated delivery of regulatory trinitrobenzene sulfonate (TNBS) colitis. The major finding of the study was that systemic administration of AAV was associated with a marked decrease in the clinical and histological severity of the TNBS induced inflammatory colitis parallel with an increase of the T lymphocytes expressing regulatory markers in the colon and thymus. Overall, this manuscript presents an interesting approach opening new possibilities.

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Results of National Colorectal Cancer Screening Program in Croatia (2007-2011)

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Abstract

AIM: To study the epidemiologic indicators of uptake and characteristic colonoscopic findings in the Croatian National Colorectal Cancer Screening Program.

METHODS: Colorectal cancer (CRC) was the sec-

ond leading cause of cancer mortality in men ($n = 1063$, 49.77/100 000), as well as women ($n = 803$, 34.89/100 000) in Croatia in 2009. The Croatian National CRC Screening Program was established by the Ministry of Health and Social Welfare, and its implementation started in September, 2007. The coordinators were recruited in each county institute of public health with an obligation to provide fecal occult blood testing (FOBT) to the participants, followed by colonoscopy in all positive cases. The FOBT was performed by hypersensitive guaiac-based Hemognost card test (Biognost, Zagreb). The test and short questionnaire were delivered to the home addresses of all citizens aged 50-74 years consecutively during a 3-year period. Each participant was required to complete the questionnaire and send it together with the stool specimen on three test cards back to the institute for further analysis. About 4% FOBT positive cases are expected in normal risk populations. A descriptive analysis was performed.

RESULTS: A total of 1 056 694 individuals (born between 1933-1945 and 1952-1957) were invited to screening by the end of September 2011. In total, 210 239 (19.9%) persons returned the envelope with a completed questionnaire, and 181 102 of them returned it with a correctly placed stool specimen on FOBT cards. Until now, 12 477 (6.9%), FOBT-positive patients have been found, which is at the upper limit of the expected values in European Guidelines for Quality Assurance in CRC Screening and Diagnosis [European Union (EU) Guidelines]. Colonoscopy was performed in 8541 cases (uptake 66%). Screening has identified CRC in 472 patients (5.5% of colonoscoped, 3.8% of FOBT-positive, and 0.26% of all screened individuals). This is also in the expected range according to EU Guidelines. Polyps were found and removed in 3329 (39% of colonoscoped) patients. The largest number of polyps were found in the left half of the colon: 64% (19%, 37% and 8% in the rectum, sigma, and descendens, respectively). The other 36% were

detected in the proximal part (17% in the transverse colon and 19% in ceco-ascending colon). Small polyps in the rectum (5-10 mm in diameter), sigmoid and descending colon were histologically found to be tubular adenomas in 60% of cases, with a low degree of dysplasia, and 40% were classified as hyperplastic. Polyps of this size in the transverse or ceco-ascending colon in almost 20% had a histologically villous component, but still had a low degree of dysplasia. Polyps sized 10-20 mm in diameter were in 43% cases tubulovillous, and among them, 32% had areas with a high degree of dysplasia, especially those polyps in the ceco-ascending or transverse part. The characteristics of the Croatian CRC Screening National Program in the first 3 years were as follows: relatively low percentage of returned FOBT, higher number of FOBT-positive persons but still in the range for population-based programs, and higher number of pathologic findings (polyps and cancers).

CONCLUSION: These results suggest a need for intervention strategies that include organizational changes and educational activities to improve awareness of CRC screening usefulness and increase participation rates.

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Key words: Colorectal cancer screening; Fecal occult blood testing; Croatian National Colorectal Cancer Screening Program; Colonoscopy; Uptake

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INTRODUCTION

The incidence of colorectal cancer (CRC) has been increasing during recent decades, and the lifetime risk for CRC in industrialized countries is about 5%^[1]. CRC is the one of major global public health problems, with approximately 950 000 newly diagnosed cases each year^[2]. CRC is a good candidate for screening, because it is a disease with high prevalence, has recognized precursor lesions, and early treatment is beneficial. Reducing the number of deaths from CRC depends on detecting and removing precancerous colorectal polyps, as well as detecting and treating the cancer in its early stages^[3]. Approximately 3000 new cases of CRC are diagnosed annually in Croatia and around 1900 people die of CRC each year. According to the Croatian Cancer Registry, in 2008 there were 1255 (54.5/100 000) women and 1819

(85.1/100 000) men with newly diagnosed CRC^[4]. The incidence and crude mortality rates from CRC have increased since the 1970s (Figure 1), and the incidence and mortality increase considerably after the age of 50 years. In Croatia it was the second leading cause of cancer mortality in men ($n = 1063$, 49.77/100 000), as well as in women ($n = 803$, 34.89/100 000) in 2009^[5]. CRC constitutes 12.9% of all newly diagnosed carcinomas in the European population (men 12.8%, women 13.1%) and accounts for 12.2% of deaths caused by malignancy^[6]. Compared to other European Union (EU) members where organized population screening programs have existed for several years, in Croatia the standardized mortality rates of CRC are higher, but still lower than those in some western countries (Figure 2). The joint-point analysis showed that CRC in men in Croatia has increasing trends of both incidence calculated as estimated annual percent change (EAPC 2.9%) and mortality (EAPC 2.1%), while in women, the increase in incidence was not significant, but mortality rates in the last 15 years showed a significant increase (EAPC 1.1%)^[7].

A number of randomized trials and one Cochrane review provided strong evidence that fecal occult blood test (FOBT) followed by colonoscopy, if offered every 2 years, reduced mortality rates associated with CRC by 16%^[8,9]. In the Czech Republic the rates were higher in a previous period, but, owing to the implementation of organized screening, in 2007 they became lower than in Croatia (Figure 3)^[10,11]. Hungary is one of the transitional countries with higher mortality than that in Croatia, and a pilot program has now been completed there and preparations for a nation-wide program are ongoing^[12,13]. The EU council recommendation of December 2003, stated that screening for CRC by FOBT in men and women aged 50-74 years met the requirements of the public health program.

The Croatian Cancer Registry is a nation wide population-based registry, covering over 90% of cancer cases in the country. The existence of cancer registry allows us to evaluate effects of screening on changes in CRC incidence, which is expected to be higher for the first few years. CRC is also expected to be increasingly detected at an earlier stage owing to the screening program. Therefore, after 5 years, it is expected that our national mortality data will show a reduction in CRC mortality. The aim of this paper is to describe the population-based screening program for CRC whose implementation started during 2008. The political decision to launch it was made by the Ministry of Health and Social Welfare at the end of 2007 according to the previously published proposal^[14]. Due to some unexpected organizational difficulties, it is planned that first round will be finished during 2011.

MATERIALS AND METHODS

Program organization: the Republic of Croatia is divided in 20 counties plus the capital city of Zagreb and there is one public health institute in each county and city. In

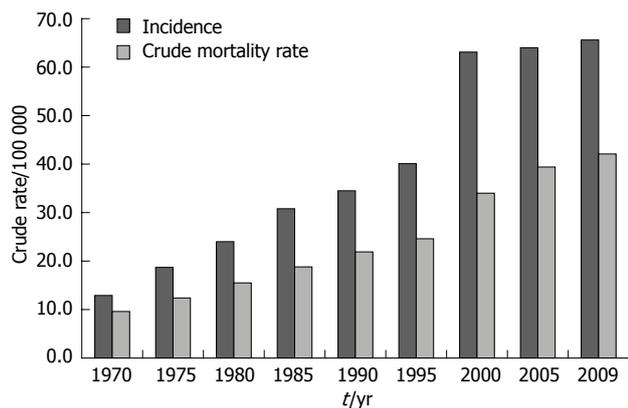


Figure 1 Incidence and crude mortality rate of colorectal cancer in Croatia from 1970 to 2009 year.

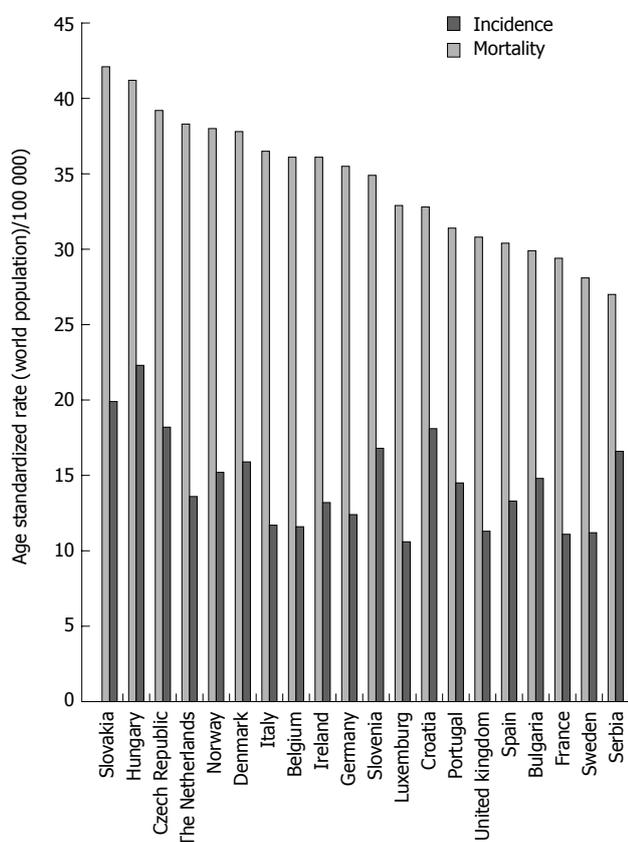


Figure 2 Incidence and mortality rates of colorectal cancer in European countries^[40].

each local public health institute there is a coordinator nominated for the National Screening Program. At the national level, a coordinator from the Croatian National Public Health Institute has been nominated, and all 22 coordinators are members of the Committee for Program Performance. An Expert Committee has also been nominated by the Minister of Health and Social Welfare with the main task to evaluate professional qualification of colonoscopists included in the National Program and to attend to other issues during the program performance. The Committee consists of project co-ordinators

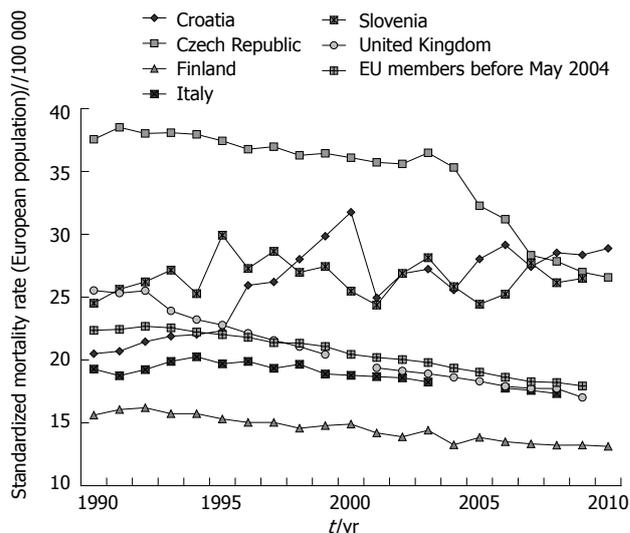


Figure 3 Comparison of standardized mortality rate of colorectal cancer in Croatia and some other European Union countries (source: World Health Organization, European Mortality Database, Health for All 2011).

from the Ministry of Health and Social Welfare, the National Co-ordinator from the Croatian National Public Health Institute, three members of the Croatian Gastroenterology Society, and representatives of oncologists and primary care physicians. The co-ordinators (medical specialists in epidemiology or in public health) in each local institute of public health are obliged to ensure performance of FOBT, followed by colonoscopy in all positive cases. They also ensure the collection of data on colonoscopic diagnosis, histopathologic findings, cancers and polyps, and to keep records about tests performed in each person. If a patient is diagnosed with CRC, the co-ordinator's obligation is to inform the patient's primary care physician. The co-ordinator also has to provide the answers to the questionnaire for each person tested in a web-based database, and to send the report with cumulative data about diagnostic findings to the national co-ordinator. Upon the receipt of the data, the national co-ordinator compiles all data and, according to results, controls the performance in each county. A national report is sent twice monthly to all members of the Expert Committee and to the member in the Ministry of Health and Social Welfare. The main indicators available from these reports include: uptake (compliance), number of persons who correctly applied specimens on cards, number of persons with positive FOBT, number of patients invited to colonoscopy, number of colonoscopies performed, number of patients in whom CRC was diagnosed, number of patients where polyp (polyps) was detected, number of patients where other disease was found (e.g., diverticula, inflammatory bowel diseases, hemorrhoids). The test and short questionnaire were planned to be delivered at the home addresses of all citizens aged 50-74 years consecutively during 3 years. So far, the population born between 1933-1945 and 1952-1957 has been invited. Each participant was re-

Table 1 Epidemiologic indicators of colorectal cancer screening uptake and diagnostic findings

Indicator	n (%)	Relative to issue
Invited	1 056 694 (84.0)	Eligible
Returned envelopes	210 239 (19.9)	Invited
Returned with specimen	181 102 (17.1)	Invited
Positive FOBT	12 477 (6.9)	Returned with specimen and examined
Colonoscopy done	8541 (66.0)	Positive FOBT
Hp confirmed Ca	472 (5.5)	Colonoscoped
Patients with polyp(s)	3329 (39.0)	Colonoscoped
Diverticula	1238 (14.6)	Colonoscoped
IBD and other findings	320 (3.7)	Colonoscoped
Hemorrhoids	2345 (27.5)	Colonoscoped

FOBT: Fecal occult blood test; Hp: Histopathologically; Ca: Carcinoma; IBD: Inflammatory bowel disease.

quired to complete the questionnaire, and send it together with the stool specimen on three test cards (each with four windows) by prepaid mail to the institute for further analysis. Most current FOBT methods are guaiac-based tests, as established more than a century ago. The pseudoperoxidase activity of heme converts colorless guaiac to a blue color in the presence of hydrogen peroxide. The FOBT is performed by guaiac-based HemoGnost card (Biognost, Zagreb, Savica-Šanci) test with a detection limit of 0.252-0.348 mg hemoglobin/g stool. Cards were designed for easy mailing, with a space to write the name of the participant and the date of fecal sampling. Each card had four windows for stool specimens. A leak proof storage and a return envelope were included. The manufacturer's instructions were closely followed concerning dietary restrictions and advice about taking samples. Dietary restrictions 3 d before and during sampling include avoiding raw meat, liver or blood dishes and large amounts of supplemental vitamin C (> 250 mg/d). Last year, in order to encourage people to take the test, printing the strip-form instruction on the back of the manufacturer's instructions was arranged. Colonoscopies were done in colonoscopic units (38 of them in Croatia) by well-trained and experienced colonoscopists.

Statistical analysis

A descriptive analysis of epidemiologic indicators was done. Results were compared with the reference range in European Guidelines for Quality Assurance in Colorectal Cancer Screening and Diagnosis^[15].

RESULTS

By the end of 2011 a total of 1 056 694 (84% of eligible) individuals (born between 1933-1945 and 1952-1957) were invited to screening. Among them, 210 239 persons (19.9%) returned the envelope with the completed questionnaire and 181 102 of them returned correctly completed FOBT cards. Until now, 12 477 positive FOBT patients have been found (6.9%). Colonoscopy was performed in 8541 cases (uptake 66%). Screening has identi-

fied CRC in 472 patients (3.6% of FOBT-positive and 0.26% of all individuals screened through the program). Polyps have been found and removed in 3329 (39% of FOBT-positive) patients (Table 1).

Unfortunately, there were great differences in the percentage of returned FOBTs between Croatian counties as well as in the number of pathologic findings (Table 2). Generally, people from continental counties returned FOBT more frequently (13.8%-30.7%), then those from the coastal areas (10.7%-21.7%). The number of CRCs identified varied from 1.0% to 12.2% of all colonoscopy participants (Table 2). There are several reasons for such differences; only partially this depends on variation in CRC and polyp incidence. Probably, the main reason can be attributed to the unequal experience and skills of the endoscopic teams, as well as the inadequate number of endoscopic instruments and equipment in some counties. An additional cause can be found in the greater response of people who had some symptoms of disease (e.g., pain, discomfort, irregular stool evacuation). Some data support this thesis because among false-positive FOBT results for CRC detection, hemorrhoids, inflammatory bowel disease and diverticula in 27.5%, 3.7% and 14.6% of cases, respectively, were found, as well as other changes which can cause positive FOBT results.

We found some differences in characteristics of polyps according to localization and size (Table 3). The largest number of polyps were found in the left half of the colon: 64% (19%, 37% and 8% in the rectum, sigmoid, and descending, respectively). The other 36% were detected in the proximal part (17% in the transverse colon, and 19% in the ceco-ascending). During screening colonoscopy, all polyps up to 15 mm in diameter were removed; 80% of those up to 20 mm in diameter, and 50% of those larger than 20 mm, were later removed by endoscopy or surgery.

Small polyps in the rectum (5-10 mm in diameter), sigmoid and descending colon were histologically tubular adenomas in 60% of cases, with a low degree of dysplasia, and 40% were classified as hyperplastic. Polyps of this size in the transverse or ceco-ascending part in almost 20% had a histologically villous component, but still were at a low degree of dysplasia. Forty-three percent of polyps 10-20 mm in diameter were tubulovillous, and among these 32% had areas with a high degree of dysplasia, especially those polyps in the ceco-ascending or transverse part.

Polyps over 20 mm in the rectum and sigmoid were tubular adenomas in 48% of cases, while others, as well as all the larger polyps in transverse or coecoascendent part were tubulovillous, villous or in 41% with high grade dysplasia, and 8% intramucosal carcinoma.

DISCUSSION

In order to ensure health-care equality, we covered 100% of the available population aged 50-74 years, and 84% of them received the test package at their home address.

Table 2 Uptake, positive fecal occult blood test and colorectal cancer diagnosed through screening program in Croatian counties

County	Invited (n)	Uptake-FOBT (%)	Positive	Percentage of pos. FOBT relative to tested persons (%)	Colonoscoped persons (n)	Diagnosed CRC (n)	Percentage of CRC relative to FOBT pos. persons (%)	Percentage of CRC relative to colonoscoped persons (%)
Bjelovarsko-bilogorska	29 763	24.0	385	7.8	223	14	3.6	6.3
Brodsko-posavska	39 586	19.2	526	8.8	431	24	4.4	5.6
Dubrovačko-neretvanska	28 971	20.2	318	7.3	41	5	1.6	12.2
Istarska	52 324	19.1	757	7.6	600	6	0.8	1.0
Karlovačka	37 301	25.4	1107	8.7	380	19	3.6	5.0
Koprivničko-križevačka	26 573	19.0	356	7.3	104	10	2.7	9.6
Krapinsko-zagorska	29 440	21.2	478	8.9	334	29	6.1	8.7
Ličko-senjska	16 711	13.8	647	23.9	281	7	1.9	2.5
Međimurska	24 965	30.7	790	11.6	703	39	4.9	5.5
Osiječko-baranjska	75 823	19.1	1369	9.9	803	58	4.2	7.2
Požeško-slavonska	19 997	28.7	401	9.8	251	11	2.7	4.4
Primorsko-goranska	78 055	21.7	905	4.1	383	41	6.3	10.7
Sisačko-moslavačka	50 441	18.3	703	8.1	585	22	3.1	3.8
Splitsko-dalmatinska	103 659	14.6	1287	9.0	848	42	3.3	5.0
Šibensko-kninska	32 845	10.7	160	5.0	140	10	6.3	7.1
Varaždinska	38 462	21.3	522	6.5	408	12	2.3	2.9
Virovitičko-podravska	21 176	18.7	299	9.1	193	7	2.3	3.6
Vukovarsko-srijemska	45 562	22.2	633	9.1	483	17	2.7	3.5
Zadarska	45 505	16.3	582	8.2	496	23	4.0	4.6
Zagreb town	190 200	22.3	361	2.9	680	62	6.4	9.1
Zagrebačka	69 335	17.2	327	3.1	174	14	3.9	8.0
Total	1 056 694	19.9	12 477	6.9	8541	472	3.8	5.5

FOBT: Fecal occult blood test; CRC: Colorectal cancer; pos.: Positive.

Table 3 Characteristics of polyps detected through colorectal cancer screening program in Croatia

Part of colon	Localization	Percent (%)	Size 5-10 mm	Size 10-20 mm	Size ≥ 20 mm
Left	Rectum	19	60% tubular adenoma, low degree of dysplasia	43% tubulovillous, among them 32% had areas with a high degree of dysplasia, especially in the ceco-ascending or transverse part	48% tubular adenomas
	Sigmoid	37	40% hyperplastic		
Right	Descending	8			Tubulovillous and villous
	Transverse	17	20% villous component, low degree of dysplasia		41% with high grade dysplasia
	Ceco-ascending	19			8% intramucosal carcinoma

Sometimes in our program, we were faced with problems such as a lack of educated colonoscopists and/or equipment in some counties. However, this also makes it possible for us to test feasibility in this part of the national health-care system and to plan program costs and the additional resources needed in leading time (mainly colonoscopies and education of population). This study of Croatian colonoscopic practice indicates that there are centers with practice of the highest quality, but considerable effort is required to improve the overall quality of colonoscopy. High quality of endoscopic service, early training, regular refresher courses, and continuous audit of standards at local and national levels must be a priority for all endoscopists performing colonoscopy^[16].

Additional efforts must be made to improve the quality of FOBT performance in public health institutes in order to avoid false positives.

Another problem is increasing the uptake, which is in our country significantly lower for CRC than for breast cancer (the uptake for mammography is up to 70%). On one hand, this issue results from unwillingness to take stool specimens and, on the other hand, from non-

compliance with avoiding complicated food and therapy restriction before testing. In order to increase the uptake, newly written recommendations by the Group for Quality Control of the International CRC Network support the decision that the procedure and prescriptions have to be simplified, so that they can be changed for the next cycle immediately^[15]. We are aware that guaiac FOBT shows notable variations in the performance characteristics between different studies^[17]. These differences most likely reflect the different populations tested and the methods for identifying neoplasia. Indeed, from the first results by Allison *et al*^[17] and Greenberg *et al*^[18] to meta-analysis by Soares-Weiser *et al*^[19], different sensitivities for the same tests in different populations or conditions have been reported^[20]. In the same reports, some advantages and disadvantages of immuno-FOBT are discussed. In the process of decision making on which test to choose for the Croatian Program, we considered the arrangement of mailing the tests, time from taking specimens to testing, and relatively high surrounding temperatures in almost half a year which all can influence the accuracy of immuno-FOBT^[21]. So, we have decided that

guaiac card-based tests are more convenient for screening in Croatia. Data about higher percentage of FOBT-positive persons indicated that the population screened so far was not really asymptomatic; people who returned tests most frequently had evident symptoms; mostly blood in stool or impairment of bowel discharge, constipation or diarrhea, with or without pain in the distal abdomen. Thus, there is a need actively to include the individuals who are in the “normal and healthy” population. This is frequently seen at the start of every screening program^[22,23]. It is well known that the effectiveness of any screening program depends not only on the diagnostic performance of the screening, but also on the uptake and general acceptance of the test by the public^[23,24]. In a field trial, urban-rural differences in the screening uptake were detected^[25]. Among some other issues, one of the important problems is to find how to improve uptake. There is clear evidence in our national program of early diagnosis of breast cancer, where we reached a 70% uptake level, that education of a focused population group can increase uptake^[25]. We must continue to improve awareness that screening for CRC can reduce the mortality associated with the disease. According to other studies, compliers with CRC screening are less deprived; they have higher education than non-compliers. There is also a need to advance knowledge and promote engagement of primary care physicians, according to other data^[21]. However, the Croatian Adult Health Survey showed that self-reported compliance for CRC screening was 4.5% for females and 6.1% for males included in study^[26]. It is obvious that the response in the National Screening Program was higher and it depended on the age group of the invited population and county. The results of a control field trial showed a significantly higher response rate to FOBT when given by primary care physicians^[27], but, unfortunately, in that study a small number of physicians was voluntarily included, precluding us from achieving conclusive results on the whole population. In addition, this experience cannot be easily implemented to the whole country due to organizational difficulties and presence or absence of willingness of physicians to be included in organized national screening. The results of a population study from Italy confirmed that there is a higher response if the FOBT kit was sent by mail, but in non-responders it incurred higher costs^[28]. Response to screening depends on population education but also on willingness of all included in the program, and it has to be carefully planned^[29-34]. We still do not have a detailed analysis of costs and possible differences if the test kit is not sent by mail, so this remains to be done for the next cycle.

There are some other reasons for nonparticipation which could be targeted in interventions aimed at increasing participation rates in Croatia. For example, it may be difficult to make arrangements for colonoscopy for people who live on islands and must travel to hospitals in the nearest city on the coast; they feel uncomfortable traveling by ferry while prepared for colonoscopy

and must be near the toilet during that journey. Hence, in that case, it might be reasonable to provide a mobile colonoscopy service.

Another problem of CRC screening is people with false-positive FOBT results, who occupy time in colonoscopic units and represent unnecessary procedures. According to our data, most false positive FOBT persons (false-positive to CRC but not to bleeding) had hemorrhoids or anal fissures, which is consistent with the other data^[35]. This can be avoided with adequate education of the population and active inclusion of primary care physicians^[36].

Successful intervention strategies include organizational changes, such as providing reminders to healthcare providers or users about screening opportunities, better financial support and educational strategies to improve awareness and attitudes toward CRC screening^[37,38]. In our future work we also must think about reducing inequalities related to socio economic position and ethnicity in the uptake of screening (e.g., Roma population).

In conclusion, the main characteristics of the Croatian CRC National Program are as follows: low percentage of returned FOBTs, a relatively higher number of FOBT-positive persons, but still in the range for population-based program; and a higher number of pathologic findings (CRC at the upper range and polyps above the upper range)^[39].

There are many possible strengthening mechanisms for this activity, which include multifactorial interventions that target more than one level of the screening process and likely can have greater effects. Firstly, much effort must be given to population education and mass campaign with whole society inclusion.

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COMMENTS

Background

Colorectal cancer (CRC) screening by either guaiac or immuno fecal occult blood test (FOBT) followed by colonoscopy of all positive patients is recommended worldwide. The main purpose of this screening is to detect CRC in early phase, or in pre-cancer phase when polyps can be easily removed by colonoscopy. This type of cancer can be prevented and/or diagnosed early, and patients can have a better quality of life, and mortality of CRC can be reduced.

Research frontiers

The implementation of the National CRC Screening Program in Croatia gives opportunity for better monitoring uptake of screening and to explore characteristic colonoscopic findings.

Innovations and breakthroughs

The CRC screening program in Croatia is organized through a network of public health institutes and represents good co-operation between public health and clinicians. But, there is still a place for improvement.

Applications

Epidemiologic data of screening uptake and characteristic colonoscopic findings compared with other data and also with expected ranges in European Guidelines for Quality Assurance in Colorectal Cancer Screening and Diagnosis

showed similar values in Croatia, but authors found some differences between counties.

Terminology

FOBT can detect very small amounts of blood in stools, which cannot be seen visually without this laboratory test. Patients who have polyps or CRC can be detected by this test. Colonoscopy is still the gold standard to make the final diagnosis and to remove polyps in order to prevent CRC.

Peer review

This study reported data on the CRC Screening Program in Croatia performed with FOBT followed by colonoscopy in positive cases. CRC detection rates widely ranged from 2.5% to 12.2% (mean 5.5%). There are several possible causes for these differences. Data on the histology of polyps removed at endoscopy showed differences between location in colon and size of polyps.

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Evaluation of magnifying colonoscopy in the diagnosis of serrated polyps

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Abstract

AIM: To elucidate the colonoscopic features of serrated lesions of the colorectum using magnifying colonoscopy.

METHODS: Broad division of serrated lesions of the colorectum into hyperplastic polyps (HPs), traditional

serrated adenomas (TSAs), and sessile serrated adenomas/polyps (SSA/Ps) has been proposed on the basis of recent molecular biological studies. However, few reports have examined the colonoscopic features of these divisions, including magnified colonoscopic findings. This study examined 118 lesions excised in our hospital as suspected serrated lesions after magnified observation between January 2008 and September 2011. Patient characteristics (sex, age), conventional colonoscopic findings (location, size, morphology, color, mucin) and magnified colonoscopic findings (pit pattern diagnosis) were interpreted by five colonoscopists with experience in over 1000 colonoscopies, and were compared with histopathological diagnoses. The pit patterns were categorized according to Kudo's classification, but a more detailed investigation was also performed using the subclassification [type II-Open (type II-O), type II-Long (type II-L), or type IV-Serrated (type IV-S)] proposed by Kimura T and Yamano H.

RESULTS: Lesions comprised 23 HPs (23/118: 19.5%), 39 TSAs (39/118: 33.1%: with cancer in one case), 50 SSA/Ps (50/118: 42.4%: complicated with cancer in three cases), and six others (6/118: 5.1%). We excluded six others, including three regular adenomas, one hamartoma, one inflammatory polyp, and one juvenile polyp for further analysis. Conventional colonoscopy showed that SSA/Ps were characterized as larger in diameter than TSAs and HPs (SSA/P vs HP, 13.62 ± 8.62 mm vs 7.74 ± 3.24 mm, $P < 0.001$; SSA/Ps vs TSA, 13.62 ± 8.62 mm vs 9.89 ± 5.73 mm, $P < 0.01$); common in the right side of the colon [HPs, 30.4% (7/23): TSAs, 20.5% (8/39): SSA/P, 84.0% (42/50), $P < 0.001$]; flat-elevated lesion [HPs, 30.4% (7/23): TSAs, 5.1% (2/39): SSA/Ps, 90.0% (45/50), $P < 0.001$]; normal-colored or pale mucosa [HPs, 34.8% (8/23): TSAs, 10.3% (4/39): SSA/Ps, 80% (40/50), $P < 0.001$]; and with large amounts of mucin [HPs, 21.7% (5/23): TSAs, 17.9% (7/39): SSA/Ps, 72.0% (36/50), $P < 0.001$]. In magnified colono-

scopic findings, 17 lesions showed either type II pit pattern alone or partial type II pit pattern as the basic architecture, with 14 HPs (14/17, 70.0%) and 3 SSA/Ps. Magnified colonoscopy showed the type II-O pit pattern as characteristic of SSA/Ps [sensitivity 83.7% (41/49), specificity 85.7% (54/63)]. Cancer was also present in three lesions, in all of which a type VI pit pattern was also present within the same lesion. There were four HPs and four TSAs each. The type IV-S pit pattern was characteristic of TSAs [sensitivity 96.7% (30/31), specificity 89.9% (72/81)]. Cancer was present in one lesion, in which a type VI pit pattern was also present within the same lesion. In our study, serrated lesions of the colorectum also possessed the features described in previous reports of conventional colonoscopic findings. The pit pattern diagnosis using magnifying colonoscopy, particularly magnified colonoscopic findings using subclassifications of surface architecture, reflected the pathological characteristics of SSA/Ps and TSAs, and will be useful for colonoscopic diagnosis.

CONCLUSION: We suggest that this system could be a good diagnostic tool for SSA/Ps using magnifying colonoscopy.

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Key words: Serrated adenoma; Sessile serrated adenoma/polyp; Hyperplastic polyps; Traditional serrated adenomas; Conventional colonoscopy; Magnifying colonoscopy; Pit patterns; Serrated lesions

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INTRODUCTION

In addition to the previously known oncogenic pathways of adenoma-carcinoma sequence and *de novo* cancer^[1], the so-called serrated neoplastic pathway has been proposed in recent years as a new oncogenic pathway from serrated lesions of the colorectum^[2-6]. Sessile serrated adenomas/polyps (SSA/Ps) are a particular focus of attention as one type of precursor lesion for the microsatellite deficiency (MSI-H) cancers that comprise around 15% of sporadic colorectal cancers^[7-11]. The low risk subtype of serrated adenocarcinomas is characterized by proximal location, BRAF-mutation, high CpG-island methylation with loss of MLH1 expression, and MSI-H phenotype^[10,12-14]. Identifying

serrated lesions of the colorectum during colorectal cancer surveillance is thus important, but few reports have described the colonoscopic characteristics of these lesions, particularly in terms of magnified colonoscopic findings, and a unified consensus has yet to be reached. We report herein our investigation of the colonoscopic characteristics of serrated lesions of the colorectum, using the surface architecture subclassification proposed by Kimura *et al*^[15] in addition to conventional pit pattern diagnosis.

MATERIALS AND METHODS

Patient characteristics

From among all suspected serrated lesions that were endoscopically resected in our hospital between January 2008 and September 2011, this study examined the 118 lesions for which magnified colonoscopic findings and histopathological specimens could be compared. The study was conducted in accordance with all rules and regulations of the St. Marianna University School of Medicine Institutional Review Board, and informed consent was obtained from each patient.

Endoscopic resection and magnifying colonoscopy

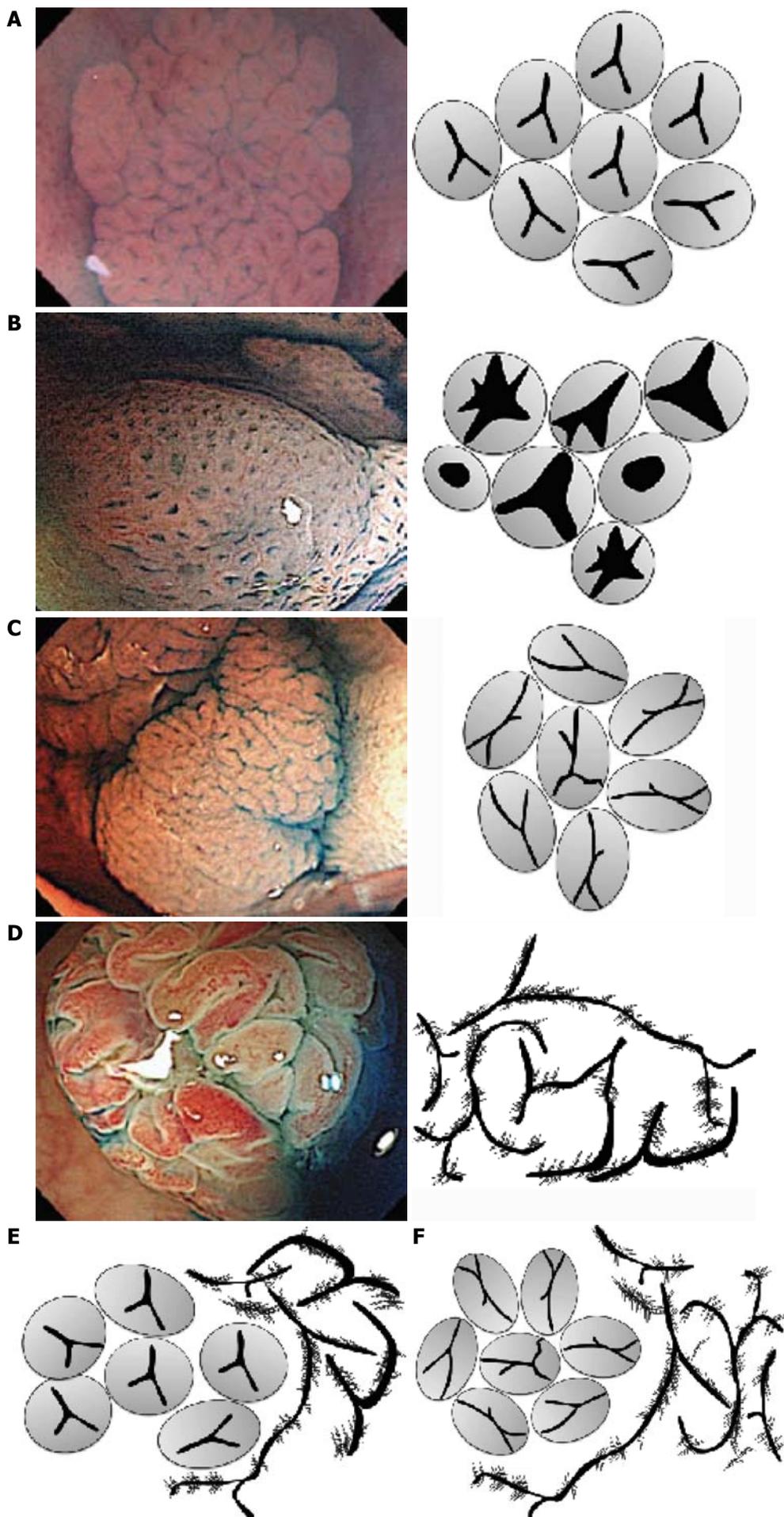
All lesions were sprayed with indigo carmine and observed under endoscopic guidance with a CF-H260AZI endoscope using the EVIS LUCERA system (Olympus, Inc., Tokyo, Japan).

Five categories of conventional colonoscopic findings (location, size, shape, color, and presence or absence of mucin) and magnifying colonoscopic findings (pit pattern diagnosis) for these lesions were interpreted by five colonoscopists with experience in over 1000 colonoscopies, and compared with histopathological diagnoses. In addition to Kudo's classification, the pit pattern was diagnosed in accordance with the subclassification proposed by Kimura *et al*^[15] and Kudo *et al*^[16] (Figure 1A-I). The endoscope was washed with an automatic washing machine and disinfectant (DISOPA Solution 0.55%, Johnson and Johnson, Langhorne, PA, United States) after each patient, according to the guidelines^[17-19].

In addition to conventional type II pit pattern according to Kudo's classification, surface architecture of the lesions was also broadly categorized as type II-Open (type II-O), type II-Long (type II-L), or type IV-Serrated (type IV-S) according to the provisional naming proposed by Kimura *et al*^[15]. If several pit patterns were present in a single lesion, the pit pattern that occupied the greatest area was regarded as the basic architecture. We categorized into two groups as SSA/Ps or others on histopathological findings. Lesions that included a diagnosis other than that of serrated lesion of the colon are indicated as "others", SSA/P, or SSA/P plus if adenoma was also present together with "SSA/Ps".

Histopathological diagnosis

Histopathological diagnosis followed conventional diagnostic criteria for regular adenomas, the diagnostic



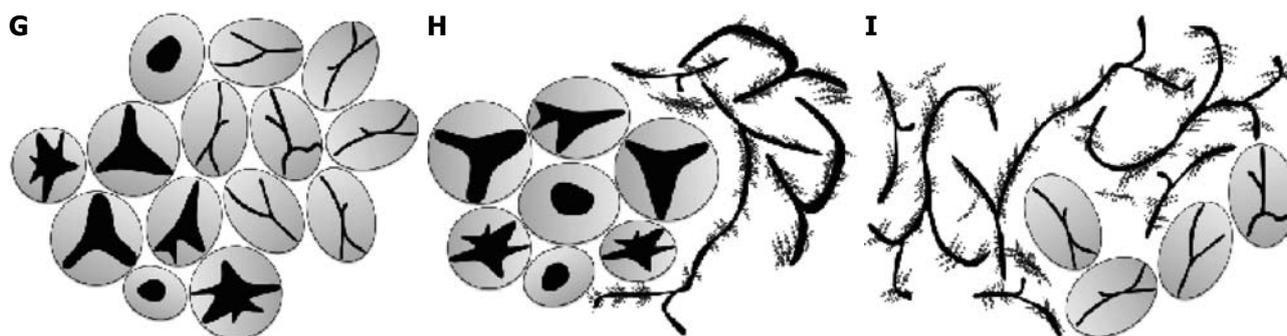


Figure 1 Type II pit patterns and subclassification of surface architectures of serrated lesions of the colon and rectum. A: Conventional type II pit pattern. Regular array of star-shaped, uniform pits with serrated architecture; B: Type II-Open pit pattern. Star-shaped pits similar to conventional type II pits, but with dilated openings of the glandular crypts; C: Type II-Long pit pattern. Similar to type II pits, but elongated without dilation; D: Type IV-Serrated pit pattern. Also called "pine cone-shaped." Villiform with a serrated architecture; E-I: Mixture of example pit patterns (E: Type II with IV-S; F: Type II-L with IV-S; G: Type II-O with II-L; H: Type II-O with IV-S; I: Type IV-S with II-L).

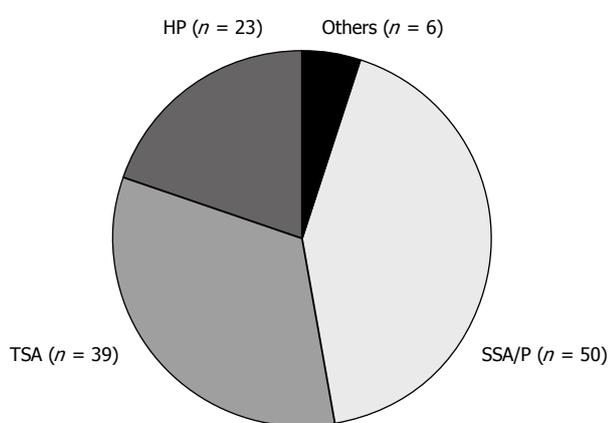


Figure 2 Classification of 118 subject lesions. Ratio of each lesions [traditional serrated adenomas (TSAs), and sessile serrated adenomas/polyps (SSA/Ps) and others].

criteria of Torlakovic *et al.*^[20] for hyperplastic polyps (HPs) and traditional serrated adenomas (TSAs). For practical purposes, according to the 2010 WHO classification, the diagnostic criteria for SSA/Ps was established by the research project "Potential of Cancerization of Colorectal Serrated Lesions" led by the Japanese Society for Cancer of the Colon and Rectum (JSCCR)^[20,21].

According to these criteria, SSA/Ps are composed of serrated cryptal epithelium with aberrant compartmentalization, essentially characterized by the architectural abnormalities listed below. If the serrated lesions had more than two findings [crypt dilation; irregularly branching crypts; horizontally arranged basal crypts (inverted T- and/or L- shaped crypts)], it can be diagnosed as SSA/Ps^[21].

Statistical analysis

Statistical testing comprised *t* tests, χ^2 tests, and Fisher's exact test, with values of $P < 0.05$ regarded as significant. All statistical analysis were performed using PRISM software for Windows, Version 4 (GraphPad Prism, Inc., San Diego, CA).

Table 1 Characteristics of the patients and conventional endoscopic findings *n* (%)

	HPs	TSAs	SSA/Ps
Cases (<i>n</i>)	23	39	50
Age (yr), mean \pm SD	58.9 \pm 12.8	61.9 \pm 13.6	63.7 \pm 10.6
Location			
Right side of colon	7 (30.4)	8 (20.5)	42 (84.0)
Left side of colon	16 (69.6)	15 (79.5)	8 (16.0)
Morphology			
Flat type	7 (30.4)	2 (5.0)	45 (90.0)
Protruded type	16 (69.6)	37 (95.0)	5 (10.0)
Color			
NC	8 (34.8)	4 (10.3)	40 (80.0)
RC	15 (65.2)	35 (89.7)	10 (20.0)
Mucin			
With	5 (21.7)	7 (17.9)	36 (72.0)
Without	18 (78.3)	32 (82.1)	14 (28.0)
Size (mm), mean \pm SD	7.74 \pm 3.24	9.89 \pm 5.73	13.42 \pm 8.62

HPs: Hyperplastic polyps; TSAs: Traditional serrated adenomas; SSA/Ps: Sessile serrated adenoma/polyps; NC: Normal-colored or pale in color of mucosa; RC: Reddish change in color.

RESULTS

Patient characteristics

Lesions comprised 23 HPs, 39 TSAs (complicated with cancer in one case), 50 SSA/Ps (complicated with cancer in three cases), and six others, including regular adenomas and juvenile polyps. We excluded these six other lesions for further analysis. Patient characteristics and conventional colonoscopic findings are shown in Table 1 and Figure 2. Mean age and sex (percentage of females) were 58.9 years/13% for HPs, 61.9 years/33.3% for TSAs, and 63.7 years/32% for SSA/Ps. No characteristic trends were evident in patient backgrounds (Table 1).

Conventional colonoscopic findings

We categorized the location of lesions as either right side of the colon (on the oral side of the transverse colon) or left side of the colon (on the anal side of the transverse colon). Results showed that HPs tended to locate on the

left side of the colon 69.6% (16/23) rather than the right side 30.4% (7/23). TSAs were more frequently located on the left side 79.5% (31/39) rather than the right side 20.5% (8/39). 84.0% (42/50) of SSA/Ps were on the right and 16% (8/50) were on the left (Table 1). SSA/Ps were found significantly more often on the right, compared with TSAs and HPs ($P < 0.001$) (Table 1).

Macroscopic morphology was categorized as either flat type (includes 0-IIa, superficial flat/elevated tumors: LST, lateral spreading tumor) or protruded type (0-Ip, protruded/pedunculated: 0-Isp, protruded/mixed sessile and pedunculated) based on JSCCR Guidelines 2010 for the treatment of colorectal cancer^[22]. Results were as follows: 30.4% (7/23) of HPs were flat type and 69.6% (16/23) were protruded type; 5.1% (2/39) of TSAs were flat type and 94.9% (37/39) were protruded type; and 90.0% (45/50) of SSA/Ps were flat type and 10.0% (5/50) were protruded type. Significantly more SSA/Ps were identified as flat surface lesions compared with TSAs and HPs ($P < 0.001$) (Table 1).

We categorized as either reddish change (RC) or normal-colored (NC) or pale mucosa. The results were as follows: 65.2% (15/23) of HPs were RC and 34.8% (8/23) were NC; 89.7% (35/39) of TSAs were RC and 10.3% (4/39) were NC; and 20.0% (10/50) of SSA/Ps were RC and 80.0% (40/50) were NC. Significantly more SSA/Ps were same to whitish change in color compared with TSAs and HPs ($P < 0.001$) (Table 1).

We categorized lesions as without mucin if they could be observed without washing or with only regular washing, or with mucin if they required several repeated washings before they could be observed. Results were as follows: 78.3% (18/23) of HPs were without mucin and 21.7% (5/23) were with mucin; 82.1% (32/39) of TSAs were without mucin and 17.9% (7/39) were with mucin; and 28% (14/50) of SSA/Ps were without mucin and 72% (36/50) were with mucin. Significantly more SSA/Ps showed abundant mucin compared with TSAs and HPs ($P < 0.001$) (Table 1).

According to the tumor size, mean lesion size was 7.74 ± 3.24 mm for HPs, 9.89 ± 5.73 mm for TSAs, and 13.62 ± 8.62 mm for SSA/Ps. SSA/Ps were significantly larger in mean size compared with TSAs and HPs (SSA/P *vs* HP, $P < 0.001$; SSA/P *vs* TSA, $P < 0.01$) (Table 1).

Magnified colonoscopic findings

A comparison of magnified colonoscopic findings and histopathological diagnoses is given in Table 2. Seventeen lesions showed either type II pit pattern alone or partial type II pit pattern as the basic architecture, with 14 HPs (82.4%) and 3 SSA/Ps (Table 2).

Fifteen lesions showed either type II-L alone or partial type II-L as the basic architecture, of which 6 were SSA/Ps, 5 were TSAs, 4 were HPs. Forty-nine lesions showed either type II-O alone or partial type II-O as the basic architecture, with SSA/Ps histology evident in 41 (83.7%) (Figure 1, Table 2). Cancer was also present in 3 lesions, in all of which a type VI pit pattern was also present within the same lesion (Figure 3C and F). There were

4 HPs and 4 TSAs.

Thirty-one lesions showed either type IV-S alone or partial type IV-S as the basic architecture, including 30 TSAs (96.8%). Cancer was present in one lesion, in which a type V₁ pit pattern was also present within the same lesion.

DISCUSSION

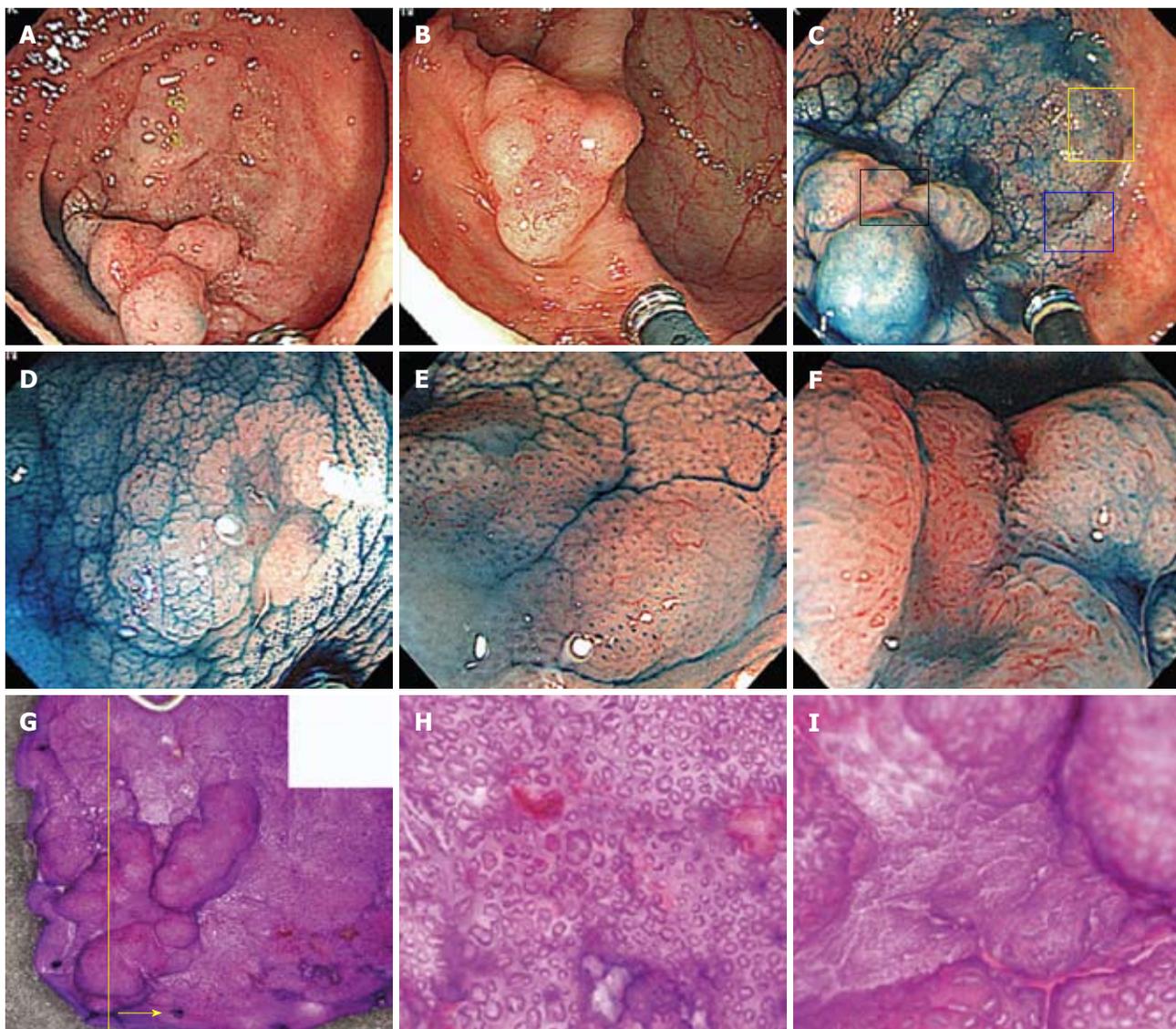
Epithelial polyps, a serrated architecture without atypical cells, were previously called HPs. Such lesions were believed not to possess cancerous potential. In 1990, Longacre *et al.*^[23] proposed that lesions with a serrated architecture and atypicalities should be known as SAs. Torlakovic *et al.*^[20,24] subsequently suggested the existence of a subtype comprising atypia admixed within HPs, for which the name SSAs was proposed. Some, however, consider the term adenoma inappropriate, because these lesions cannot be recognized as tumorous. The latest 2010 WHO classification uses the term SSA/Ps, incorporating both adenoma and polyp^[21,25]. The concept of serrated lesions, particularly SSA/Ps, is therefore comparatively new, with terminology and classifications that have only recently been standardized. Although this transition has yet to be fully adopted, the fact that it is widely recognized that there is no risk of cancer developing from HPs is one reason for the lack of detailed reports on the colonoscopic characteristics of these lesions.

Based on the WHO classification, serrated lesions of the colorectum can be broadly divided into HPs, TSAs, and SSA/Ps^[25]. The present study investigated the characteristics of these three types from conventional colonoscopic findings and magnified colonoscopic findings (pit pattern diagnosis), which are routinely used in everyday colonoscopic evaluation^[25]. To start with conventional colonoscopic findings, the characteristics of HPs are that they tend to occur in the left side of the colon, particularly in the rectum, are commonly less than 5 mm in size, and are NC or pale in color and flat^[26]. In this study, HPs did not necessarily fit these characteristics. This may have been because the lesions investigated in the present study had been removed colonoscopically, and HPs that exhibited typical characteristics were not indicated for resection. HPs thus included many atypical lesions. The characteristics of TSAs are that they are Isp or Ip protruded lesions that tend to occur on the left side of the colon, vary in size from less than 5 mm to over 10 mm (although most are less than 10 mm)^[23], and are often reddish in color. A further characteristic that was not included in this study is that some flat lesions show a surface architecture that can be described as highly protruding double elevation, pinecone-shaped, or coral-shaped^[15,27,28]. In the present study, TSAs mostly satisfied these characteristics. SSA/Ps have been reported as a subclassification of HPs^[20,24], and the colonoscopic findings are mostly similar. However, SSA/Ps tend to occur more often in the right colon compared with HPs; many are large, with a diameter exceeding 10 mm, and coloration may be yellowish due to mucin^[29]. Our findings in the present study were similar.

Table 2 Comparison of the magnified colonoscopic findings and pathological diagnoses

Pit pattern	<i>n</i>	HPs (<i>n</i> = 23)	TSAs (<i>n</i> = 38)	TSAs + cancer (<i>n</i> = 1)	SSA/Ps + α (<i>n</i> = 47)	SSA/Ps + cancer (<i>n</i> = 3)
Type II	17	14			3	
Alone	16	13			3	
With IV-S	1	1				
Type II-L	15	4	5		6	
Alone	10	4	1		5	
With IV-S	5		4		1	
Type II-O	49	4	4		38	3
Alone	30	3			27	
With II-L	9		1		8	
With IV-S	7	1	3		3	
With V ₁	3					3
Type IV-S	31	1	29	1		
Alone	28	1	27			
With II-L	2		2			
With V ₁	1			1		

HPs: Hyperplastic polyp; TSAs: Traditional serrated adenoma; SSA/Ps: Sessile serrated adenoma/polyp; SSA/Ps + α : SSA/Ps or SSA/Ps + adenoma, others except serrated lesions.



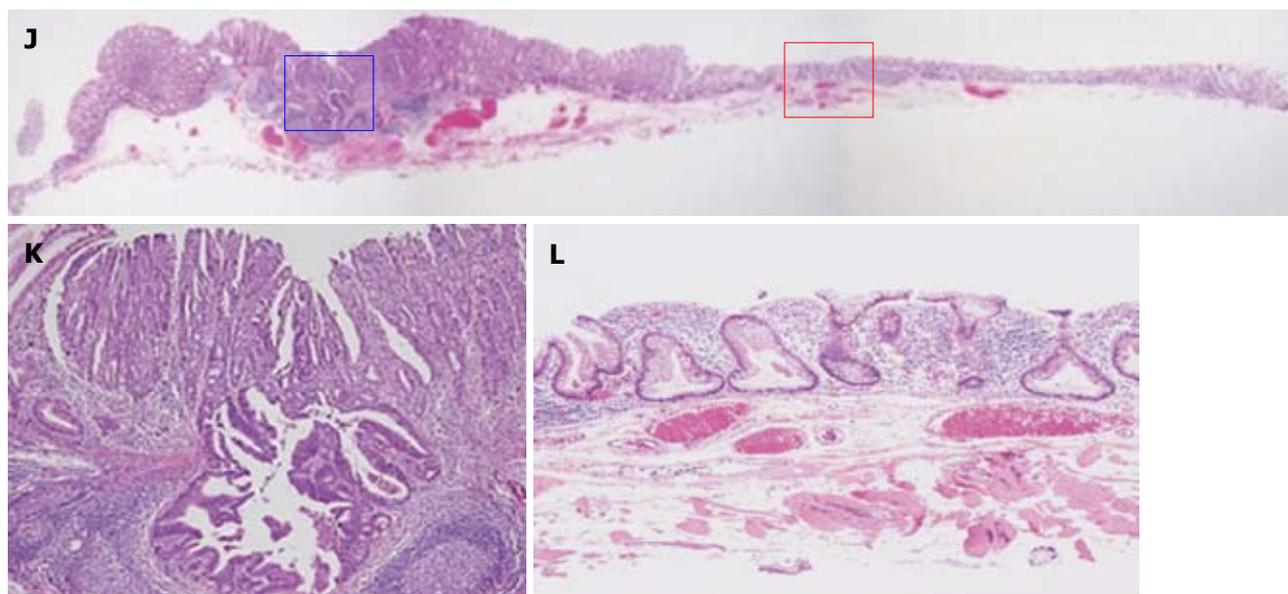


Figure 3 Adenocarcinoma (tub1) in sessile serrated adenomas/polyps case. A 60-year-old woman had a tumor lesion in the cecum, which was flat and elevated, that is a so-called lateral spreading tumor (LST), 45 mm in diameter. A, B: Standard view. A 45-mm LST lesion with large amounts of mucin is evident in the cecum. The flat portion is somewhat discolored compared with the surrounding mucosa. The protruded portion on the anal side is reddish in the center; C: Indigo carmine staining. The center of the protruded portion on the anal side is flat; D: Indigo carmine staining, enlarged image (yellow square in 3C). Type II-Long pit pattern is evident; E: Indigo carmine staining, enlarged image (blue square in 3C). Type II-Open pit pattern is evident; F: Indigo carmine staining, enlarged image (black square in 3C). We diagnosed as type VI pit pattern, because high density of crypts and irregular pit pattern were evident; G: Comparison of stereomicroscopic and colonoscopic images; H: Enlarged image of flat portion. Although basically type II, dilated duct openings are evident; I: Enlarged image of protruded portion on the anal side. An irregular surface architecture is evident; J: HE magnifying glass image (yellow line in Figure 3G). We examined HE staining (Figure 3J-L) using the yellow cutting line of endoscopically resected tissue (yellow arrow side tissue); K: Central part of the protrusion on the anal side (blue square in Figure 3J). Highly differentiated ductal cancer corresponding to type VI pits is evident; L: Dilatation of crypts and deformation in the horizontal direction at the bottom of the crypts are evident in the flat portion (red square in Figure 3J). Sessile serrated adenomas/polyps was diagnosed.

In magnified colonoscopic findings, Rembacken *et al*^[30] reported the value of SA diagnosis of lesions with serrated architecture with type III_L tubiform pit pattern as type III_H pits, and type IV villiform pit pattern enlarged to resemble pine cones as type IV_H pit pattern. These pits may be considered to correspond to the type IV-S pits of the present study. In this study, TSAs accounted for 76.9% (30/39) of lesions with a basic architecture of type IV-S pit pattern, and lesions without type IV-S pit pattern could be classified as non-TSAs with 96.7% sensitivity and 88.9% specificity. Lesions previously regarded as SAs are now considered to be TSAs, and type IV-S pit pattern can be considered a characteristic finding of TSAs.

Pit patterns reflect the pathological characteristics of lesions, and if the characteristics differ, different pit patterns should also be apparent. HPs are characterized histologically by serrated crypts, with straight crypts evenly distributed throughout^[31]. There should thus be no argument that the type II pit pattern of Kudo's classification reflect this characteristic. Diagnostic criteria for SSA/Ps, however, have yet to be standardized, and the classifications of Higuchi *et al*^[32,33] have mainly been used in Japan. The JSCCR diagnostic criteria used in the present study have extracted three categories from among those defined by Higuchi *et al*^[32,33] that can be regarded as important expressions of the nature of SSA/Ps. Figure 3 shows a typical case of SSA/Ps complicated with cancer treated in our hospital. In terms of serrated crypt, as in HPs, "dilatation of crypts" can be named as an observ-

able colonoscopic finding. This finding may be considered to correspond to the type II-O pit pattern proposed by Kimura *et al*^[15]. The present study also found SSA/P histology in 41 of 50 lesions (82.0%) with type II-O pit pattern as the basic architecture. Lesions without type II-O pit pattern could be classified as non-SSA/Ps with 83.7% sensitivity and 85.7% specificity. We could not deny that we diagnosed as HPs some lesions that even had a tiny SSA/P component at the glandular base. Although it is therefore not possible to diagnose all SSA/Ps solely on the basis of type II-O pit pattern, at least this finding is useful for the diagnosis of SSA/Ps with dilatation of crypts. Even if not diagnosed as SSA/Ps according to current diagnostic criteria, lesions with type II-O pit pattern reportedly possess molecular biological commonalities and regarding these as a single population may be preferable^[9,15].

TSAs show a greater number of characteristic findings compared with HPs and SSA/Ps, facilitating colonoscopic diagnosis. Magnified colonoscopic assessment of the histological characteristics of SSA/Ps is difficult when they do not exhibit dilatation of the crypts, and distinguishing them from HPs is difficult at this point. Our findings suggest, however, that type II-O pit pattern may be of assistance in the colonoscopic diagnosis of lesions with dilatation of crypts.

The concept of serrated lesions of the colorectum is relatively recent, and criteria for pathological diagnosis have yet to be standardized. As TSAs and SSA/Ps are

undoubtedly precursor lesions of colon cancer, however, colonoscopists are faced with the task of establishing colonoscopic diagnoses and elucidating lesions for which treatment is indicated. Identifying serrated lesions of the colorectum during colorectal cancer surveillance is thus important. Rex *et al* recommend that all serrated lesions proximal to the sigmoid colon and all serrated lesions in the rectosigmoid > 5 mm in size, be completely removed^[34-37]. However, many issues still remain, including whether all TSAs and SSA/Ps should be indicated for treatment as precursor lesions for colon cancer and the probability of progression to a cancerous state. We hope that the present findings will be useful as a reference for the accumulation of larger numbers of cases in further studies to elucidate the oncogenic pathways in serrated lesions of the colon and rectum.

COMMENTS

Background

Broad division of serrated lesions of the colorectum into hyperplastic polyps, traditional serrated adenomas (TSAs), and sessile serrated adenomas/polyps (SSA/Ps) has been proposed on the basis of recent molecular biological studies. However, few reports have examined the colonoscopic features of these divisions, including magnified colonoscopic findings. In this article, the authors verified the diagnostic potency for SSA/Ps using the pit pattern classifications by magnifying colonoscopy.

Research frontiers

The pit patterns were categorized according to Kudo's classification, but a more detailed investigation was also performed using the subclassification [type II-Open (type II-O), type II-Long (type II-L), or type IV-Serrated (type IV-S)] proposed by Kimura T and Yamano H. However, it has yet to be standardized. In this study, the authors performed herein the investigation of the colonoscopic characteristics of serrated lesions of the colorectum, using the surface architecture subclassification proposed by Kimura T and Yamano H in addition to conventional pit pattern diagnosis

Innovations and breakthroughs

The authors successfully confirmed that the pit pattern diagnosis using magnifying colonoscopy, particularly magnified colonoscopic findings using subclassifications of surface architecture, reflected the pathological characteristics of SSA/Ps and TSAs.

Applications

This study offers a better understanding of pathological characteristics of SSA/Ps and TSAs using subclassifications of surface architecture by magnified colonoscopy.

Terminology

Pit patterns reflect the pathological characteristics of lesions, and if the characteristics differ, different pit patterns should also be apparent. Hyperplastic polyps are characterized histologically by serrated crypts, with straight crypts evenly distributed throughout. There should thus be no argument that the type II pit pattern of Kudo's classification reflect this characteristic.

Peer review

In magnified colonoscopic findings, lesions without type II-O pit pattern could be classified as non-SSA/Ps with high sensitivity and specificity. It is therefore not possible to diagnose all SSA/Ps solely on the basis of type II-O pit pattern, at least this finding is useful for the diagnosis of SSA/Ps with dilatation of crypts.

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Circular smooth muscle contributes to esophageal shortening during peristalsis

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Abstract

AIM: To study the angle between the circular smooth muscle (CSM) and longitudinal smooth muscle (LSM) fibers in the distal esophagus.

METHODS: In order to identify possible mechanisms for greater shortening in the distal compared to proximal esophagus during peristalsis, the angles between the LSM and CSM layers were measured in 9 cadavers. The outer longitudinal layer of the muscularis propria was exposed after stripping the outer serosa. The inner circular layer of the muscularis propria was then revealed after dissection of the esophageal mucosa and the underlying muscularis mucosa. Photographs of each specimen were taken with half of the open esophagus folded back showing both the outer longitudinal and inner circular muscle layers. Angles were measured every one cm for 10 cm proximal to the squamocolumnar junction (SCJ) by two independent investigators. Two human esophagi were obtained from organ transplant donors and the angles between the circular and longitudinal smooth muscle layers were measured using micro-computed tomography (micro CT) and Image J software.

RESULTS: All data are presented as mean \pm SE. The CSM to LSM angle at the SCJ and 1 cm proximal to SCJ on the autopsy specimens was 69.3 ± 4.62 degrees vs 74.9 ± 3.09 degrees, $P = 0.32$. The CSM to LSM angle at SCJ were statistically significantly lower than at 2, 3, 4 and 5 cm proximal to the SCJ, 69.3 ± 4.62 degrees vs 82.58 ± 1.34 degrees, 84.04 ± 1.64 degrees, 84.87 ± 1.04 degrees and 83.72 ± 1.42 degrees, $P = 0.013$, $P = 0.008$, $P = 0.004$, $P = 0.009$ respectively. The CSM to LSM angle at SCJ was also statistically significantly lower than the angles at 6, 7 and 8 cm proximal to the SCJ, 69.3 ± 4.62 degrees vs 80.18 ± 2.09 degrees, 81.81 ± 1.75 degrees and 80.96 ± 2.04 degrees, $P = 0.05$, $P = 0.02$, $P = 0.03$ respectively. The CSM to LSM angle at 1 cm proximal to SCJ was statistically significantly lower than at 3, 4 and 5 cm proximal to the SCJ, 74.94 ± 3.09 degrees vs 84.04 ± 1.64 degrees, 84.87 ± 1.04 degrees and 83.72 ± 1.42 degrees, $P = 0.019$, $P = 0.008$, $P = 0.02$ respectively. At 10 cm above SCJ the angle was 80.06 ± 2.13 degrees which is close to

being perpendicular but less than 90 degrees. The CSM to LSM angles measured on virtual dissection of the esophagus and the stomach on micro CT at the SCJ and 1 cm proximal to the SCJ were 48.39 ± 0.72 degrees and 50.81 ± 1.59 degrees. Rather than the angle of the CSM and LSM being perpendicular in the esophagus we found an acute angulation between these two muscle groups throughout the lower 10 cm of the esophagus.

CONCLUSION: The oblique angulation of the CSM may contribute to the significantly greater shortening of distal esophagus when compared to the mid and proximal esophagus during peristalsis.

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Key words: Esophageal shortening; Gastroesophageal junction; Circular smooth muscle; Gastroesophageal reflux disease; Esophageal contraction

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INTRODUCTION

The mechanisms of contraction and bolus transportation of the esophagus have been of great interest^[1]. Peristalsis in the smooth muscle portion of the esophagus involves the interaction of central and peripheral neural mechanisms and the interaction between these neural mechanisms and the smooth muscle^[2,3]. Different instruments have been devised to measure the motions of esophageal peristalsis^[4,5]. The longitudinal muscle contraction of the human esophagus has been inferred from longitudinal shortening measured using widely spaced metal clips attached to the esophageal mucosa. The measured degrees of shortening vary depending upon how widely the clips are placed. The more widely spaced the clips, the more the measurement will under predict true longitudinal shortening. Furthermore, the relative motion between a clip on the mucosal surface and the underlying muscle layer introduces additional unknown errors into the estimate of longitudinal shortening^[6-8]. We recently demonstrated that the mucosa shortens independent of the muscularis propria, upon cholinergic stimulation and with peristaltic contraction^[9,10]. Nicosia *et al*^[11] avoided this variability associated with mucosal clips, by developing a procedure to accurately measure local longitudinal shortening of the esophageal wall using simultaneous

high-resolution endoluminal ultrasound and manometry during peristaltic contraction.

In a follow-up study we measured cross sectional area and manometry at four levels in the esophagus: 5 cm, 10 cm, 15 cm and 20 cm above the upper border of the distal esophageal high-pressure zone. We found that (1) the shortening of the circular smooth muscle (CSM) and longitudinal smooth muscle (LSM), at 5 cm was significantly greater than at 20 cm above the distal esophageal high pressure zone; and (2) the CSM and LSM both shortened in the longitudinal direction but the CSM contribution towards longitudinal esophageal shortening at the distal esophagus was greater than at the proximal esophagus^[12]. This was surprising, as it was commonly assumed that the LSM of the esophagus was completely responsible for esophageal shortening, while the CSM was responsible for the narrowing of the lumen and the pressure generated within the esophageal lumen. Before this finding it was assumed that CSM contraction did not actively contribute to the longitudinal esophageal shortening and that any shortening within the CSM occurred by longitudinal smooth muscle drag.

We proposed several hypotheses to explain this physiologic phenomenon: (1) the LSM dragged the CSM with it during shortening causing the CSM to shorten. While this could account for some of the CSM shortening it could not account for the CSM shortening more than what is accounted for by the LSM. This also cannot explain the regional differences in shortening along the length of the esophagus; (2) the entire esophagus is tonically stretched at rest (under axial tension) and during swallowing there is a decrease in this tension due to deglutitive inhibition causing the circular smooth muscle to shorten, as a spring would shorten when the tension was released. Once again this could account for some of the CSM shortening but could not account for the differences in CSM shortening along the length of the esophagus; and (3) the CSM fibers actually spiral down the esophagus at an angle other than perpendicular to the longitudinal axis leading to subsequent shortening of the CSM during peristaltic contraction. This seemed the most likely explanation for the CSM shortening more than that contributed by LSM in the distal esophagus. In addition we hypothesized that if this was the case, one would observe that the distal spiral angle would be greater than the proximal spiral angle.

The aim of this study was to provide an explanation to the functional observation obtained by Dai *et al*^[12], that the CSM shortens more than can be explained by longitudinal drag of the LSM alone during peristaltic contraction, especially in the distal esophagus.

MATERIALS AND METHODS

Study design and conduct

This research study was approved by the Temple University Institutional review board. Data management, analysis and interpretation and preparation of the manuscript

were performed by the authors who had full access to all the study data. All authors had responsibility for the decision to submit the manuscript for publication, and vouch for the accuracy, integrity, and completeness of the data as reported.

Assessments and outcome measurements

In the current study, we used fine dissection of cadaveric esophagi to define the LSM and CSM layers in detail. Angles of the CSM fibers relative to the axial direction (longitudinal muscle fibers) of the esophagus were measured. In addition, we used micro-computed tomography (micro CT) scan to virtually dissect the human esophagi obtained from organ transplant donors to evaluate the angle of the CSM relative to LSM.

Cadaveric esophageal measurements

Nine human esophagi were procured from formaldehyde preserved cadavers previously used for medical education purposes. Each esophagus was removed from the pharyngo-esophageal junction to the cardia of the stomach. The outer longitudinal layer of the muscularis propria was exposed after carefully stripping the outer serosa. Minimal tension was applied to straighten the esophagus and the esophagus was then longitudinally cut open in a straight line following the orientation of the longitudinal muscles. The squamocolumnar junction (SCJ) was identified on the mucosa and its location was marked on the specimen. The inner circular layer of the muscularis propria was then revealed after careful dissection of the esophageal mucosa and the underlying muscularis mucosa. Photographs of each specimen were taken with half of the open esophagus folded back showing both the outer longitudinal and inner circular muscle layers. Although the entire esophagus is much longer only the distal 10 cm of the esophagus was used to make measurements. A ruler was also included in the pictures with a mark placed at the SCJ, which was identified earlier. The ruler was also used to mark the esophagus at every centimeter proximal to the SCJ. Special attention was given to ensure that the folded-back longitudinal layer was in its *in vivo* position relative to the circular layer by carefully aligning the marks made along the incision line on the esophagus before it was cut open (Figure 1).

Image Pro Plus (version 6.1, Media Cybernetics, Inc. MD) was used to analyze each digital image. Angles between the longitudinal and the circular muscles were measured by two independent investigators, blinded to the other investigators measurements. Measurements were made starting at the SCJ and moving proximally measuring every 1 cm for 10 cm.

High-resolution micro-computed tomography scan of the gastroesophageal junction

Two human esophagi and stomachs were obtained from organ transplant donors (National Disease Research Institute and the International Institute for the Advancement of Medicine). The specimens were fixed by sub-

mersion in 4% paraformaldehyde for 10 d, followed by immersion in a 2% phosphotungstic acid, 0.02% potassium permanganate, 0.1% hematoxylin (PTAH) solution for 48 h after fixative wash out using running tap water for several hours. The specimen was removed carefully, washed and then dissected down in size so that a region of 5 cm diameter \times 5 cm in height remained. This block of tissue was supported by a piece of Styrofoam that was shaped to match the shape of the specimen and wrapped in Parafilm and sealed closed to maintain hydration.

SkyScan 1172 high-resolution cone-beam micro-CT scanner (SkyScan, Ltd, Kontich, Belgium), with a Hamamatsu C9300 11Mp camera, was used to scan the specimens using the double-size and oversize sample options. The following settings were used: a camera pixel size of 8.76 μm , an image pixel size of 11.89 μm , voltage of 80 kV, current of 124 μA , aluminum 0.5 mm as well as copper filters, rotation step of 0.50 degrees, 180 degrees of rotation, a frame averaging of 11 335 rows, 3872 columns, and a scan duration of 3 h and 45 min. The image slices were reconstructed using cone-beam reconstruction software (SkyScan NRecon) based on the Feldkamp algorithm, on 3 linked servers, a process that yielded 3492 tomographic sections, each with a thickness of 11.89 μm in thickness, in the axial (transverse) plane, for each sample. A ring artifact correction of 10, and a beam hardening correction of 40% was applied to both samples.

Three dimensional (3D) image constructs were made using 3D imaging software (SkyScan CTvox). Virtual dissection of these 3D constructs was performed in order to image the circular smooth muscle and the longitudinal smooth muscle in the same plane, using sphere-shaped and cylinder-shaped virtual “cutting boxes” (Figure 2). Image J software was used to measure the angle between the muscle fibers of the LSM and the CSM. Although the entire esophagus is much longer only the distal 5 cm of the esophagus was used due to the limitations of the micro-CT scanner.

Statistical analysis

All data are presented as mean \pm SE. Statistical analysis was performed using one way analysis of variance with post hoc pair wise comparisons using the Bonferroni method.

RESULTS

All data are presented as mean \pm SE. The CSM to LSM angle at the SCJ and 1 cm proximal to SCJ on the autopsy specimens was 69.3 ± 4.62 degrees *vs* 74.9 ± 3.09 degrees, $P = 0.32$. The CSM to LSM angle at SCJ were statistically significantly lower than at 2 cm, 3 cm, 4 cm and 5 cm proximal to the SCJ, 69.3 ± 4.62 degrees *vs* 82.58 ± 1.34 degrees, 84.04 ± 1.64 degrees, 84.87 ± 1.04 degrees and 83.72 ± 1.42 degrees, $P = 0.013$, $P = 0.008$, $P = 0.004$, $P = 0.009$ respectively. The CSM to LSM angle at SCJ was also statistically significantly lower than the angles at

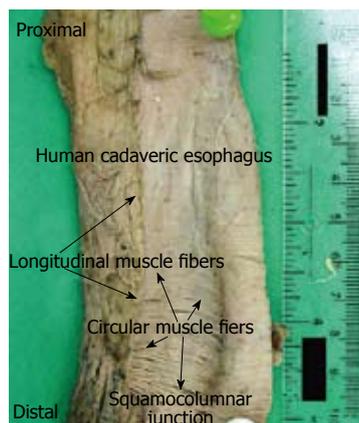


Figure 1 A photograph of a dissected cadaveric esophagus. The esophagus is cut longitudinally along the longitudinal muscle fibers after dissecting the serosa. The mucosa and sub mucosa were later dissected. The esophagus was later folded back so that the longitudinal fibers were on top of the circular muscle fibers. Measurements of the angle of the circular smooth muscle fibers with respect to the longitudinal smooth muscle fibers were made starting from the squamocolumnar junction.

6, 7 and 8 cm proximal to the SCJ, 69.3 ± 4.62 degrees *vs* 80.18 ± 2.09 degrees, 81.81 ± 1.75 degrees and 80.96 ± 2.04 degrees, $P = 0.05$, $P = 0.02$, $P = 0.03$ respectively. The CSM to LSM angle at 1 cm proximal to SCJ was statistically significantly lower than at 3 cm, 4 cm and 5 cm proximal to the SCJ, 74.94 ± 3.09 degrees *vs* 84.04 ± 1.64 degrees, 84.87 ± 1.04 degrees and 83.72 ± 1.42 degrees, $P = 0.019$, $P = 0.008$, $P = 0.02$ respectively. At 10 cm above SCJ the angle was 80.06 ± 2.13 degrees which is close to being perpendicular but less than 90 degrees (Figure 3A). The CSM to LSM angles measured on virtual dissection of the esophagus and the stomach on micro CT at the SCJ and 1 cm proximal to the SCJ were 48.39 ± 0.72 degrees and 50.81 ± 1.59 degrees. Rather than the angle of the CSM and LSM being perpendicular in the esophagus we found an acute angulation between these two muscle groups throughout the lower 10 cm of the esophagus. (Figure 3B).

DISCUSSION

A number of prior studies looking at longitudinal shortening were performed using metallic clips or wires. Dodds *et al*^[6] measured the axial motion of four tantalum wires inserted into the cat esophageal mucosa in vivo. Their results suggested the progression of a wave of longitudinal shortening into the lower esophagus roughly coincident with the bolus tail. Pouderoux *et al*^[8] placed three clips at 4-cm intervals in the distal 8-10 cm of the human esophagus and concluded: “contracting longitudinal segment was advancing ahead of the contracting circular muscle segment.” An earlier study with mucosal clips by Edmundowicz *et al*^[7] measured the change in length of upper and lower halves of the human esophagus and observed that early in the swallow the lower half lengthened while the upper half shortened, consistent with the clip motions in the cat esophagus measured by Dodds *et al*^[6]. Their measurements were also consistent with the later

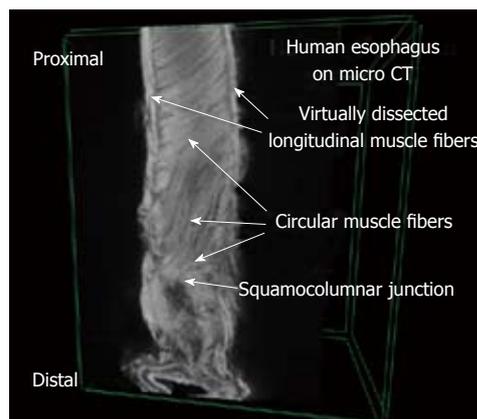


Figure 2 A human esophagus obtained using micro-computed tomography. The longitudinal muscle fibers were virtually dissected to expose the underlying circular muscle fibers. Measurements were made starting from the squamocolumnar junction.

clip data of Pouderoux *et al*^[8], who observed that two adjacent 4-cm segments of the distal esophagus initially lengthen and later shorten in peristalsis-like fashion. Dai *et al*^[13], Liu *et al*^[14] and Miller *et al*^[15-19] pioneered the use of endoluminal ultrasound to evaluate esophageal motility. Miller *et al*^[20-22] combined the use of endoluminal ultrasound with manometry to study peristaltic contraction. Nicosia *et al*^[11] used this new method to measure local longitudinal esophageal shortening in which we calculated shortening for both the circular CSM and LSM using the equation $\text{Cross Sectional Area}_{\text{rest}} / \text{Cross Sectional Area}_{\text{contract}} = \text{Length}_{\text{contract}} / \text{Length}_{\text{rest}}$. It is thought that there is a mechanical advantage of local longitudinal shortening on peristaltic transport in the human esophagus. The peristaltic wave of local longitudinal muscle contraction coordinated with the circular muscle contraction wave has both a physiological advantage (concentrating circular muscle fibers), and a mechanical advantage (reducing the level of contractile force required to transport the bolus), which combine to greatly reduce circular muscle tone during esophageal peristalsis^[23].

In a follow-up study evaluating local longitudinal shortening in different regions of the esophagus, we identified regional differences in local longitudinal shortening. The distal esophagus shortened more than the proximal esophagus. In addition the local longitudinal shortening of the CSM and total muscularis propria at 5 cm above the LES were significantly greater than at 20 cm. The shortening of LSM at 5 cm *vs* 20 cm was not statistically significant. These surprising results imply that the CSM was actually responsible for the greater shortening of the distal esophagus. We hypothesized that the increase in shortening of the CSM was due to the spiral nature of the muscle fibers within the distal CSM^[12].

An extensive literature search yielded only a handful of publications that suggested the oblique/spiral nature of the CSM in the body of the esophagus. Netter illustrated in his atlas a distal circular smooth muscle layer oriented in a spiral fashion^[24]. Floch in Netter’s Gastroenterology textbook described, “In the upper esophagus, the circular muscle closely approximates the encircling lower

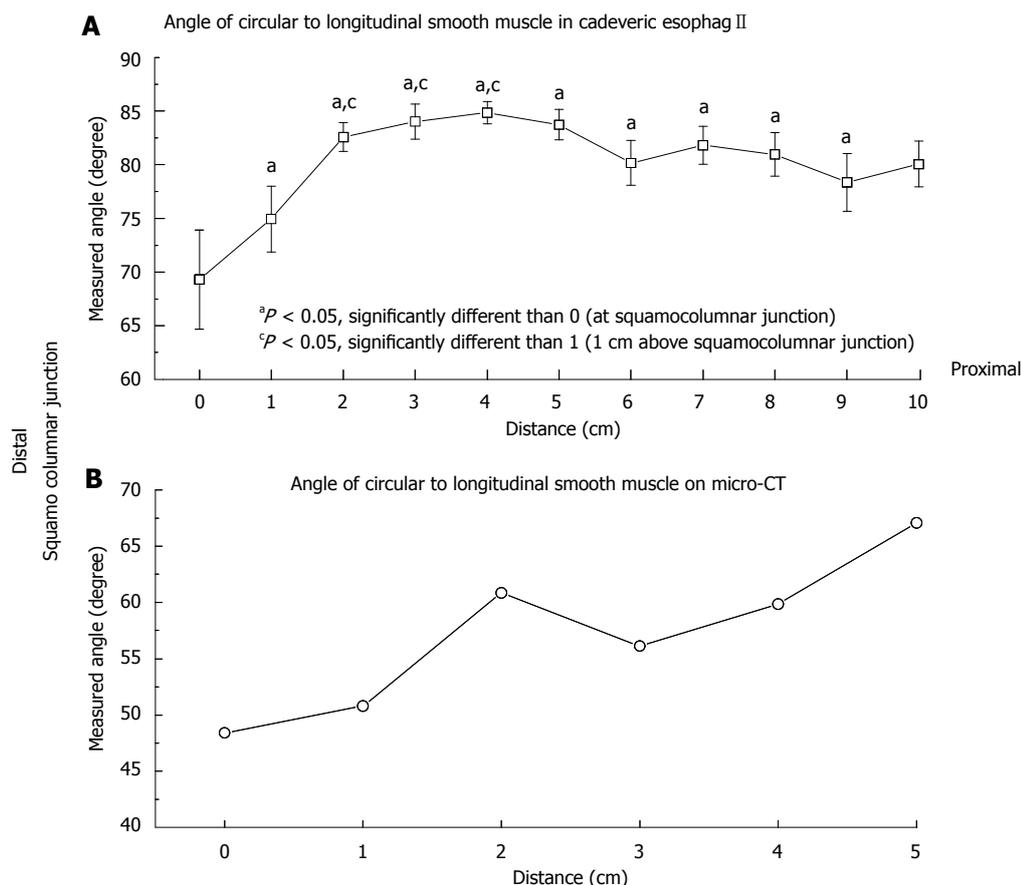


Figure 3 The average angle of the circular smooth muscle with respect to the longitudinal smooth muscle. A: On cadaveric esophagi. The x-axis represents the distance from the squamocolumnar junction with 0 being the squamocolumnar junction. At 90 degrees the circular smooth muscle and longitudinal smooth muscle are perpendicular to each other. Therefore the smaller the angle the greater the deviation from the perpendicular and the greater the resulting shortening during peristaltic contraction; B: Measured using micro computed tomography. The x-axis represents the distance from the squamocolumnar junction with 0 being the squamocolumnar junction. CT: Computed tomography.

fibers of the cricopharyngeus muscle. The upper esophageal fibers are not circular but elliptical, with the anterior part of the ellipse at a lower level to the posterior part. The ellipses become more circular as the esophagus descends, until the start of its middle third, where the fibers run in a horizontal plane. In a 1-cm segment, the fibers are truly circular. Below this point, the fibers become elliptical once again, but they now have a reverse inclination - that is, the posterior part of the ellipse is located at a lower level than the anterior part. In the lower third of the esophagus, the fibers follow a spiral course down the esophagus²⁵. Gray's anatomy text also mentioned the oblique nature of the CSM²⁶.

The study used formalin fixed specimens. The measurements might have been somewhat different if fresh non-contracted tissues were used. Formalin fixation can cause shortening in the length of the esophagus and the shortening associated with formalin fixation will change the angle measured between the CSM vs LSM. However, this does not change the validity of the comparisons between angles in different parts of the esophagus, but may alter the absolute numbers measured.

In the current study we found that rather than the angle of the circular smooth muscle and longitudinal

smooth muscle being perpendicular to each other in the esophagus, a significant difference was found in the angle of the muscle fibers between these two smooth muscle groups. We also found a significant difference between the muscle angles of the CSM at and 1 cm above the SCJ when compared with the muscle angles further above the SCJ. In conclusion, we believe that this spiral or oblique angulation of the CSM to LSM along with drag from the LSM causes shortening of the esophagus which is greater than can be explained by shortening due to the LSM alone. Furthermore we believe that acute angulation of the CSM at the SCJ may be responsible for the greater distal esophageal shortening compared to the proximal esophagus during peristaltic contraction.

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COMMENTS

Background

In a previous study, the distal esophagus shortened more than the proximal

esophagus. In addition the local longitudinal shortening of the circular smooth muscle (CSM) and total muscularis propria at 5 cm above the lower esophageal sphincter were significantly greater than at 20 cm. It was concluded that the CSM shortened longitudinally more than that which was accounted for by the longitudinal smooth muscle (LSM) shortening. These surprising results implied that the CSM was actually responsible for the greater shortening of the distal esophagus. Authors hypothesized that the increase in shortening of the CSM was due to the spiral nature of the muscle fibers within the distal CSM.

Research frontiers

It is thought that the LSM of the esophagus is completely responsible for esophageal shortening during peristaltic contraction. The role of CSM in esophageal shortening has not adequately been evaluated. In this study, the authors demonstrate that the CSM, which is at an oblique angle to the LSM, contributes to esophageal shortening.

Innovations and breakthroughs

Recent reports have highlighted the importance of longitudinal shortening of the esophagus in peristaltic contraction. This is the first study to evaluate the role of the CSM in longitudinal shortening due to the orientation of the CSM fibers.

Applications

By understanding how the CSM is oriented in the esophagus this study may increase the readers' knowledge of the anatomy, physiology of peristaltic contraction. In the future, understanding of the mechanism of esophageal shortening may help in the explanation of peristaltic contractile dysfunction.

Terminology

The esophageal wall consists of multiple layers. The main muscular component of the distal esophagus is the muscularis propria which is composed to two smooth muscle layers, the CSM and LSM. The CSM is the inner layer of muscularis propria which is closest to the lumen of the esophagus. The CSM is thought to generate the luminal pressure during peristaltic contraction. The LSM is the layer of muscularis propria which is separated from the CSM by an intramuscular connective tissue layer which contains neural tissue. The LSM is the outer layer of the muscularis propria which is thought to be responsible for esophageal shortening during peristaltic contraction.

Peer review

The authors examined the relative angles of the CSM and LSM in the distal esophagus to explain how the CSM contributes to shortening of the distal esophagus. The authors found that the CSM in the distal esophagus is oriented at an oblique angle to the LSM, thus contributing to distal esophageal shortening. The results are interesting and may have a role in the pathophysiology of esophageal peristalsis.

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Screening *Helicobacter pylori* genes induced during infection of mouse stomachs

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Abstract

AIM: To investigate the effect of *in vivo* environment on gene expression in *Helicobacter pylori* (*H. pylori*) as it relates to its survival in the host.

METHODS: *In vivo* expression technology (IVET) systems are used to identify microbial virulence genes. We modified the IVET-transcriptional fusion vector, pIVET8, which uses antibiotic resistance as the basis for selec-

tion of candidate genes in host tissues to develop two unique IVET-promoter-screening vectors, pIVET11 and pIVET12. Our novel IVET systems were developed by the fusion of random Sau3A DNA fragments of *H. pylori* and a tandem-reporter system of chloramphenicol acetyltransferase and beta-galactosidase. Additionally, each vector contains a kanamycin resistance gene. We used a mouse macrophage cell line, RAW 264.7 and mice, as selective media to identify specific genes that *H. pylori* expresses *in vivo*. Gene expression studies were conducted by infecting RAW 264.7 cells with *H. pylori*. This was followed by real time polymerase chain reaction (PCR) analysis to determine the relative expression levels of *in vivo* induced genes.

RESULTS: In this study, we have identified 31 *in vivo* induced (*ivi*) genes in the initial screens. These 31 genes belong to several functional gene families, including several well-known virulence factors that are expressed by the bacterium in infected mouse stomachs. Virulence factors, *vacA* and *cagA*, were found in this screen and are known to play important roles in *H. pylori* infection, colonization and pathogenesis. Their detection validates the efficacy of these screening systems. Some of the identified *ivi* genes have already been implicated to play an important role in the pathogenesis of *H. pylori* and other bacterial pathogens such as *Escherichia coli* and *Vibrio cholerae*. Transcription profiles of all *ivi* genes were confirmed by real time PCR analysis of *H. pylori* RNA isolated from *H. pylori* infected RAW 264.7 macrophages. We compared the expression profile of *H. pylori* and RAW 264.7 coculture with that of *H. pylori* only. Some genes such as *cagA*, *vacA*, *lpxC*, *murI*, *tlpC*, *trxB*, *sodB*, *tnpB*, *pgi*, *rbfA* and *infB* showed a 2-20 fold upregulation. Statistically significant upregulation was obtained for all the above mentioned genes ($P < 0.05$). *tlpC*, *cagA*, *vacA*, *sodB*, *rbfA*, *infB*, *tnpB*, *lpxC* and *murI* were also significantly upregulated ($P < 0.01$). These data suggest a strong correlation between results obtained *in vitro* in the macrophage cell

line and in the intact animal.

CONCLUSION: The positive identification of these genes demonstrates that our IVET systems are powerful tools for studying *H. pylori* gene expression in the host environment.

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Key words: *Helicobacter pylori*; *In vivo* expression technology; Virulence genes; Mice; Infection

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative bacterium that infects a large percentage (50% to 90%) of the world's population and is a causative agent for gastritis, ulcer disease and some gastric cancers. To date, the mechanism of *H. pylori* pathogenesis is not completely understood. *H. pylori* can infect and survive in the stomachs of mice and macrophages. *H. pylori* infection can last for a lifetime, suggesting that the microbes successfully evade the host immune response to infection. Although previously considered an extracellular organism, several recent *in vitro* studies suggest that *H. pylori* may be a facultative intracellular bacterium^[1]. The intracellular habitation offers a plausible explanation for the evasion of host immune response and thus a life-long persistence in the host.

Characterization of microbial genes that are specifically induced during infection is critical to the understanding of the mechanism by which microbial pathogens cause disease. Many different techniques have been developed to study bacterial genes that are expressed during growth in specific niches^[2-4]. A useful tool for identifying genes involved in virulence is *in vivo* expression technology (IVET)^[5,6]. IVET was designed to identify genes of pathogens that are preferentially expressed during infection and has been used extensively^[7,8]. It is a promoter trapping strategy used for identifying bacterial promoters that are upregulated in the host by using an auxotrophic mutant strain or with various reporter systems. This technique allows the identification of genes that may be expressed only under *in vivo* conditions. Such genes are difficult to identify during growth under laboratory conditions, but are likely to play an important role

in survival inside the host. This technology has not been exhaustively utilized in *H. pylori* because of limitations imposed by the genetic intractability of this bacterium. Recently, recombination-based IVET has been utilized to identify *H. pylori* genes important for host colonization^[9]. In this study, we developed an antibiotic-based IVET tool (a variant of IVET^[10]) that is specific for screening *H. pylori* genes that are specifically expressed *in vivo* in mice and macrophage hosts.

MATERIALS AND METHODS

Bacterial strains and growth media

All bacterial strains used in this study are listed in Table 1. The *H. pylori* strains used in this study were: Sydney strain 1 (SS1)^[11] and strain HP1061^[12]. The strains were grown for 16 h to 18 h at 37 °C in a microaerophilic atmosphere in bisulfiteless Brucella broth (BLBB)^[13] containing 5% fetal bovine serum (Hyclone, Logan, UT). For BLBB solid medium, 1.7% agar was added. Unless stated otherwise, the antibiotics used in BLBB solid or liquid medium were: kanamycin (kan) 15 mg/L, Glaxo Selective Supplement A (GSSA) (5 mg/L of Amphotericin-B, 20 mg/L of Bacitracin, 1.07 mg/L of Nalidixic acid, 0.33 mg/L of Polymyxin-B, and 10 mg/L of Vancomycin)^[14]. BLBB 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) plates were supplemented with X-gal at 40 mg/L. *Escherichia coli* (*E. coli*) strains, TAM1λ pir and DH5α λ pir were grown in L broth (LB) medium^[15].

Animal housing and diet

Mice were maintained in a National Institutes of Health (NIH) animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (Rockville, MD).

They were maintained in a specific-pathogen-free animal care holding room and were confirmed to be free of the following microorganisms: cilium-associated respiratory bacillus, ectromelia, mouse rotavirus, mouse encephalomyelitis virus, lymphocytic choriomeningitis virus, murine cytomegalovirus, mouse hepatitis virus, mouse adenovirus, minute virus of mice, *Mycoplasma pulmonis*, parvovirus, polyomavirus, pneumonia virus of mice, reovirus, and Sendai virus. Mice were housed in 7.5- by 11.5- by 5-in. sterilized ventilated Thoren cages (Thoren Caging System, Inc., Hazleton, PA) on Tek Fresh bedding (Harlan Teklad, Madison, WI). Cages were changed weekly. The animal holding room was maintained under environmental conditions of 20 °C, 40% to 70% relative humidity, 15 air changes/h and a 12-h/12-h light-dark cycle. Mice were fed an autoclaved pelleted rodent diet (rodent NIH-31 autoclavable NA; Zeigler Brothers, Inc., Gardners, PA) ad libitum and provided sterilized individual water bottles for an ad libitum water source. Upon arrival, the mice were acclimated for a minimum of 7 d prior to being used in the experiments. Mice were identified by numerical stainless steel rodent ear tags (National Band and Tag Co., Newport, KY). This study was

Table 1 Strains and plasmids used in this study

Strains or phenotype	Relevant genotype or plasmids	Source or reference
<i>Helicobacter pylori</i>		
SS1		Lee <i>et al</i> ^[11]
HP1061		Goodwin <i>et al</i> ^[12]
<i>Escherichia coli</i>		
DH5 α pir ⁺	<i>Apir</i> , <i>supE44</i> , Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15), <i>hsdR17</i> , <i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>relA1</i>	Stanley Maloy, San Diego State University
TAM1 λ pir ⁺	<i>Apir</i> , <i>mcrA</i> , Δ (<i>mrrhsdRMSmcrBC</i>), ϕ 80 <i>lacZ</i> Δ M15, Δ <i>lacX74</i> , <i>recA1</i> , <i>ara</i> Δ 139(<i>ara-leu</i>)7697, <i>galU</i> , <i>rpsL</i> , <i>endA1</i> , <i>nupG</i>	Active Motif, Inc., Carlsbad, CA 92008
Plasmids		
pIVET8		Slauch <i>et al</i> ^[52]
pIVET9		This study
pIVET10		"
pIVET11		"
pIVET12		"

SS1: Sydney strain 1.

reviewed and approved by the NIH Institutional Animal Care and Use Committee. All procedures and use of animals were in compliance with the Public Health Service Guide for the Care and Use of Laboratory Animals^[16].

Isolation of DNA from bacterial strains

Plasmid DNA was isolated from *E. coli* using the QIAprep Miniprep or QIAfilter plasmid maxi kit (QIAGEN, United States) in accordance with manufacturer's recommended protocols. Genomic DNA was extracted from *H. pylori* strains, SS1 and 1061 using the Wizard Genomic DNA Purification Kit (Promega, United States) as described by the manufacturer.

Plasmid construction

Two novel *H. pylori* specific plasmids, pIVET11 and pIVET12 (Figure 1.) were constructed by modifying plasmid pIVET8^[10]. pIVET8 is a suicide vector containing *oriR6K* origin that requires, in trans, a host-encoded Pi protein for replication^[17-19]. It also contains an ampicillin resistance gene and a promoterless *cat* and *lacZY* gene fusion. The gene encoding kanamycin resistance was amplified by PCR from pCR II (Stratagene), with primers KanF and KanR (Table 2). The amplified fragment was cloned at the *Aat*II site of pIVET9. A *Bgl*II restriction site in the kanamycin sequence was removed by Quick change site-directed mutagenesis kit (Stratagene). In so doing, we produced pIVET 10 which contained the unique *Bgl*II cloning site immediately upstream of promoterless *cat* and *lacZY* genes. We produced pIVET11 by removing the mob RP4 sequence which accounted for the conjugal transfer functions necessary for mobilization. Finally, pIVET12 plasmid was generated by the removal of ampicillin gene by *Bsp*BI restriction and the transfer of the kanamycin gene from the *Aat*II site to a unique *Bam*HI

site in pIVET11. The kanamycin gene sequence, inserted at the *Aat*II site in pIVET11, disrupts *lacZ* gene without affecting its activity. *H. pylori* is sensitive to ampicillin and therefore we could not use this antibiotic in our system.

Construction of genomic library

H. pylori Sydney strain 1 (SS1) genomic DNA was isolated using the Wizard Genomic DNA purification kit (Promega, United States). The genomic DNA was partially digested with *Sau*3AI. After agarose gel electrophoresis, fragments of 1-3kb were purified using QIAquick Gel Extraction Kit (Qiagen, United States) and ligated into *Bgl*II digested and dephosphorylated pIVET11 or pIVET12 using the Rapid DNA Ligation kit (Roche, United States). Ligation samples were transformed into DH5 α λ pir competent cells which provided the Pi protein, in trans, for plasmid replication. Transformants were replica plated in a 6 \times 8 pattern on LB agar plates containing kanamycin. Forty eight colonies from the replica plates were pooled in TE buffer, pH 8.0 and plasmid DNA purification was carried out using the QIAprep Miniprep kit (QIAGEN, United States).

Preparation of electro-competent *H. pylori*

An overnight-grown 100 mL *H. pylori* culture was chilled for 10 min on ice. The cells were pelleted at 4360 \times g for 5 min. The centrifugations and all subsequent procedures were done at 4 $^{\circ}$ C. The pellets were washed three times, twice with 40 mL ice cold water and finally with 40 mL ice cold water containing 5% glycerol. The washed pellets were placed on ice, covered with 2 mL ice cold 10% glycerol, incubated for 10 min and later resuspended in the same solution. 200 μ L samples were placed in cold screw capped tubes and after quickly freezing on dry ice samples were stored at -80 $^{\circ}$ C.

Electrotransformation of *H. pylori* and merodiploid selection

Electro-competent *H. pylori* strain 1061 was transformed with 0.5-2 μ g recombinant plasmid DNA pools by electroporation (12.5 ms, 2.5 kV, 25 μ F, 600 ohm, 0.4 cm gap, Bio-Rad gene pulser, United States). Electrotransformed *H. pylori* were screened for kanamycin resistance on BLBB GSSA Kan agar plates. Selection for kanamycin resistance requires the integration of the recombinant plasmids into the chromosome by homologous recombination, using the cloned *Helicobacter* DNA as the source of homology (wild type *H. pylori* does not have *cat* and *lacZY* genes). This integration event duplicates a small portion of the *H. pylori* genomic DNA leading to the generation of a merodiploid. Kanamycin resistant colonies/merodiploids were replica plated in a 6 \times 8 pattern on BLBB GSSA Kan agar plates. After 72 h of microaerophilic incubation, forty eight colonies from the replica plates were pooled in 1 mL of BLBB, GSSA, and Kan. This suspension was inoculated in 10 mL BLBB, GSSA, Kan and grown under microaerophilic conditions. After 24 h of incubation, cultures were subcultured 1:100 in 10 mL of BLBB, GSSA,

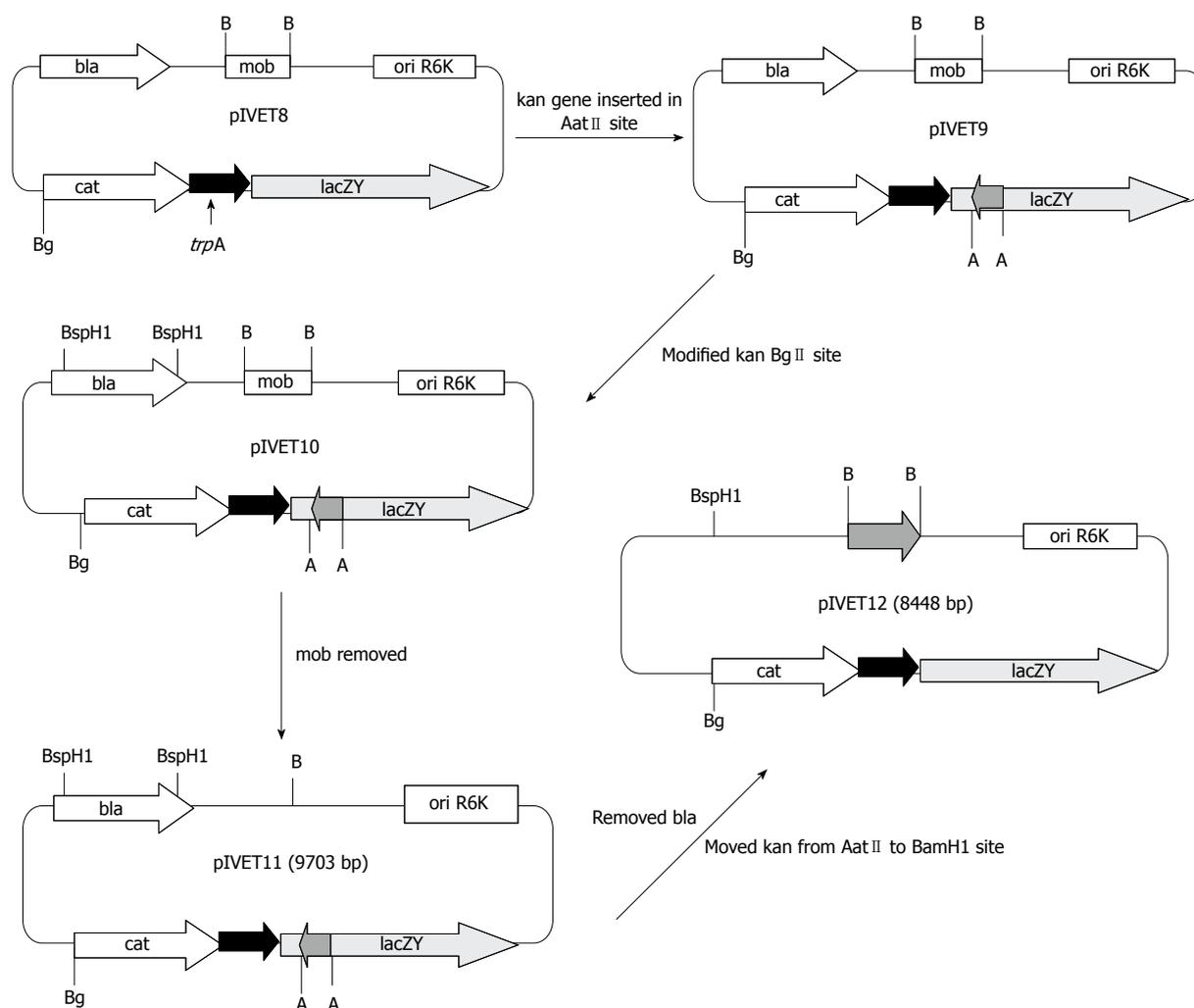


Figure 1 Construction of *Helicobacter pylori* specific antibiotic-based *in vivo* expression technology plasmids, pIVET11 and pIVET12. These plasmids are derivatives of plasmid pIVET8^[10]. A: Aat II; B: BamHI; Bg: BglII; bla: β-lactamase; kan: Kanamycin gene; mob: Plasmid mobilization; trpA: Tryptophan synthase α-subunit.

Kan and grown for 16-18 h under microaerophilic conditions. These resulting merodiploids were used to infect mice or macrophage cultures (see below).

Inoculation of mice with *H. pylori* merodiploids

Six to eight week old *Helicobacter* and pathogen-free female C57BL/6 mice (Jackson Laboratory Maine, United States) were used, in compliance with guidelines and protocol approved by the Animal Care and Use Committee of the National Institutes of Health, United States. Using a 20-gauge ball-point metal feeding tube (Harvard Apparatus, Inc., Holliston, MA), mice were inoculated intragastrically with 0.1 mL of *H. pylori* merodiploid pooled cultures (10^8 colony-forming-units per milliliter-CFU/mL) once a day every other day for a total of three inoculations. Each pair of mice was inoculated with a fresh 18 h culture. Control mice were inoculated with BLBB medium.

Isolation and analysis of DNA from fecal pellets

Five days post-infection, fecal pellets were collected from

the infected and control mice. DNA was isolated from fecal pellets (2 pellets per mouse) by using the QIAamp DNA stool mini kit (QIAGEN, United States). DNA samples extracted from the fecal pellets were analyzed by PCR as done previously^[20].

Selection for chloramphenicol resistant *H. pylori* in mice

Seven days post-infection, mice were treated with chloramphenicol. 100 μL chloramphenicol (0.9 g/L, apple flavored, Foer's Pharmacy, United States) was given to each mouse by oral gavage twice a day for three days. Chloramphenicol was also added to the water in the mouse cages to a concentration of 0.1 g/L. Following the last chloramphenicol treatment, the mice were sacrificed, and the stomachs were excised.

H. pylori IVET selection in mouse stomachs

The excised stomach was cut into two longitudinal sections. One half was added to 1 mL BLBB medium containing 20% glycerol and frozen at -80 °C. The other half of the stomach was added to 1 mL BLBB Kan medium

Table 2 Primers used in this study

Genes	Primer sequences 5'-3'
HP1	CTGGAGAGACTAAGCCCTCC
HP2A	AACATTACTGACGCTGATTG
Kan Aat II F	GATTTAGACGTCTCAGGGCGCAAGGGCT
Kan Aat II R	TTCCTTTGACGTCTCAGAAGAATCGTCAA
Kan Bgl II F (Mut)	AGG GGA TCA AGG TCA GAT CAA GAG A
Kan Bgl II R (Mut)	TCT CTT GAT CTG ACC TTG ATC CCC T
MCS BamHI F (Mut)	GTC GAC CGA CCC CAA GCT TCT AGA GGT ACC G
MCS BamHI R (Mut)	CGG TAC CTC TAG AAG CTT GGG GTC GGT CGA C
Kan BamHI F (pIVET12)	GAT TTA GGA TCC TCA GGG CGC AAG GGC T
Kan BamHI R (pIVET12)	TTC CTT TGG ATC CTC AGA AGA ACT CGT CAA
CAT	CAA CGG TGG TAT ATC CAG
MCAT	GCC ATT GGG ATA TAT CAA CGG TGG TAT ATC C
NMCAT	CTC CAT TTT AGC TTC CIT AGC TCC TGA AAA TCT CG
CagA F	GATGGCGTGATGTTTGTGATT
CagA R	CGTAGACCCACACCCCTATC
CysS F	TGTTATCCCCACCATGAAA
CysS R	GCAAGCTCCACGCCAAAG
Flhf F	TGTIGCCCTGAAGATTGAATTT
Flhf R	TAGCGCGCGGCTAATTTAG
FliA F	AAGCAACAACACCACCATCAAG
FliA R	GCTGGGCAAGCGCTCTT
FliM F	CGCCAGGTGATGCAAAATTT
FliM R	AGCGACGATTTGGACCACAT
FucT F	TGAATGTGCATGATTCAACAAC
FucT R	GCTTTCCCATCAAGGGTGTTT
HsdM F	CTGGGACATTCAAATCAATTATGG
HsdM R	CGTCTTGCAAGCGTTTAAAGATCT
InfB F	GAGTCGCTCACACGGGAAGCT
InfB R	CAATGAAAGACACCCATTTGTCA
LpxC F	CAAGCCCATCATTTTGTCTTTAGTAA
LpxC R	CAAGTAATTCACTTCTTGCAAAAACC
MinC F	TGCGCAAAACACAGCTTTT
MinC R	ATGGCGCTCATAAAATCGTTGT
MurC F	GGCTCATGGAAAGAGCAGTACA
MurC R	GCCCAATAATCGCTCCAA
MurD F	GCGGCACTAACGGGAAAA
MurD R	CCCACTCACAGCCTTAAAATCITC
MurI F	AAAAGCGACGATTCAAATCCAA
MurI R	AAGTAGCTAAAATGCGAAAATGTTCAAA
Nth F	TGAATTTATGGTGGCGACCAT
Nth R	TTGGGCGTTATTTGATTCACCTCT
Omp26 F	CAAATCGGCACCGTTACCA
Omp26 R	CATGAAATCCCCTAGAAAGCA
Pgi F	AGCCACACAGGGTGTTT
Pgi R	CTCCAATATTGCTTGGTGAATCTT
PriA F	GGAGGAGCGCTAGGCAAAT
PriA R	AGTTCGCACCTTTCTTGCAATT
RbfA F	GCGAGTGAATTCITTAAGCGTTAC
RbfA R	GACCTCAGCTTTTTTCAATTTAGA
RecG F	GCGATCAACAAAACGCCATTA
RecG R	GCACCCACATCGCCTATAA
SodB F	TGCTAAAGACAGCATGGGAGATT
SodB R	TTCACATAAGTTTIGATGGIGTTTCC
TlpC F	TGTTAGCGGATTATACGAA
TlpC R	GGGTAGGCGATTTTGAATCC
TnpB F	TIGGTGTTTGAATGCGGGTATAG
TnpB R	TTTCTACCCCAAAAAGACTTAACC
TrxB F	CGCCATTGCTTTGTGCAA
TrxB R	TGATGCGGCTGAATCTTTTT

Type II R / M F	TTCTATAACAGCACCCGCTGACATT
Type II R / M R	CGCGTATATTGTTAGAAGTGATGAAA
VacA F	GGGTTATGCCAGACAAAATGATTG
VacA R	TCTTATGCTCTAAACTGGCTATGTTGTT
VirB4 F	GACCATAGCCCTTATTGTTTAATTTTC
VirB4 R	CTCCTATAATCATGGTATGTCCCACTAC
Ycf5 F	AGCGCTATCAATGAAATGAGCAT
Ycf5 R	CCACACCCCGCTAAGAAA
HP0423 F	TCGCTCCTTAAGGTTACACGATT
HP0423 R	TCAAAGCCACCATCAATAACAAA
HP0424 F	CGCTGTTTTTAGTTTTAGAGGCTTTC
HP0424 R	GGCTAGGGAAGTGGCTCAAA
HP0426 F	TGCGGTATAGGCTTTCATGAAC
HP0426 R	AAGGTGTTCAAAGACAGCAAAAAA
HP0427 F	TTGCGGTGTTGGTTAATGAA
HP0427 R	GCAACGCTACCATACTTTTATCACIT

and homogenized with a sterile motorized Polytron homogenizer (Kinematica AG, United States). The resulting homogenate was spread on BLBB, GSSA, Kan plates at 10, 100 and 1000 fold dilutions. The plates were incubated under microaerophilic conditions at 37 °C for 3-4 d. Kanamycin resistant colonies were replica plated in a 6 × 8 pattern on BLBB GSSA Kan agar plates. Forty eight colonies from the replica plates were pooled and grown overnight for the second round of mice infections.

H. pylori IVET selection in macrophages

The IVET selection in macrophage was performed as described previously with some modifications^[10]. The mouse macrophage cell line, RAW 264.7 was grown in Dulbecco's modified Eagle medium (DMEM; Gibco-BRL) supplemented with 10 % heat-inactivated fetal calf serum (FCS; Gibco-BRL) and GSSA antibiotics at 37 °C in a 50 mL/L CO₂ humidified atmosphere. The day before each *in vivo* selection assay RAW 264.7 cells were seeded in 24-well tissue culture plates to 2 × 10⁵ cells per well. *H. pylori* merodiploid pools were grown for 16 h to 18 h under microaerophilic conditions at 37 °C in BLBB Kan medium. 30 µL of the bacterial cultures were added per 1 mL of DMEM medium. The monolayers were infected with 1 mL of the bacteria suspension and centrifuged for 5 min at 600 r/min to synchronize bacterial contact with the monolayers^[21,22]. The infected macrophage monolayers were incubated for 2 h at 37 °C in a microaerophilic atmosphere. The monolayers were washed three times with phosphate buffered saline (PBS) and then were incubated with 2 mL of DMEM containing 100 mg/L gentamicin for 2 h at 37 °C in a microaerophilic atmosphere to kill extracellular bacteria. Following extracellular killing, the monolayers were washed three times with 2 mL of PBS and then the infected cells were incubated overnight in DMEM containing 1 mg gentamicin/L and 20 mg chloramphenicol/L. After the incubation period the macrophage-monolayers were washed three times with PBS and then lysed by adding 1 mL of sterile water per well. The resulting lysate was spread on BLBB, GSSA, Kan plates at 10, 100 and 1000 fold dilutions. The plates were incubated under microaerophilic conditions at 37 °C for 3-4 d and the kanamycin resistant colonies were pooled and grown

overnight for the second round of infections.

Screening of chloramphenicol resistant *H. pylori* for β -galactosidase expression *in vitro*

Kanamycin resistant colonies recovered from the stomach homogenates and RAW 264.7 cell lysates were replica plated in a 6 × 8 pattern on BLBB GSSA Kan X-gal plates. These plates were then incubated under microaerophilic conditions at 37 °C for 24 h, and screened for blue or white color. White colonies were used to inoculate BLBB, GSSA medium for preparing genomic DNA.

Identification of *ivi* gene fusions

The genomic DNA samples prepared from white colonies were tested for the presence of *H. pylori* 16S DNA^[20]. The presence of pIVET11/pIVET12 in the genome of co-integrate strains was confirmed by PCR analysis using KanF and KanR primers. To sequence regions of genomic DNA flanking the inserted plasmid, we performed Vectorette PCR according to the manufacturer's instructions (Sigma-Genosys). Genomic DNA from the co-integrate strains was digested separately with *Eco*RI, *Bam*HI and *Hin*DIII. Following this, compatible vectorette linkers were ligated to the ends of the genomic DNA fragments and PCR was then performed using a primer (MCAT) homologous to the 5' end of *cat* gene in pIVET11/pIVET12 and a primer unique to the vectorette linker. The resulting PCR products were sequenced using MCAT primer or cloned into pSC-A-amp/kan, PCR AU cloning vector (Stratagene, United States) and sequenced subsequently.

RNA isolation

RAW 264.7 macrophages were infected with *H. pylori* as described above and incubated for 2 h at 37 °C in a microaerophilic atmosphere. An identical amount of *H. pylori* was added to a flask without RAW 264.7 cells and incubated in the same way as the *H. pylori*-RAW 264.7 cell coculture. A non-infected flask of RAW 264.7 cells served as a negative control for RNA isolation to ensure that no contaminating signals derived from eukaryotic RNA were present. After 2 h of incubation, the *H. pylori*-RAW cell coculture was washed three times with 2 mL of PBS to remove extracellular *H. pylori* cells. Finally, the *H. pylori* infected RAW cell cultures were incubated in 2 mL of medium containing 100 mg/L gentamicin for 2 h at 37 °C in a microaerophilic atmosphere to kill extracellular bacteria. Following extracellular killing, the coculture was washed three times with 2 mL of PBS and then the infected cells were treated with the TRIzol reagent for RNA isolation as described by the manufacturer (Invitrogen, United States). Further RNA purification was performed with an RNeasy mini kit (Qiagen, United States). The culture containing only *H. pylori* was centrifuged and washed twice with phosphate buffered saline, and TRIzol was directly applied to the pellet and the preparation was subsequently treated in the same fashion as *H. pylori* and RAW cells coculture described above for RNA purification.

Separation of eukaryotic and prokaryotic mRNA

H. pylori RNA from coculture was enriched by removal of the eukaryotic 18S and 28S rRNAs and polyadenylated mRNAs using the MICROBEnrich kit (Ambion) according to manufacturer's instructions.

Real time PCR

Primers were designed for 100-150 bp regions of *in vivo* induced genes obtained after sequencing (Table 3). Primer design was aided by Primer Express 3.0 software (Applied Biosystems, United States). Standard PCR was performed with *H. pylori* SS1 genomic DNA as the template to check that all the primer pairs resulted in the amplification of a single product. RNA was reverse transcribed using Superscript III first strand synthesis system for RT-PCR (Invitrogen, United States). Real-time PCR was done using Power SYBR Green PCR Master mix (Applied Biosystems, United States). 16S RNA was used in each set of reaction for normalization. Each reaction was repeated thrice with three independent RNA samples in an Applied Biosystems 7500 real time PCR system. Melt curve analysis was done to confirm the specificity of the amplified product. Relative expression levels were determined using the 2-delta-delta Ct method^[23]. Results were expressed as fold induction of expression in *H. pylori* and RAW 264.7 coculture as compared to *H. pylori* only.

Statistical analysis

Data were presented as mean ± SE in Microsoft Excel. Differences between the *H. pylori* alone, and *H. pylori* and RAW 264.7 co-culture group means were analyzed by the Student's *t* test. The threshold significance level for the mean difference between groups was $P < 0.05$.

RESULTS

Construction of *H. pylori* specific IVET vectors and vector validation with known *H. pylori* promoter

As a means to identify Helicobacter promoters specifically expressed under defined conditions, we have successfully developed two promoter trap vectors, pIVET11 and pIVET12 (Figure 1). These vectors are suicide vectors containing *oriR6K* origin that requires a host-encoded Pi protein, in trans, for replication. They contain two promoterless reporter genes, one encoding the *cat* gene and the other *lacZY* genes. These two genes are organized into a single transcription unit located immediately downstream from a unique *Bgl*/II site, allowing for the cloning of promoter libraries. In Figure 2, we summarized an overview of the IVET strategy used in these studies. To validate the *H. pylori* specific promoter trap vector system, we placed the *cat-lacZY* fusion of pIVET11 under the control of the *H. pylori vacA* promoter. We transformed the resulting plasmid, pIVET11/*vacA*, through electroporation into electro-competent *H. pylori* strain 1061. Transformed *H. pylori* were screened

Table 3 *Helicobacter pylori* *in vivo* induced genes

	Locus	Function or enzyme	Role in virulence
<i>ivi</i> -232	<i>flhF</i>	Regulation of flagella biosynthesis	Motility
<i>ivi</i> -122	<i>fliA</i>	Flagellar motor switch protein	
<i>ivi</i> -121	<i>fliM</i>	Flagella motor switch protein	
<i>ivi</i> -364	<i>tlpC</i>	Methyl-accepting chemotaxis protein	
<i>ivi</i> -11	<i>trxB</i>	Thioredoxin reductase	Oxidative stress protection
<i>ivi</i> -134	<i>nth1</i>	Endonuclease III	
<i>ivi</i> -322	<i>ycf5</i>	Cytochrome C biogenesis protein	
<i>ivi</i> -352	<i>sodB</i>	Iron-dependent superoxide dismutase	Acid resistance
<i>ivi</i> -161	<i>hsdM</i>	DNA methyltransferase	Nucleic acid metabolism
<i>ivi</i> -162	<i>hsdM/R</i>	Type II restriction enzyme R and M protein	
<i>ivi</i> -123	<i>recG</i>	ATP-dependent DNA helicase	
<i>ivi</i> -351	<i>priA</i>	Primosome protein replication factor	
<i>ivi</i> -361	<i>tnpB</i>	IS606 Transposase	
<i>ivi</i> -171	<i>lpxC</i>	UDP-3-O-acyl N- acetylglucosamine deacetylase	Cell envelope structures
<i>ivi</i> -172	<i>minC</i>	Septum site directing protein	
<i>ivi</i> -1722	<i>murD</i>	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthase	
<i>ivi</i> -1721	<i>murC</i>	UDP-N-acetylmuramoyl alanine-D-glutamate ligase	
<i>ivi</i> -363	<i>murI</i>	Glutamate racemase	
<i>ivi</i> -321	<i>fucT</i>	Alpha 1, 3-fucosyltransferase	
<i>ivi</i> -351	<i>omp26</i>	Outer membrane protein	
<i>ivi</i> -112	<i>pgi</i>	Glucose-6-phosphate isomerase	Sugar metabolism
<i>ivi</i> -1132	<i>rbfA</i>	Ribosome binding factor A	Translation and regulation
<i>ivi</i> -1131	<i>infB</i>	Translation initiation factor IF-2	
<i>ivi</i> -2721	<i>cysS</i>	CysteinyI-tRNA synthetase	Protein and peptide synthesis
<i>ivi</i> -110	<i>vacA</i>	Vacuolating toxin	Virulence factors
<i>ivi</i> -362	<i>cagA</i>	Cytotoxin-associated gene	
<i>ivi</i> -192	<i>virB4</i>	Type IV secretion system	Transport <i>cagA</i> protein
<i>ivi</i> -241		Predicted coding region HP0426	<i>H. pylori</i> hypothetical protein
<i>ivi</i> -242		Predicted coding region HP0427	<i>H. pylori</i> hypothetical protein
<i>ivi</i> -3101		Predicted coding region HP0423	<i>H. pylori</i> hypothetical protein
<i>ivi</i> -3102		Predicted coding region HP0424	<i>H. pylori</i> hypothetical protein

H. pylori: *Helicobacter pylori*.

for kanamycin resistance (See materials and Methods). Selection for kanamycin resistance requires the integration of the recombinant plasmid into the chromosome by homologous recombination, where the source of homology is the cloned helicobacter DNA *vacA* promoter sequence. The integrated sequences partially duplicate the *H. pylori* genomic DNA, leading to the generation of a merodiploid. With this merodiploid, one copy of the promoter drives the expression of *cat-lacZY* fusion, while the other promoter copy drives the expression of a wild-type *ivi* gene. Kanamycin resistant merodiploids were used to infect mice and macrophages. After infection, chloramphenicol resistant colonies were recovered and subjected to blue or white screening to analyze β -galactosidase expression *in vitro*. We identified 728 cm^R white colonies and analyzed their genomic DNA. The region of genomic DNA flanking the inserted plasmid was identified using the universal vectorette system as described by Sigma-Genosys. Sequence analysis of the PCR product showed the presence of the *vacA* promoter upstream of *cat-lacZY* fusion. These results indicate that the *vacA* promoter is capable of driving the expression of promoterless *cat-lacZY* genes *in vivo*. The expression results also serve to validate the efficacy of our promoter trap systems to detect and identify *H. pylori* promoters expressed *in vivo*.

IVET selection in mouse model

We generated IVET vectors that contained a library of *Sau3AI* digested *H. pylori* chromosomal DNA. These IVET vectors were transformed into *H. pylori* 1061 to generate a library of merodiploid (co-integrated) strains. These strains are plasmid recombinants characterized by integration into different loci of the *H. pylori* genome through homologous recombination. The recombinant strains along with wild type *H. pylori* strain were used to infect mice. Infected mice were then subjected to chloramphenicol treatment. Chloramphenicol effectively kills the intragastric wild type *H. pylori* strain. The surviving chloramphenicol resistant *H. pylori* are merodiploid which were recovered from the stomachs of mice infected with these strains. We pooled the resistant colonies and repeated the second round of infection in mice. Of a total of 702 merodiploid strains that survived chloramphenicol challenge, 38 (approximately 6%) were found to be negative for β -galactosidase activity during *in vitro* screens. In the pre-selection pool, 15% (86/596) of the *H. pylori* genomic DNA *cat-lacZY* fusions were LacZ⁺ *in vitro* (light blue) and 85% (510/596) were LacZ⁻ (white). In contrast, after two rounds of the antibiotic selection, 94% (664/702) were LacZ⁺, and 6% (38/702) were LacZ⁻. These LacZ⁺ strains presumably carried gene fusions that expressed chloramphenicol transacetylase *in vivo* in order

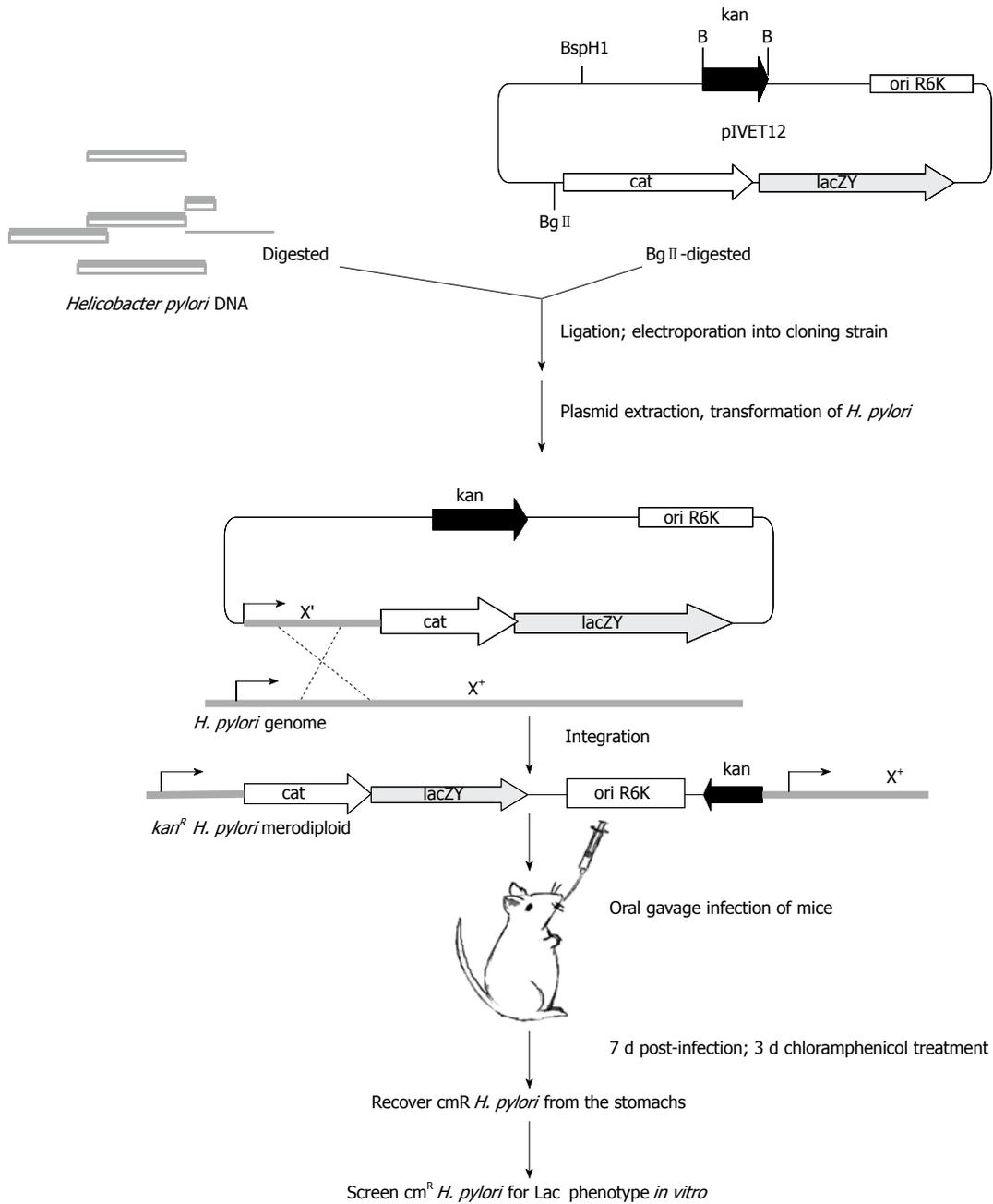


Figure 2 *Helicobacter pylori* specific *in vivo* expression technology strategy. *H. pylori*: *Helicobacter pylori*; B: *Bam*HI; Bg: *Bgl*II ; kan: Kanamycin gene.

to survive the systemic antibiotic treatment. However, the enzyme was expressed poorly when these strains were grown *in vitro* on BLBB Kan X-gal medium. Operating on this premise, we focused our efforts on the characterization of these gene fusions because they may represent genes that are specifically induced *in vivo* (*ivi* genes).

IVET selection in cultured macrophages

RAW 264.7 macrophages were infected with *H. pylori* as described in the methods section. Chloramphenicol resistant bacteria were recovered from the lysates of RAW cells infected with merodiploid strains, but not from those infected with the wild type *H. pylori*. Chloramphenicol resistant colonies were pooled and the pools were used for the second round of infection of RAW 264.7

cells. Of a total of 231 merodiploid strains that survived chloramphenicol challenge, 15 (approximately 7%) were found to be inactive in β -galactosidase *in vitro* screens. In the pre-selection pool, 20% (41/206) of the *cat-lacZY* fusions were $LacZ^+$ *in vitro* (light blue) and 80% (165/206) were $LacZ^-$ (white). In contrast, after two rounds of chloramphenicol selection, 93% (216/231) were $LacZ^+$ and 7% (15/231) were $LacZ^-$. These strains likely contain fusions of *cat-lacZY* to *H. pylori* promoters which are active in macrophages but were not induced during *in vitro* growth.

Functional validation of IVET

To validate that the *in vivo* expressed pIVET11 and 12 proteins were under the control of *H. pylori* promoters,

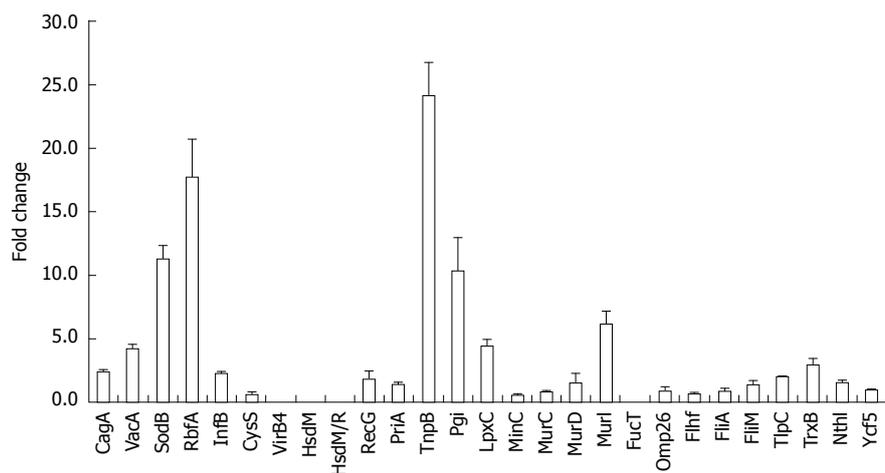


Figure 3 Gene expression of *Helicobacter pylori* induced by phagocytosis. Up-regulation and down-regulation of *Helicobacter pylori* *in vivo* induced genes expressed by macrophage engulfed bacteria.

we analyzed the nucleotide sequences and real time PCR results from infected mice (38 clones) and from infected RAW cells (15 clones). These clones contain the *cat-lacZY* reporter genes that are fused with genes in the *H. pylori* genome. The reporter genes should express in the host but not *in vitro*. Genomic DNA isolated from these clones was digested with *EcoRI*, *BamHI* and *HinDIII* and ligated to the compatible vectorette linkers. To sequence regions of genomic DNA flanking the inserted plasmid, we performed PCR using a primer homologous to the 5' end of *cat* gene in the IVET vectors and a primer unique to the vectorette linker. The resulting PCR products were sequenced directly or cloned and then sequenced.

Based on analysis of the nucleotide sequences of the individual inserts and comparison with the annotated genes of *H. pylori* in the GenBank database, we identified 31 genes. The list of the genes is shown in Table 3. The 31 genes included genes for virulence, cell envelope structures, motility, oxidative stress, nucleic acid and sugar metabolism, translation, protein synthesis and type IV secretion system. Four *ivi* conserved gene sequences did not show significant homology with any known genes in the Genbank database.

The real time PCR primers used to screen for *ivi* genes were tested using standard PCR conditions. Using *H. pylori* SS1 genomic DNA as the template, we found that only single PCR products resulted from each primer set. RNA was isolated from: (1) RAW 264.7 and *H. pylori* coculture; (2) *H. pylori* only; and (3) RAW 264.7 cells only. No product was obtained with the RNA of RAW 264.7 cells alone, confirming that there was no cross-reactivity that might have confounded the interpretation of data. Using 16s RNA as an internal control, we compared the expression profile of *H. pylori* and RAW 264.7 coculture with that of *H. pylori* only. The expression levels of genes differed from those observed in mice. We observed a 2-20 fold upregulation in *cagA*, *vacA*, *lpxC*, *murI*, *tlpC*, *trxB*, *sodB*, *tnpB*, *pgi*, *rbfA* and *infB* (Figure 3). In contrast, *hsdM*, *hsdM/R*, *fucT*, *virB4*, HP0426 and HP0427 were not

upregulated. The expression levels of the remaining *ivi* genes remained the same.

DISCUSSION

Characterization of microbial genes that are specifically induced during infection is important to the understanding of the mechanisms by which microbial pathogens cause disease. Intracellular pathogens have to evolve strategies to overcome the unfavorable environment met inside the host, which is very different in a culture broth outside the host. *H. pylori* colonizes the gastric mucosa during infection and synthesizes defense molecules to survive in the acidic gastric environment. Therefore, it is important to identify the genes of *H. pylori* that are up-regulated in the intracellular environment of the host.

IVET has previously been attempted in *Salmonella typhimurium*, *Vibrio cholerae* and *Pseudomonas aeruginosa*^[5,10,24-27]. In the present investigation, an antibiotic-based IVET has been applied in *H. pylori* for the first time. Novel *H. pylori* specific plasmids, pIVET11 and pIVET12 (Figure 1), were constructed by modifying the plasmid pIVET8^[10] and then used to construct the *H. pylori* library. Although this library does not contain the entire *H. pylori* genome, it will likely give insight into the type of genes up-regulated and hence necessary for the bacterium to evade host immune defenses. On the basis of chloramphenicol selection, 31 genes were identified (Table 3). These include genes responsible for a broad and varied group of cellular structures and functions: virulence, cell envelope structures, motility, oxidative stress, nucleic acid and sugar metabolism, translation, protein synthesis, type IV secretion system and few conserved and hypothetical proteins. Virulence genes such as *cagA* and *vacA* were induced and upregulated *in vivo*. *CagA* is translocated into gastric epithelial cells and induces numerous alterations in cellular signaling^[28-30]. Several *H. pylori* factors are known to interact directly with immune cells and modulate immune responses to *H. pylori*. One of these factors is *vacA* which

alters the function of T lymphocytes, B cells, macrophages and mast cells^[31,32].

H. pylori null mutant strains defective in the production of flagella are unable to colonize animal models^[33]. Flagella facilitate bacterial motility resulting in bacterial penetration of the mucus layer. Hence, upregulation of *flhF*, a global regulator of flagella biosynthesis and *fliA*, *fliM*, flagella motor switch proteins and *tlpC*, a methyl accepting chemotaxis protein is significant.

During host infection, animal pathogens are exposed to reactive oxygen species, such as superoxides, hydrogen peroxides, or organic peroxides, as a result of the release of lysosomal contents within inflammatory cells^[34]. In our IVET screen, proteins involved in oxidative stress protection, *trxB*, thioredoxin reductase, *sodB*, iron-dependent superoxide dismutase, *nth1*, endonuclease III and *ycf5*, cytochrome c biogenesis were upregulated. Thioredoxins have been implicated in a variety of physiological processes and biological pathways. In addition, they play a role in defense against oxidative stress, either by reducing protein disulfide bonds produced by various oxidants or by scavenging reactive oxygen species^[35]. Superoxide dismutase has been demonstrated to play an important role in oxidative stress defense mechanisms to counter iron-promoted DNA damage in *H. pylori*^[27].

Bacterial surface structures (adhesins, pilins, lipopolysaccharide, capsules) are often involved in direct contact with host cells, signaling molecules, and/or immune defenses (e.g., antibody). Hence the production and/or modification of many of these surface structures *in vivo* is often hypervariable in order to facilitate dissemination and to avoid immune defense mechanisms^[36]. In our system, several cell envelope structure-related proteins were identified. These included *fucT*, *lpxC*, *minC*, *murD*, *murC*, *murI*, and *omp26*.

Our IVET screening revealed the host-induced expression of several genes involved in nucleic acid metabolism, including *hsdM/R*, *hsdM*, *recG*, *priA* and *tnpB* (encodes the *H. pylori* IS606 transposase). This class of host-induced genes is involved in DNA synthesis and modification. Bacterial type II restriction-modification systems involve a restriction endonuclease and, a methyltransferase^[37,38]. The coordinated action of these enzymes mimics primitive immune defense mechanisms and protects bacterial cells from foreign DNA invasion^[39,40]. In addition, DNA methylation may play a role in gene regulation by inhibiting the interaction between regulatory proteins and their target DNA sequences^[41]. It may also be involved in the regulation of chromosomal DNA replication and gene expression^[42], transposon movement^[43], or DNA mismatch repair^[44]. A potential role for *recG* in recombination and in the rescue of stalled replication forks has been suggested^[45-47]. Additionally, recent studies suggest that *recG* provides a more general defense against pathological DNA replication^[19]. Cells lacking *priA* show a reduced viability and an increased sensitivity to DNA damage, phenotypes that are generally attributed to the deficiency in rescuing stalled or damaged forks^[48]. Genes involved in transposition have been upregulated in mi-

croorganisms during interaction with a eukaryotic host. *IS600* and *tnpF* genes were upregulated during interaction of *S. flexneri* with epithelial cells and HeLa monolayer respectively^[49,50].

Our IVET screens showed that genes involved in sugar metabolism (*pgi*), translation and regulation (*rbfA* and *infB*), as well as, protein and peptide synthesis (*cysS*) were also upregulated. We also detected upregulation of four hypothetical proteins: HP0426, HO0427, HP0423 and HP0424. These genes encode putative proteins with unknown functions and do not show significant homology to known proteins. This finding has been observed in most genome-wide analyses, including IVET studies^[7]. In a recent IVET study of *V. cholerae*, the largest class of *ivi* genes was found to encode hypothetical proteins^[51]. These results indicate that the function of many genes required for growth and survival in complex niches remain uncharacterized and additional functional analyses of these genes are needed.

Transcription profiles of all *ivi* genes were confirmed by real time PCR of *H. pylori* RNA isolated from *H. pylori* infected RAW 264.7 macrophages. These experiments were conducted to determine how well *in vitro* *ivi* genes in macrophages mirror *in vivo* *ivi* genes inside the host. The expression levels of several *ivi* genes in macrophages varied from the levels observed in mice. For example, *cagA*, *vacA*, *lpxC*, *murI*, *tlpC*, *trxB*, *sodB*, *tnpB*, *pgi*, *rbfA* and *infB* showed a 2-20 fold upregulation (Figure 3). However, *hsdM*, *hsdM/R*, *fucT*, *virB4*, HP0426 and HP0427 were not upregulated in the macrophage cell line, and there was no change in the expression of the remaining *ivi* genes. These data suggest a strong correlation between results obtained *in vitro* in the macrophage cell line and in the intact animal. Thus, the macrophages are suitable for the study of initial stages of host cell and bacterium interaction. However, the *in vivo* animal IVET screenings provide a broader and more comprehensive picture of *ivi* genes necessary for infection and colonization. In this study, we identified novel *H. pylori* *in vivo* induced genes that belonged to several functional gene families, including several well known virulence factors that are expressed by bacterium in infected mouse stomachs. The positive identification of these genes demonstrates that our IVET systems are powerful tools for studying *H. pylori* gene expression in the host environment, and points to potential *H. pylori* specific targets that allow *H. pylori* to circumvent host immune defenses.

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COMMENTS

Background

Helicobacter pylori (*H. pylori*) chronically infect 50% to 90% of the world's population. Gastritis and ulcers are seen in 15% to 20% of the infected popula-

tion and gastric cancers occur in 1% to 2% of the same group. Identification of bacterial genes (virulence factors) accounting for *H. pylori* survival in the host is fundamental to understanding the mechanisms of pathogenesis.

Research frontiers

Several methods such as signature-tagged mutagenesis, selective capture of transcribed sequences, differential fluorescence induction and microarray analyses have been used to study bacterial genes that are expressed during infection of animal hosts. These strategies are often limited by their inability to reproduce the complex environments encountered by pathogens in their hosts. To overcome these limitations, *in vivo* expression technology (IVET) has been developed. IVET has resulted in the identification of bacterial genes involved in infection, survival and pathogenesis.

Innovations and breakthroughs

IVET has been utilized extensively in *Salmonella typhimurium*, *Vibrio cholerae* and *Pseudomonas aeruginosa* to identify potential virulence factors. This technology has not been exhaustively utilized in *H. pylori* because of limitations imposed by the genetic intractability of this bacterium. Recombination-based *in vivo* expression technology (RIVET) approach has been used with *Vibrio cholerae*, *Lactobacillus plantarum*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, and *Bordetella pertussis*. RIVET is a variant of the original IVET in which a promoter transcriptional event is captured permanently as a conversion of the infecting strain from antibiotic resistant to antibiotic sensitive. Recently, RIVET has been utilized to identify *H. pylori* genes important for host colonization. In this study, authors have developed IVET approach for screening *H. pylori* genes that are specifically expressed *in vivo*.

Applications

The study results suggest that this IVET approach may provide powerful tools for studying *H. pylori* gene expression in the host environment. Identification of *H. pylori in vivo* induced genes will provide an improved understanding of metabolic, physiological, and genetic factors that contribute to survival and virulence of this pathogen. It may also lead to the identification of possible vaccine targets.

Terminology

IVET is a genetic method used to determine which bacterial genes are upregulated when bacteria invade the stomach of a host.

Peer review

This study demonstrated the efficacy of *in vivo* expression technology for screening *H. pylori* genes that are expressed *in vivo* in mice and macrophage hosts. In this study, genes responsible for a broad group of functions were identified. Although no screen of this type can provide an exhaustive account of all genes induced *in vivo*, it will likely give insight into the type of genes upregulated and hence necessary for the survival of *H. pylori* in gastric mucosa.

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Temporal trends in the relative prevalence of dysphagia etiologies from 1999-2009

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Abstract

AIM: To examine the relative prevalence and temporal variation of dysphagia etiologies in patients undergoing upper endoscopy (EGD) over the past decade.

METHODS: EGDs with the indication of dysphagia at an urban, university medical center in 1999, 2004 and 2009 were retrospectively identified from the electronic medical record. The entire patient chart, including EGD, pathology, manometry, radiographic and clinician reports, was reviewed for demographic and clinical data and to determine the etiology of dysphagia. The number of EGDs in which an esophageal biopsy was performed was also noted. Gastroesophageal reflux disease (GERD) as a cause of dysphagia independent of peptic stricture was defined by symptoms with erosive esophagitis or symptom response to proton pump inhibition (PPI). Cases of eosinophilic esophagitis (EoE) were defined by an appropriate clinical history and his-

tological criteria of ≥ 15 eosinophils per high powered field. PPI-responsive esophageal eosinophilia was not routinely reported prior to 2008. Statistical analysis was performed using one-way analysis of variance to analyze for trends between 1999, 2004 and 2009 and a post-hoc Tukey analysis was performed following a significant main effect.

RESULTS: A total of 1371 cases (mean age 54 years, 43% male) met pre-specified inclusion criteria with 191, 504 and 675 cases in 1999, 2004 and 2009, respectively. Patients were older in 2004 compared to 2009 (mean \pm SD, 54.0 \pm 15.7 years *vs* 52.3 \pm 16.8 years, $P = 0.02$) and there were more males in 1999 compared to 2004 (57.5% *vs* 40.8%, $P = 0.005$). Overall, GERD (27.6%) and EoE (7.7%) were the most common identifiable causes of dysphagia. An unspecified diagnosis accounted for 21% of overall cases. There were no significant differences in the relative prevalence of achalasia or other motility disorders, peptic stricture, Schatzki's ring, esophageal cancer or unspecified diagnoses over the 10-year time period. There was, however, a decrease in the relative prevalence of GERD (39.3% *vs* 24.1%, $P < 0.001$) and increases in the relative prevalence of EoE (1.6% *vs* 11.2%, $P < 0.001$) and oropharyngeal disorders (1.6% *vs* 4.2%, $P = 0.02$) from 1999 to 2009. Post-hoc analyses determined that the increase in relative prevalence of EoE was significant between 1999 and 2009 as well as 2004 and 2009 (5.4% *vs* 11.6%, $P < 0.001$), but not between 1999 and 2004 (1.6% *vs* 5.4%, $P = 0.21$). On the other hand, the decrease in relative prevalence of GERD was significant between 1999 and 2009 and 1999 and 2004 (39.3% *vs* 27.7%, $P = 0.006$), but not between 2004 and 2009 (27.7% *vs* 24.1%, $P = 0.36$). There were also significantly more EGDs in which a biopsy was obtained in 1999 compared to 2009 (36.7% *vs* 68.7%, $P < 0.001$) as well as between 2004 and 2009 (37.5% *vs* 68.7%, $P < 0.001$). While total EGD volume did increase over the 10-year time period, the percentage of EGDs for the indication of dysphagia remained stable making increasing

upper endoscopy an unlikely reason for the observed increased prevalence of EoE.

CONCLUSION: EoE has emerged as a dominant cause of dysphagia in adults. Whether this was due to a rise in disease incidence or increased recognition is unclear.

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Key words: Eosinophilic esophagitis; Gastroesophageal reflux disease; Dysphagia; Endoscopy; Esophagitis; Esophageal diseases

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INTRODUCTION

Dysphagia is a common indication for referral to a gastroenterology specialist, and is classified based on location as either oropharyngeal or esophageal^[1,2]. Common causes of oropharyngeal dysphagia include cerebrovascular accidents (CVA), radiation injury, and Parkinson's disease^[2]. Frequently identified etiologies of esophageal dysphagia include structural lesions such as peptic strictures, Schatzki's rings, and neoplasm as well as non-obstructive disorders such as gastroesophageal reflux disease (GERD) and esophageal motility disorders^[1,2]. Upper endoscopy (EGD) has largely supplanted upper gastrointestinal radiologic examination for the initial evaluation of dysphagia^[1]. Little published data exists reporting the frequency of the various dysphagia etiologies for patients undergoing EGD.

Recent cross-sectional studies have demonstrated that eosinophilic esophagitis (EoE) is an important and emerging cause of dysphagia^[3-6]. These studies, examining patients undergoing an EGD in the evaluation of dysphagia, report an EoE prevalence between 6.5% and 15%^[3-5]. The studies did not report the frequency of additional etiologies of dysphagia nor whether the prevalence of EoE has changed over time. A histologic analysis of 296 esophageal biopsies from children in Australia demonstrated an 18 fold increase in the prevalence of EoE from 1995 to 2004, but did not include analysis of clinical or endoscopic data^[7]. A population based study from Olten County, Switzerland recently reported a 12 fold increase in the prevalence of EoE from 1989 to 2009^[8].

The aims of this study were to examine the prevalence of various etiologies of dysphagia amongst patients undergoing EGD and to determine the relative prevalence of EoE over the past decade.

MATERIALS AND METHODS

We performed a retrospective review of patients undergoing EGD for the indication of dysphagia at a single, urban, academic medical center, with the goal of determining the relative prevalence of dysphagia-related diagnoses. Endoscopy at this medical center is performed by both academic and private practice gastroenterologists (approximately two-thirds academic faculty and one-third private practice).

Patient population

Patients were identified by a query of the electronic medical record (EMR) for all adult inpatients and outpatients who had an EGD ordered with an associated ICD-9 code for dysphagia (787.2) from 1999 through 2010. For ease of data analysis, our search was narrowed to the years of 1999, 2004 and 2009. The total number of EGDs performed for all indications was extracted to assess endoscopy volume. Clinician office and EGD reports were reviewed to confirm that dysphagia was the actual indication for the EGD and that an EGD was, in fact, performed. Cases were excluded if the indication for the EGD was found not to be dysphagia, if a scheduled EGD was never performed, or if the EGD report was absent from the EMR. If a particular patient had multiple EGDs during this time period, that patient was included in the earliest year but final diagnosis was still determined based on the entirety of their medical record. The medical center completed its transition to an EMR in 2003 and medical records prior to this date were uploaded retrospectively to the database, leading to numerous absences of reports prior to 2003.

Data collection

Pertinent demographic data were collected for the patients who met inclusion criteria. EGD, pathology, manometry, radiographic, and clinician consultation notes were reviewed to determine the etiology of dysphagia. When necessary and available, records from subsequent years were reviewed to determine the final diagnosis. The number of EGDs in which an esophageal biopsy was performed was also noted.

Non-obstructive dysphagia related to GERD was diagnosed by the exclusion of esophageal stricture and response of dysphagia to treatment of acid reflux with proton pump inhibition (PPI)^[9]. All-GERD related dysphagia was the sum of non-obstructive GERD and peptic strictures. Non-obstructive dysphagia related to GERD was diagnosed by the exclusion of esophageal stricture and response of dysphagia to treatment of acid reflux. Cases of EoE were defined by an appropriate clinical history (i.e., dysphagia) and ≥ 15 eosinophils per high powered field (EOS/HPF) on histological review. PPI-responsive esophageal eosinophilia was not routinely reported prior to 2008 and was therefore not analyzed separately from GERD as recommended by the 2007 and 2011 consensus statements on EoE^[10,11]. Achalasia was defined by

characteristic manometric features as well as supportive endoscopic and radiologic abnormalities. Additional abnormal esophageal dysmotility assessed by manometry, such as nutcracker esophagus, diffuse esophageal spasm, or aperistalsis, was grouped as “other motility disorder” and considered the etiology of dysphagia only if subsequent clinician notes specifically attributed dysphagia to esophageal dysmotility. Schatzki’s rings were considered the etiology of dysphagia only if the EGD report explicitly stated that they contributed to the dysphagia; in cases where the EGD or clinician report specifically stated that the Schatzki’s ring was widely open, non-obstructing, or not likely to be contributing to the dysphagia, an alternative diagnosis was considered. Post-operative and iatrogenic etiologies included anastomotic strictures, marginal ulcers, and symptomatic type III paraesophageal hernias. Oropharyngeal disorders included CVA, traumatic brain injury, and neurologic disorders such as amyotrophic lateral sclerosis or multiple sclerosis in which patients had an unremarkable endoscopic exam along with either an abnormal radiologic swallow study and, or an abnormal swallow evaluation by a speech and language pathologist. Functional dysphagia was deemed the final diagnosis after all other organic causes were ruled out based on negative EGD, pathology reports, and motility studies, and if the clinician’s reports indicated that the patient’s dysphagia was functional. Given its low prevalence, it was included in the “other” category. In cases where review of the chart was unable to determine an etiology, the diagnosis was considered “unspecified”.

Statistical analysis

Data were analyzed using statistical software, SPSS version 20 (IBM SPSS Inc, Chicago, IL). The number of excluded EGDs was expressed as a percentage of the total number of EGDs for dysphagia in that year. The total number of included EGDs for dysphagia was the difference between the total number of EGDs for dysphagia and the excluded EGDs for dysphagia. The number of EGDs in which an esophageal biopsy was performed and the relative prevalence of the various etiologies for dysphagia were expressed as a percentage of the included EGDs for dysphagia in the given year.

One-way analysis of variance was used to analyze for trends between 1999, 2004 and 2009. Following the significant main effect, a Tukey *post-hoc* analysis was performed. A *P* value of less than 0.05 was considered statistically significant. The study was approved by the Northwestern University Institutional Review Board.

RESULTS

A total of 1478 patients were identified who had an EGD ordered for dysphagia, with 237, 513 and 728 cases in 1999, 2004 and 2009, respectively. A total of 1371 cases met inclusion criteria with 191, 504 and 675 cases in 1999, 2004 and 2009, respectively (Table 1). In 1999, 46 cases (19.4%) were excluded because EGD reports were

Table 1 Baseline demographic data and relative prevalence of dysphagia etiologies from 1999-2009 *n* (%)

	1999	2004	2009	<i>P</i> value
Total EGDs performed	2456	5944	9071	--
Total included EGDs for dysphagia	191	504	675	--
Excluded EGDs for dysphagia	46 (19.4)	9 (1.8)	53 (7.3)	< 0.001
Age (yr), mean (SD)	55.5 (16.1)	54.0 (15.7)	52.3 (16.8)	NS
Male sex (%)	57.5	40.8	40.7	0.005
EGD with biopsy performed	70 (36.7)	189 (37.5)	464 (68.7)	< 0.001
Diagnosis				
Non-obstructive GERD	75 (39.3)	140 (27.7)	163 (24.1)	< 0.001
Eosinophilic esophagitis	3 (1.6)	27 (5.4)	76 (11.2)	< 0.001
Achalasia	9 (4.7)	26 (5.1)	27 (4.0)	NS
Other motility disorder	4 (2.1)	30 (5.8)	30 (4.4)	NS
Peptic stricture	2 (1.0)	11 (2.1)	18 (2.6)	NS
Schatzki's ring	10 (5.2)	19 (3.7)	25 (3.7)	NS
Esophageal cancer	8 (4.2)	19 (3.7)	15 (2.2)	NS
Post-operative	6 (3.1)	31 (6.1)	25 (3.7)	NS
Oropharyngeal dysphagia	3 (1.6)	11 (2.1)	32 (4.7)	0.02
Radiation esophagitis	6 (3.1)	17 (3.4)	15 (2.2)	NS
Infectious esophagitis	5 (2.6)	13 (2.5)	19 (2.8)	NS
Unspecified	47 (24.6)	103 (20.4)	138 (20.4)	NS
Other ¹	13 (6.8)	54 (10.7)	88 (13.0)	NS

¹Including: Cricopharyngeal bar, globus, functional dysphagia, diverticulum, scleroderma and epidermolysis bullosa with proximal stricture. Statistical analysis performed using one-way analysis of variance. GERD: Gastroesophageal reflux disease; EGDs: Upper endoscopies; NS: Non-significant.

unavailable in the EMR (44 cases) or the indication for EGD was found to be upper gastrointestinal bleeding. In 2004, 9 cases (1.8%) were excluded because review of the EMR revealed the indication for EGD was either an upper gastrointestinal bleed or iron-deficiency anemia. In 2009, 53 cases (7.3%) were excluded because an EGD was not performed (46 cases) or because the indication was found not be dysphagia on review of the chart. There was a significant difference in the number of excluded EGDs between 1999, 2004, and 2009 (*P* < 0.001).

The mean age of the patients included in the study was 53.5 years (SD = 16.3 years) with 43% of the patients being men (*n* = 591). Patients were older in 2004 compared to 2009 (*P* = 0.04) and there were more men in 1999 compared to 2004 (*P* = 0.003).

Overall, non-obstructive GERD (27.6%) and EoE (7.7%) were the most common identifiable causes of dysphagia over the ten year period. An unspecified diagnosis accounted for 21.0% of cases. Other motility disorders (4.7%), achalasia (4.5%) and dysphagia secondary to a post-operative/miscellaneous etiology (4.5%) were other important identifiable causes.

The relative prevalence of the various etiologies for dysphagia over the ten year time period is shown in Table 1 and Figure 1. When analyzing for temporal trends, there was a decrease in the relative prevalence of non-obstructive GERD (39.3% in 1999 and 24.1% in 2009; *P* < 0.001), and increases in the relative prevalence of EoE (1.6% in 1999 and 11.2% in 2009, *P* < 0.001) and

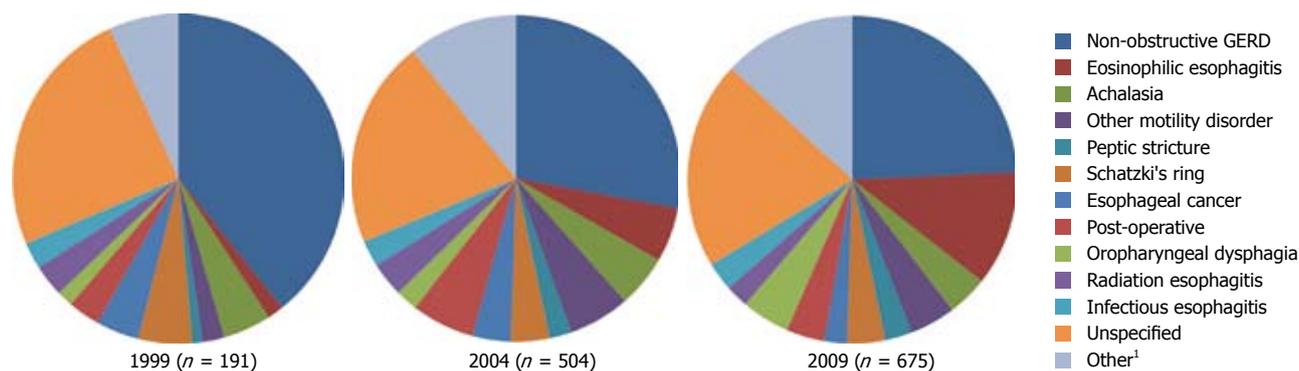


Figure 1 Trends in relative prevalence of dysphagia etiologies from 1999-2009. GERD: Gastroesophageal reflux disease. ¹Including cricopharyngeal bar, globus, functional dysphagia, diverticulum, scleroderma, and epidermolysis bullosa with proximal stricture.

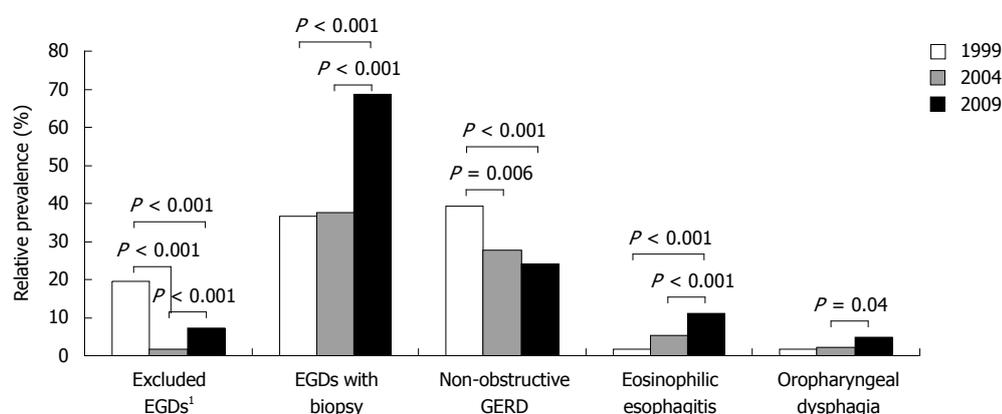


Figure 2 Selected post-hoc Tukey analysis. ¹Expressed as percentage of total upper endoscopies for dysphagia in the given year. GERD: Gastroesophageal reflux disease; EGD: Upper endoscopy.

oropharyngeal disorders (1.6% in 1999 and 4.7% in 2009; $P = 0.02$). There was no significant difference in the relative prevalence of all-GERD related dysphagia (non-obstructive GERD and peptic strictures) over the 10-year time period ($P = 0.07$).

Select post-hoc Tukey analyses are shown graphically in Figure 2. There was a decrease in the relative prevalence of non-obstructive GERD from 1999 to 2004 ($P = 0.006$) as well as from 1999 to 2009 ($P < 0.001$). The increase in the relative prevalence of EoE between 1999 and 2009 was significant ($P < 0.001$) as was the increase between 2004 and 2009 ($P < 0.001$). Of the three patients in 1999 ultimately diagnosed with EoE who had an EGD for dysphagia, one patient was actually diagnosed with EoE in 2003 and another patient was diagnosed in 2005. As specified in the methods section, these patients were included in the 1999 cohort. The increase in relative prevalence of oropharyngeal disorders was significant between 2004 and 2009 ($P = 0.04$). There was no significant difference in the relative prevalence of achalasia, other esophageal motility disorders, peptic strictures, Schatzki's rings, esophageal cancer, or unspecified diagnosis over the ten year time period (Table 1 and Figure 1).

The percentage of EGDs in which an esophageal biopsy was obtained increased over the time period ($P < 0.001$). As shown in Figure 2, this increase was significant

between 1999 and 2009 ($P < 0.001$) as well as between 2004 and 2009 ($P < 0.001$); there was no significant difference in the proportion of EGDs in which a biopsy was performed in 1999 compared to 2004 ($P = 0.98$). Total EGD volume at the medical center also increased in the ten year time period with 2456 EGDs performed in 1999, 5944 in 2004 and 9071 in 2009. The percentage of EGDs performed for the indication of dysphagia remained stable at 8%-9%.

DISCUSSION

This is the first study to describe the relative prevalence of distinct etiologies of dysphagia in an adult patient population undergoing EGD. In our cohort, non-obstructive GERD was the most common, identifiable cause of dysphagia but its relative prevalence decreased over the past decade while EoE was the second most common identifiable cause and its relative prevalence increased from 1.6% in 1999 to 11.2% in 2009. Interestingly, the relative prevalence of all-GERD related dysphagia remained constant. The number of EGDs performed for the indication of dysphagia as a percentage of total EGD volume remained stable at 8%-9%. However, the proportion of EGDs with esophageal biopsies obtained did increase from 36.7% in 1999 to 68.7% in 2009.

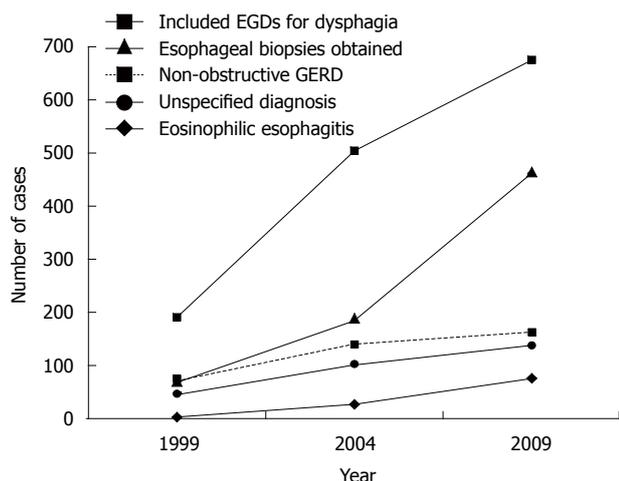


Figure 3 Trends in upper endoscopy volume, esophageal biopsies, and dysphagia diagnoses. GERD: Gastroesophageal reflux disease; EGDs: Upper endoscopies.

The emergence of EoE as a leading cause of dysphagia is of interest and supports earlier studies. The relative prevalence of EoE in 2009 of 11.2% is consistent with the results of recent prospective, short-term, cross-sectional studies^[3-5] that reported an EoE prevalence ranging from 10%-15% in patients with dysphagia undergoing EGD. While these studies reported EoE prevalence, they did not report the other etiologies of dysphagia in the non-EoE patients. In these studies, EoE was defined by histological criterion of ≥ 20 EOS/HPF, which was more stringent than our definition of ≥ 15 EOS/HPF. In two of the studies^[4,5], all patients underwent esophageal biopsies regardless of endoscopic findings while 59% of the patients in the other study^[3] underwent esophageal biopsy. In all of the studies, a specific protocol for obtaining multiple biopsies along the length of the esophagus (midesophagus *vs* proximal and distal) was employed; in our retrospective study, a standardized biopsy strategy could not be used. Regardless of the differences in study designs, it is interesting to note that the high prevalence of EoE in our study was similar to that reported in other prospective studies of patients with dysphagia undergoing EGD.

The rise in the relative prevalence of EoE over the past decade is another interesting finding of this study. In a retrospective epidemiological study of Olmsted County, Prasad *et al*^[6] reviewed pathology specimens that mentioned “eosinophils” and/or “esophagitis” from 1976 to 2005 and found that there was an increase in the incidence of EoE over time ($P < 0.001$). The histological criterion used in that study was ≥ 15 EOS/HPF and esophageal symptoms were assessed by review of the medical record. However, the authors noted a parallel increase in endoscopy utilization over the three decades and raised the possibility that recognition bias from increased endoscopy volume contributed to the rise in EoE diagnoses. In contrast to our cohort, the absolute number of just 78 EoE cases in the Olmsted County population

was quite small. Additionally, the population in the study was not limited to patients with dysphagia.

The largest EoE prevalence study to date utilized a national pathology database to identify 363 cases of EoE from biopsy specimens taken between January 2002 and May 2006 with “eosinophilic” in the diagnosis and/or comment text^[12]. In their subset analyses of pathology specimens from 12 465 patients who underwent an EGD for the indication of dysphagia, they found an increasing prevalence of EoE from 2002 to 2005 ($P < 0.001$), with a prevalence of 0.1% in 2002 and 1.9% in 2005. This study had many important differences from ours. Cases were identified from a pathology database and the timeframe studied was shorter. Additionally, dysphagia as the indication for EGD was not confirmed by review of the medical chart, which, as seen in our study, would have potentially excluded a number of cases. Lastly, only EGDs for dysphagia in which a biopsy was obtained were included in their study, which is only a subset of the total number of EGDs performed for dysphagia although it is surprising that the reported prevalence was much lower than that of the present study.

It is difficult to ascertain whether the increase in relative prevalence of EoE seen in our study is due to a true rise in population prevalence or secondary to heightened recognition from increased numbers of biopsies or increased clinician awareness of the disorder. Interestingly, the prevalence of an “unspecified” cause of dysphagia remained constant at 20%-24% over the measured time period implying that EoE was not simply misclassified as “unknown” a decade ago. It is possible that EoE was initially misclassified as non-obstructive GERD and then properly classified as EoE with increased awareness of the disease, although each patient’s entire EMR was reviewed so that if a subsequent diagnosis of EoE was made at our medical center it would have been detected. However, another plausible explanation for the decrease in prevalence of non-obstructive GERD is the increasing utilization of PPIs by both patients (over-the-counter) and primary care physicians so that only a smaller percentage of PPI-refractory patients are undergoing EGDs.

Increased recognition of EoE may explain the rise in prevalence given the increase in esophageal biopsies obtained, as shown in Figure 3. Lending credibility to this theory is the fact that as the number of biopsies increased between 1999 and 2009 as well as between 2004 and 2009, the prevalence of EoE also increased (Figure 2). On the other hand, when there was not a significant increase in biopsies, such as between 1999 and 2004, the relative prevalence of EoE also did not significantly increase. In contrast to the study by Prasad *et al*^[6] which suggested that increased endoscopy volume may have contributed to increased recognition of EoE, our study provides more specific insight into the issue of increased recognition by identifying trends in the number of biopsies taken.

The stable and relatively low overall prevalence of esophageal cancer (3.1%) was surprising given the rise

in esophageal adenocarcinoma seen in the past five decades^[13,14]. One possible explanation for the low prevalence of esophageal cancer seen in our study is that our medical center is a tertiary care center and patients with esophageal cancer causing dysphagia were being diagnosed in the community and referred directly to oncology and surgery.

The mechanism for non-obstructive dysphagia in GERD is not clear. Several studies have demonstrated that non-obstructive dysphagia in GERD is common and improves with therapy directed at acid reflux^[15,16]. Manometric abnormalities are commonly cited as explanations for this entity^[17], but the role of altered visceral sensation as well as diminished esophageal wall compliance owing to inflammation have not been adequately examined. In a study by Triadafilopoulos^[18], dysphagia was more commonly associated with severity of acid reflux on pH monitoring or erosive esophagitis with only a minority having abnormal motility.

The primary mechanism for dysphagia in EoE is esophageal remodeling secondary to subepithelial fibrosis that is identified in over 90% of patients^[11]. Additionally, structural alterations of the esophageal luminal diameter in the form of focal strictures, esophageal rings or narrow caliber esophagus can be identified in most adults with EoE. In fact, the impact of these structural alterations in EoE have been verified and shown to decrease esophageal mural compliance and lead to significantly reduced esophageal distensibility in EoE patients compared to normal controls^[19]. Furthermore, there is indirect evidence of the role of fibrostenotic complications in the pathogenesis of dysphagia in EoE given the effectiveness of esophageal dilation in the treatment of dysphagia in EoE^[20,21].

There were several important limitations to this study. The large number of excluded cases in 1999 was due to unavailable data in the EMR (given the transition from paper charting in 2003) while the excluded cases in 2009 were because the EGDs were not performed; both of these pitfalls were largely attributable to the retrospective nature of this study. The large number of excluded cases and the difference in age of the patients in 2004 compared to 2009 raise the question of whether the groups being analyzed were subsets of the same larger population. The retrospective nature of the study did not allow for a standardized protocol for esophageal biopsies, which may have led to confounding by indication since biopsies were more likely to be obtained when the EGD showed the classic EoE features^[3,11] of rings, linear furrows, and exudates. The setting of the study was a single, urban, tertiary care center with active esophageal motility and EoE research, so there was the potential for loss to follow-up outside of the medical center in addition to referral bias that limits the generalizability of the results to other practice settings. The utilization of EGD as the primary modality for the evaluation of dysphagia may affect the generalizability of the results to populations where dysphagia may be assessed with barium esophagrams. In addition, many referred patients may have had

a diagnostic endoscopy for dysphagia performed at an outside facility that already established the etiology. When undergoing a follow up EGD at our medical center, such patients may have had listed indications for EGD such as “GERD” or “unspecified esophagitis” and would have been potentially excluded in our analysis.

Another potential problem with this study was how EoE was defined. It is likely that PPI-responsive esophageal eosinophilia was included in the EoE cohort^[11]. Our definition of EoE did not require the exclusion of GERD with a trial of PPI therapy or a normal pH monitoring study as suggested by the 2007 consensus definition^[10] and its 2011 update^[11], since this criterion was not applied widely prior to 2008. The understanding of interactions between GERD and EoE has become increasingly complex and remains controversial^[22,23]. However, significant esophageal eosinophilia (≥ 15 EOS/HPF) is uncommon in GERD, being demonstrated in less than 2% of patients in one large study^[24]. While a significant proportion of patients with suspected EoE respond to PPI therapy, this response may not be specific for acid reflux^[25] and may occur with an allergic pattern of inflammation^[26].

In summary, the relative prevalence of EoE in patients undergoing EGD for dysphagia increased from 1.6% to 11.2% over the past decade. EoE has emerged as one of the dominant, identifiable causes of dysphagia in adults, second only to GERD. Prospective, long-term studies are needed to discern whether this is due to a true increase in disease prevalence or increased recognition.

COMMENTS

Background

Dysphagia is a commonly encountered clinical problem and limited data exist regarding the prevalence of dysphagia etiologies. Recently, cross-sectional studies have demonstrated that eosinophilic esophagitis (EoE) is an important cause of dysphagia.

Research frontiers

Upper endoscopy (EGD) is critical in the evaluation and management of patients with dysphagia. To the knowledge, this is the first study to integrate clinical, pathological, EGD, manometry, and imaging reports in order to systematically report the relative prevalence of all dysphagia-related diagnoses in a large series of patients undergoing EGD, which provides an evidence-based differential diagnosis for the practicing gastroenterologist. Cross-sectional studies have reported on the prevalence of EoE in patients with dysphagia, however these studies have not looked at EoE prevalence over time and with relation to other dysphagia diagnoses. This study demonstrated that between 1999 and 2009, the relative prevalence of gastroesophageal reflux disease (GERD) decreased while the relative prevalence of EoE increased.

Innovations and breakthroughs

Recent studies have reported an EoE relative prevalence between 10%-15% amongst patients undergoing EGD for dysphagia. This study shows that the relative prevalence of EoE has risen significantly from 1.6% to 11.2% over the past decade making it the second most common identifiable cause of dysphagia. Furthermore, the authors' *post-hoc* analysis show that in contrast to previous studies, the proportion of EGDs for dysphagia remained stable over the measured time, but the percentage of EGDs in which a biopsy was performed did increase significantly, providing a plausible mechanism for increased recognition of EoE.

Applications

By providing an evidence based differential diagnosis, this study informs clinicians' decision making in the evaluation of dysphagia. Given the possible inclu-

sion of proton pump inhibition (PPI)-responsive esophageal eosinophilia in the EoE cohort and the increasing proportion of EGDs with biopsies, prospective, long-term studies would be beneficial to discern whether the findings were due to a true increase in EoE prevalence or increased recognition.

Terminology

EoE is a chronic, immune/antigen driven inflammatory disease of the esophagus defined by clinical symptoms of esophageal dysfunction as well as pathological criteria of ≥ 15 eosinophils per high powered field. Current guideline recommendations require exclusion of other causes of esophageal eosinophilia, such as GERD, to make the diagnosis of EoE. By acknowledging the accumulating body of evidence showing that PPIs have effects beyond acid suppression alone, a new disease entity termed "PPI-responsive esophageal eosinophilia", which is distinct from GERD, has been recognized.

Peer review

The authors examined the relative prevalence and temporal variation of dysphagia etiologies in patients undergoing EGD over the past decade and found a decrease in the prevalence of GERD and increases in the prevalence of EoE and oropharyngeal disorders. This is an interesting research.

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National trends in resection of the distal pancreas

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Abstract

AIM: To investigate national trends in distal pancreatectomy (DP) through query of three national patient care databases.

METHODS: From the Nationwide Inpatient Sample (NIS, 2003-2009), the National Surgical Quality Improvement Project (NSQIP, 2005-2010), and the Surveillance Epidemiology and End Results (SEER, 2003-2009) databases using appropriate diagnostic and procedural codes we identified all patients with a diagnosis of a benign or malignant lesion of the body and/or tail of the pancreas that had undergone a partial or distal pancreatectomy. Utilization of laparoscopy was defined in NIS by the International Classification of Diseases, Ninth Revision correspondent procedure code; and in NSQIP by the exploratory laparoscopy or unlisted procedure current procedural terminology

codes. In SEER, patients were identified by the International Classification of Diseases for Oncology, Third Edition diagnosis codes and the SEER Program Code Manual, third edition procedure codes. We analyzed the databases with respect to trends of inpatient outcome metrics, oncologic outcomes, and hospital volumes in patients with lesions of the neck and body of the pancreas that underwent operative resection.

RESULTS: NIS, NSQIP and SEER identified 4242, 2681 and 11 082 DP resections, respectively. Overall, laparoscopy was utilized in 15% (NIS) and 27% (NSQIP). No significant increase was seen over the course of the study. Resection was performed for malignancy in 59% (NIS) and 66% (NSQIP). Neither patient Body mass index nor comorbidities were associated with operative approach ($P = 0.95$ and $P = 0.96$, respectively). Mortality (3% vs 2%, $P = 0.05$) and reoperation (4% vs 4%, $P = 1.0$) was not different between laparoscopy and open groups. Overall complications (10% vs 15%, $P < 0.001$), hospital costs [44 741 dollars, interquartile range (IQR) 28 347-74 114 dollars vs 49 792 dollars, IQR 13 299-73 463, $P = 0.02$] and hospital length of stay (7 d, IQR 4-11 d vs 7 d, IQR 6-10, $P < 0.001$) were less when laparoscopy was utilized. One and two year survival after resection for malignancy were unchanged over the course of the study (ductal adenocarcinoma 1-year 63.6% and 2-year 35.1%, $P = 0.53$; intraductal papillary mucinous neoplasm and neuroendocrine 1-year 90% and 2-year 84%, $P = 0.25$). The majority of resections were performed in teaching hospitals (77% NIS and 85% NSQIP), but minimally invasive surgery (MIS) was not more likely to be used in teaching hospitals (15% vs 14%, $P = 0.26$). Hospitals in the top decile for volume were more likely to be teaching hospitals than lower volume deciles (88% vs 43%, $P < 0.001$), but were no more likely to utilize MIS at resection. Complication rate in teaching and the top decile hospitals was not significantly decreased when compared to non-teaching (15% vs 14%, $P = 0.72$) and lower volume hospitals (14% vs 15%, $P = 0.99$).

No difference was seen in the median number of lymph nodes and lymph node ratio in N1 disease when compared by year ($P = 0.17$ and $P = 0.96$, respectively).

CONCLUSION: There appears to be an overall underutilization of laparoscopy for DP. Centralization does not appear to be occurring. Survival and lymph node harvest have not changed.

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Key words: Laparoscopic distal pancreatectomy; Trends; Nationwide Inpatient Sample; National Surgical Quality Improvement Project; Surveillance epidemiology and end results

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INTRODUCTION

The first open distal pancreatectomy was reported by Billroth in 1884^[1,2]. In the early 1900s, Finney^[1] and Mayo^[3] reported the first open case series and description of their respective techniques. The laparoscopic approach was first introduced to general surgery in the 1980s for gallbladder resection, and quickly adopted as an operative approach for resection of other solid organs. This adoption of laparoscopy has been slower in pancreatic surgery due to the complexity of the procedure, low number of cases, the level of technical skills required by the surgeon in manipulating the important vascular structures, and the lower acceptance of this approach in the resection of malignant lesions.

The use of laparoscopy in distal pancreatectomy was first described in a porcine model by Soper *et al*^[4] in 1994. Soon after this reports of laparoscopy in human patients were reported^[5], though for many years laparoscopy was used principally for staging of malignancy. Multiple reports have shown that laparoscopic pancreatic resection is safe and feasible for benign and malignant pancreas lesions^[6-9]. The known benefits of laparoscopic approach such as lower intraoperative blood loss, less pain and analgesic requirements, earlier return of bowel function, shorter recovery and hospital stay have also been shown in pancreas surgery, including high quality clinical trials^[9-13]. Importantly, it has been reported that complication rates are lower after laparoscopic resection^[9,14].

There has been recent interest in improving the oncologic aspects of distal pancreas resection with attention

to margins and lymph node harvest, oncological surrogate markers for oncological resection. During open or laparoscopic distal pancreatectomy in patients with peripancreatic and retropancreatic invasion, negative surgical margins and appropriate lymph node harvest can be accomplished if wider and deeper dissection techniques are used^[15,16]. It is unclear if this attention to a more radical resection has affected the practice of oncological surgical resection. Strasberg *et al*^[16] reported that after open radical antegrade modular pancreatectomy procedure for patients with adenocarcinoma of the body and tail of the pancreas outside the capsule, a negative tangential margin was accomplished in 91% of the patients.

Recent reports have not shown any difference between open and laparoscopic operations in regards to these oncological surrogate markers^[8,10,11,15,16].

Due to the association of higher hospital procedure volumes and improved clinical outcomes in complex oncologic operations, there has been interest in centralizing pancreas resections to high-volume hospitals. Current reports have documented the trend towards centralization of pancreatic procedures in the last decade, and report an improvement in perioperative mortality when these procedures are performed in high-volume hospitals^[17-20]. It has not been determined whether this centralization has occurred with distal pancreas resection.

We sought to determine whether these concepts have gained any traction and are reflected in the trends of distal pancreas resections in the United States. Therefore we analyzed three major national databases with respect to inpatient outcome metrics, oncologic outcomes, and volume trends of patients with lesions of the neck and body of the pancreas that underwent a surgical resection.

MATERIALS AND METHODS

From the Nationwide Inpatient Sample (NIS, 2003-2009) and the National Surgical Quality Improvement Project (NSQIP, 2005-2010) databases we identified all patients with a diagnosis of a benign or malignant lesion of the body and/or tail of the pancreas that had undergone a partial or distal pancreatectomy. From the Surveillance Epidemiology and End Results (SEER, 2003-2009) database we identified patients with diagnosis of a malignant lesion in the body and tail of the pancreas that underwent an operative resection. Patients from the NIS database were identified by the correspondent International Classification of Diseases, ninth revision (ICD-9) diagnosis and procedure codes (Table 1). In the NSQIP database patients were identified by a combination of ICD-9 diagnosis codes and current procedural terminology (CPT) codes. Utilization of laparoscopy was defined in NIS by the ICD-9 correspondent procedure code; and in NSQIP by the exploratory laparoscopy or unlisted procedure CPT codes. In SEER patients were identified by the International Classification of Diseases for Oncology, third edition (ICD-O-3) diagnosis codes and the SEER Program Code Manual, Third Edition procedure

Table 1 International classification of diseases, ninth revision, and current procedural terminology codes used

ICD-9 codes	Description
Diagnosis	
Malignant neoplasms	
157.1	Body of pancreas
157.2	Tail of pancreas
157.3	Pancreas duct (Santorini and Wirsung)
157.4	Islets of Langerhans
157.8	Other specified sites of the pancreas (ectopic pancreatic tissue, malignant neoplasms of contiguous or overlapping sites of pancreas)
157.9	Pancreas, part unspecified
Benign neoplasms	
211.6	Benign neoplasms of pancreas except Langerhans
211.7	Benign neoplasm of islets of Langerhans
Procedure	
52.5	Partial pancreatectomy
52.52	Distal pancreatectomy
52.53	Radical subtotal pancreatectomy
52.59	Other partial pancreatectomy
54.21	Laparoscopy utilization
CPT codes	
48140	Pancreatectomy, distal subtotal, with or without splenectomy; without pancreaticojejunostomy
48145	Pancreatectomy, distal subtotal, with or without splenectomy; with pancreaticojejunostomy
48999	Unlisted procedure
49320	Exploratory laparoscopy
ICD-O-3 codes	
25.1	Body of the pancreas
25.2	Tail of the pancreas
SEER codes	
30	Partial pancreatectomy, NOS (distal pancreatectomy)
80	Pancreatectomy, NOS
90	Surgery, NOS

ICD-9: International Classification of Diseases, ninth revision; CPT: Current procedural terminology; ICD-O-3: International Classification of Diseases for Oncology, third edition; SEER: Surveillance Epidemiology and End Results; NOS: Not otherwise specified.

codes. Diagnoses and procedures codes are summarized in Table 1. Neither database enabled differentiation of whether laparoscopy was utilized for resection or diagnosis, and if diagnostic laparoscopy was followed by an open resection.

Patients' demographic characteristics included age, gender and body mass index. The comorbidities considered in NIS were acquired immune deficiency, alcohol abuse, iron deficiency anemia, rheumatoid arthritis, collagen vascular diseases, chronic blood loss anemia, congestive heart failure, chronic pulmonary disease, coagulopathy, uncomplicated diabetes, diabetes with chronic complications, hypertension, liver disease, fluid and electrolyte disorders, obesity, peripheral vascular disorders, renal failure and cardiac valve disease. Postoperative complications of interest in NSQIP were superficial surgical site infection, deep incisional surgical site infection, organ space surgical site infection, pneumonia, unplanned intu-

bation, pulmonary embolism, renal insufficiency, stroke with neurological deficit, coma, peripheral nerve injury, cardiac arrest requiring CPR, myocardial infarction, intra-operative or postoperative transfusions, deep vein thrombosis requiring therapy, sepsis and septic shock. The type of hospital (teaching or non-teaching) was assessed by a specific variable in the NIS database. We defined high volume hospitals as the top decile for volume (with ≥ 11 cases per year) over the course of this study (NIS).

Statistical analysis

In SEER, patient identification was performed using the Surveillance Research Program, National Cancer Institute SEER Stat software (seer.cancer.gov/seerstat) version 7.0.5.

In NIS and NSQIP patient identification and data analysis were performed with SAS software version 9.2. Statistical analysis of categorical variables was done with Fischer's exact test. Continuous variables were analyzed with Wilcoxon test as data distribution for non-parametric data. Kendall correlation was done to assess for significance between body mass index (BMI) and year of operation. Linear regression was used to analyze for correlation between mortality and morbidity with operation approach. Logistic regression was used to analyze for trend significance of laparoscopy utilization and pancreas procedures performed in high volume hospitals or teaching hospitals during the course of the study. Survival was analyzed through Kaplan-Meier method.

RESULTS

Demographics

From the NIS database 4242 patients were identified of which 2618 (62%) were female and 1612 (38%) were male; from NSQIP 2681 total patients were identified, 1581 (62%) female and 1093 (41%) male, and from SEER 1090 were identified, 599 (55%) female and 491 (45%) male. The mean age was 60.8 ± 15.1 years in NIS, 61.9 ± 13.8 years in NSQIP and 63.4 ± 13.3 years in SEER (mean \pm SD). In NSQIP the BMI was 28 ± 6.6 kg/m² (mean \pm SD), and 335 (13%) of the patients had a BMI ≥ 35 kg/m². The overall BMI increased significantly during the course of the study (NSQIP, $P = 0.04$). There were 72% patients in NIS that had one or more comorbidities.

Pathology of primary tumor and lymph nodes

In NIS there were 2486 (59%) malignant lesions and 1756 (41%) benign lesions, whereas in NSQIP 1759 (66%) were malignant and 922 (34%) benign. In SEER, when compared by year of operation, no difference was seen in the median number of lymph nodes resected and the lymph node ratio in N1 disease ($P = 0.17$ and $P = 0.96$, respectively). Results by year are summarized in Table 2. Overall, when grouped by lymph node ratio, 45% were $> 0-0.20$ LNR, 28% were $> 0.20-0.40$ LNR and 27% with LNR > 0.40 . The most common neoplasm types in patients that underwent an operation were adenocarcinoma

Table 2 Number of patients and lymph nodes resected per year from 2003 to 2008 (Surveillance Epidemiology and End Results database)

Year	Number of lymph nodes resected				Lymph node ratio in N1 disease			
	<i>n</i>	Median	1QR	3QR	<i>n</i>	Median	1QR	3QR
2003	88	8	2	14	31	0.27	0.14	0.50
2004	133	7	4	13	66	0.23	0.13	0.50
2005	133	7	3	14	62	0.23	0.10	0.50
2006	160	8	5	13	71	0.21	0.14	0.45
2007	179	8	4	15	85	0.25	0.13	0.36
2008	315	9	5	16	94	0.21	0.13	0.40

QR: Quartile range.

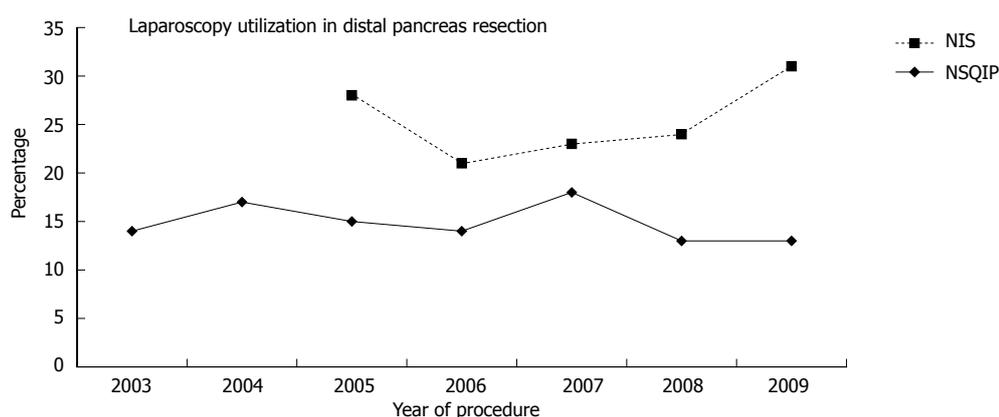


Figure 1 Yearly percentage of laparoscopic utilization in distal pancreas resection by both Nationwide Inpatient Sample and National Surgical Quality Improvement Project. NIS: Nationwide Inpatient Sample; NSQIP: National Surgical Quality Improvement Project.

in 52%, neuroendocrine (NE) in 27% and intraductal papillary mucinous neoplasm (IPMN) in 12% (SEER). Survival at 1 and 2 years was unchanged for resected patients with ductal adenocarcinoma of the pancreas (1-year 63.6% and 2-year 35.1%, $P = 0.53$ and $P = 0.21$, SEER). Over the time covered in the study neither the IPMN nor NE 1 and 2 year survival changed (1-year 90%, and 2-year 84%, $P = 0.15$ and $P = 0.25$, SEER).

Operative approach

Overall, laparoscopy was utilized in 15% (NIS) and 27% (NSQIP) patients and 85% NIS and 73% NSQIP patients were performed through an open approach. During the course of the study the overall use of laparoscopy did not increase in either NIS ($P = 0.51$) or NSQIP ($P = 0.54$) datasets (Figure 1).

In patients with a comorbidity there was no difference in the utilization of laparoscopy (15%) in comparison to patients without any medical comorbidities (NIS, $P = 0.96$). Patients' BMI was not associated with an operative approach (NSQIP, $P = 0.95$). Laparoscopy was utilized significantly more frequently in malignant lesions (21%) in comparison to benign lesions (8%; NIS, $P < 0.001$). In patients with malignant lesions spleen resection was performed more commonly (75%) in comparison to spleen preservation (25%; NIS, $P < 0.001$).

Utilization of laparoscopy did not correlate with type of hospital; utilization of laparoscopy occurs in 15% of

resection in teaching hospitals and 14% were performed in non-teaching hospitals (NIS, $P = 0.26$).

Outcome metrics

The rate of reoperation was not different between laparoscopy utilization (4%) and open approach (4%, NIS, $P = 1.0$).

Overall, postoperative complications were less frequent after laparoscopy utilization (10%) in comparison to open approach (15%, NIS, $P < 0.001$). Postoperative complications occurred more commonly after resection of malignant lesions (12% *vs* 8%, NIS, $P < 0.001$). After resection of a malignant lesion complications were less frequent after laparoscopy utilization when compared to open approach (9% *vs* 18%, NIS, $P < 0.001$). Overall, the most frequent complications were intra-abdominal infection in 9%, sepsis in 7% and superficial site infection in 4% (NSQIP).

The overall length of stay was shorter when laparoscopy was utilized in comparison to open approach both in NIS [laparoscopy: 7 d, interquartile range (IQR) 4-11 *vs* open: 7 d, IQR 6-10, $P < 0.001$] and NSQIP (laparoscopy: 6 d, IQR 4-8 *vs* open: 7 d, IQR 5-9, $P < 0.001$; Figure 2). In both NIS and NSQIP the overall median length of stay was shorter after resection of a benign lesions (NIS, 7 d, IQR 5-9 *vs* NSQIP, 6 d, IQR 5-8) in comparison to malignant lesion (NIS, 8 d, IQR 6-11 *vs* NSQIP, 7 d, IQR 5-9, $P < 0.001$), irrespective of approach.

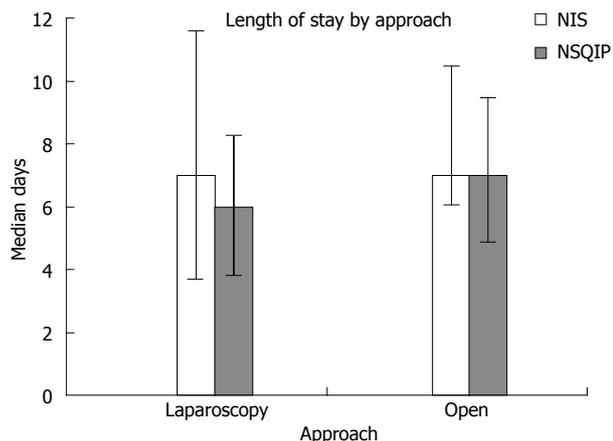


Figure 2 Median length of stay by approach. Bars represent interquartile ranges. NIS: Nationwide Inpatient Sample; NSQIP: National Surgical Quality Improvement Project.

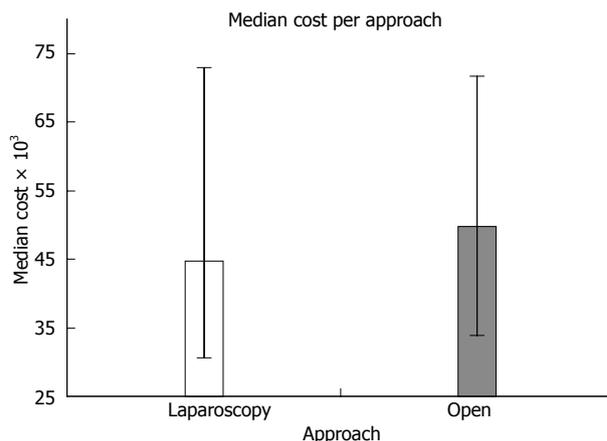


Figure 3 Median cost of procedures by approach (Nationwide Inpatient Sample, $P = 0.02$). Bars represent interquartile ranges.

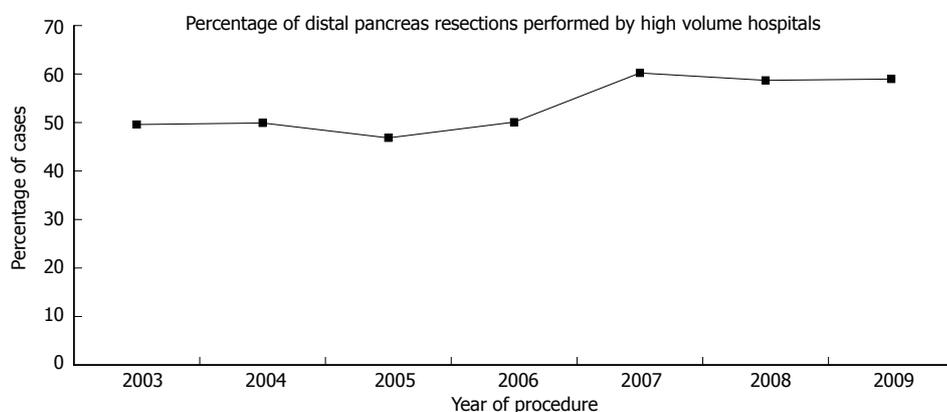


Figure 4 Percentage of distal pancreas procedures performed by high volume hospitals.

Regarding cost of care, there was a significantly lower total charge with laparoscopy utilization (median cost was 44 741 dollars, IQR 28 347-74 114 dollars) when compared to open approach (median cost was 49 792 dollars, IQR 13 299-73 463, NIS, $P = 0.02$) (Figure 3).

The hospital mortality rate was not different between laparoscopy utilization and open approach respectively (3% *vs* 2%, NIS, $P = 0.05$).

Hospital volume

Of the 903 hospitals included in NIS, the top decile for volume included hospitals that performed greater than 11 cases per year. Overall, the fraction of total procedures of the distal pancreas performed by high volume hospitals did not increase significantly during the course of the study (NIS, $P = 0.43$) (Figure 4). A significant majority of the teaching hospitals (88%) were in the top decile of hospitals for volume (NIS, $P < 0.001$).

No significant difference was seen in the frequency of postoperative complications when analyzed by deciles of hospital volume; the complication rate for resection in the top decile hospitals was 14% and 15% in the lower decile hospitals (NIS, $P = 0.99$). Neither was a significant

difference seen when analyzed by type of hospital; teaching hospitals and non-teaching hospitals had similar complication rate (15% *vs* 14% respectively, NIS, $P = 0.72$).

DISCUSSION

The NIS database is directed to determine health care utilization, access, charges, quality and outcomes^[21]. NSQIP focuses on perioperative complications, with a mandate to determine the quality of surgical care^[22] and SEER focuses on the incidence, prevalence and survival of cancer^[23]. Taking into consideration the goal of each database, we amalgamated the available information from these respected datasets to obtain an integrated and broad nationwide analysis of distal pancreas resection. Specifically we found NIS useful to assess laparoscopy utilization and comorbidities, comparison of costs and analysis by hospital type (teaching and non-teaching). On the other hand, the NSQIP database is more appropriate for detailed analysis of perioperative complications and patient risk factors. Oncological metrics and outcomes are best assessed by the SEER database. All of these have limitations, but we believe that the nationwide databases

are complementary to each other and it is helpful to present an analysis of them together. The limiting factor we encounter in both NIS and NSQIP is that procedures are grouped in broad categories, limiting the ability to distinguish the role of laparoscopy in each procedure. Though using the current coding system allowed for a general procedure analysis, an improvement in coding system that further categorizes procedures will facilitate a more detailed analysis.

Overall, we found that in patients where laparoscopy was utilized, the median length of stay was shorter. This reinforces the findings of single center studies^[8,12,15,24]. Recently, Venkat *et al*^[9] in a meta-analysis reported that patients that underwent a laparoscopic distal pancreatectomy had not just a lower blood loss and hospital length of stay, but also had fewer complications and surgical site infections. Importantly, no difference was found in operative time, margin positivity, incidence of postoperative pancreatic fistula or mortality. Additionally, we found that utilization of laparoscopy reduced overall expenses; this finding is likely secondary to the shorter length of stay and lower complication rate.

Other recent reports have confirmed the advantages of laparoscopic distal pancreatectomy over open approach, in terms of lower intraoperative blood loss, pain and analgesic requirements, earlier return of bowel function, shorter recovery and hospital stay^[9,10,14,25,26]. Therefore, the subset of patients with medical comorbidities could potentially benefit more than healthier patients, but we did not find that patient morbidity was associated with use of minimally invasive surgery.

Despite the known and reported benefits, overall laparoscopy was utilized in 15% (NIS) and 27% (NSQIP) of the distal pancreas resection cases. This represents all patients that had laparoscopy performed for diagnosis, staging, resection or a combination of these procedures. The rate of utilization of laparoscopy has remained stable over most of the past decade, although we could not determine in each case the specific technique of laparoscopy used. The finding of no increase in utilization of laparoscopy could be explained by the low number of pancreas surgeons that perform laparoscopic pancreatic resection. Also, another potential explanation for this stable rate could be that more laparoscopic resections and fewer diagnostic staging procedures are being performed over the study course, but these changes could not be detected by our analysis.

In our analysis, we showed that laparoscopy was utilized more frequently in malignant lesions, which could be due to utilization of laparoscopy for diagnosis and/or staging purposes. Interestingly, we did not find that laparoscopy was utilized and performed more frequently in teaching or high volume hospitals, though we expected that teaching hospitals and specialized medical centers would perform more MIS resection cases. It is not surprising that NSQIP hospitals have a higher rate of laparoscopy utilization than NIS hospitals, because NSQIP hospitals have volunteered for the program in an effort

to improve quality. But it would not be appropriate to further compare data across different databases.

No consensus exists for or against the use of laparoscopic approach for distal pancreatectomy in malignant lesions, though recent publications support this approach in selected patients^[7]. It has also been reported that after laparoscopic distal pancreatectomy in patients with invasion, a resection to negative margins and adequate lymph node harvest can be accomplished^[15]. We also found that postoperative complications were more common after resection of malignant lesions, but even in malignant lesions use of laparoscopy was associated with a decrease in complications. The reported laparoscopic distal pancreatectomy morbidity ranges from 20% to 47% and the reported mortality rate ranges from 3% to 5%^[14,27,28]. Our study shows that nationwide mortality is at or below the benchmark reported data. The most common reported complications after laparoscopic distal pancreatectomy are pancreatic fistula, intra-abdominal abscesses, wound infection, sepsis, malabsorption, electrolyte disturbance and hemorrhage^[14,27,28]. The rate of intra-abdominal infection we report (9%) is also at or below reported benchmarks. This may reflect inaccuracy of coding.

Regarding oncological outcomes, no increase was seen in the number of lymph nodes harvested and lymph node ratio in N1 disease. More importantly the 1-year and 2-year survival did not increase during the course of this study.

The oncological surrogate markers associated with survival in pancreas cancer such as tumor size, tumor differentiation, surgical margins, lymph node status and lymph node ratio have been well documented^[8,10,11,15,29]. We speculate that surgeons might opt for open operation to enable a more radical oncologic resection. This however, does not appear to be the case, as survival and oncologic surrogate markers were unchanged over the course of the study. We believe that published data support using MIS to perform a more radical oncologic resection, but these data show this is not a common practice across the country^[8,15,16,30].

Multiple reports support centralization of oncological pancreas procedures towards high-volume hospitals, and as a result of this, a decrease in mortality has also been reported^[17-20]. Stitzenberg *et al*^[18] analyzed the NIS database to assess cancer surgery centralization for pancreas, esophagus and colon cancer. Interestingly, during the course of the study there was a decrease in total number of hospitals performing pancreas procedures, but a statistical significant increase in the number of high-volume hospitals and a decrease in low-volume hospitals performing pancreatectomy procedures. All the high-volume centers were teaching hospitals. A total of 8.3% patients with a diagnosis of pancreas malignancy were admitted through an emergency room, and were more likely to have surgery done in a low-volume hospital.

Forces opposing the trend for centralization include the technological advances in imaging, resulting in diagnosis of pancreas lesions at earlier stages. Smaller le-

sions could potentially be managed in a non-specialized hospital, and only complicated cases referred to specialized hospitals. On the other hand, recent reports and guidelines support the use of endoscopic ultrasound with guided fine-needle aspiration for diagnosis and staging of body and tail pancreatic neoplasms^[31,32]. This procedure is usually performed in specialized medical centers, which would support the concept of centralization. In our analysis, centralization of distal pancreas procedures over the last decade has not occurred.

Even though the definition of utilization of laparoscopy in our analysis was broad, it appears that at a nationwide level, laparoscopy is underutilized for distal pancreas resection despite sufficient evidence of its benefit. Centralization of distal pancreas resections to high volume centers does not appear to be occurring in the United States, but this does not appear to affect overall quality. During the course of this study survival and extent of lymph node harvest has not changed.

We can conclude that there is room for improvement in distal pancreas resection in the United States.

COMMENTS

Background

Laparoscopy utilization in pancreas operations has progressed at a slower pace when compared to procedures for other solid organs. Current reports have documented the trend towards centralization of proximal pancreatic procedures in the last decade, and improvement in perioperative mortality when performed in high-volume hospitals.

Research frontiers

Database analysis allows assessment of trends across the spectrum of surgical practice in a country. Trends of distal pancreas resections in the United States were studied with respect to concepts of centralization of care, surgical technique, and clinical and oncological outcome metrics.

Innovations and breakthroughs

In patients where laparoscopy was utilized, there were fewer complications and surgical site infections, and decreased length of stay. Additionally, utilization of laparoscopy reduced overall expenses. The rate of utilization of laparoscopy has remained stable over most of the past decade. Interestingly, they did not find that laparoscopy was utilized and performed more frequently in teaching or high volume hospitals. Postoperative complications were more common after resection of malignant lesions. Regarding oncological outcomes, no increase was seen in the number of lymph nodes harvested and lymph node ratio in N1 disease. More importantly, the 1-year and 2-year survival did not increase during the course of this study. There was no trend for centralization of care for resection of lesions of the distal pancreas.

Applications

Utilization of laparoscopy during distal pancreas resection is associated with improved outcomes at a national level, when compared to open resection. These findings confirm results of recent multi-center studies.

Terminology

From the Health Care Cost and Utilization Project, the Nationwide Inpatient Sample database is directed to determine health care utilization, access, charges, quality and outcomes. The National Surgical Quality Improvement Project is a database from the American College of Surgery that focuses on perioperative complications, with a mandate to determine the quality of surgical care. The Surveillance Epidemiology and End Results by the National Cancer Institute focuses on the incidence, prevalence and survival of cancer.

Peer review

Each database shows unique aspects of the trends in distal pancreatectomy, demonstrating their individual advantages and weaknesses. There appears to be an overall underutilization of laparoscopy for distal pancreatectomy across the United States despite the benefits demonstrated on multiple published series.

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Chronic methadone use, poor bowel visualization and failed colonoscopy: A preliminary study

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Abstract

AIM: To examine effects of chronic methadone usage on bowel visualization, preparation, and repeat colonoscopy.

METHODS: In-patient colonoscopy reports from October, 2004 to May, 2009 for methadone dependent (MD) patients were retrospectively evaluated and compared to matched opioid naive controls (C). Strict criteria were applied to exclude patients with risk factors known to cause constipation or gastric dysmotility. Colonoscopy reports of all eligible patients were analyzed for degree

of bowel visualization, assessment of bowel preparation (good, fair, or poor), and whether a repeat colonoscopy was required. Bowel visualization was scored on a 4 point scale based on multiple prior studies: excellent = 1, good = 2, fair = 3, or poor = 4. Analysis of variance (ANOVA) and Pearson χ^2 test were used for data analyses. Subgroup analysis included correlation between methadone dose and colonoscopy outcomes. All variables significantly differing between MD and C groups were included in both univariate and multivariate logistic regression analyses. *P* values were two sided, and < 0.05 were considered statistically significant.

RESULTS: After applying exclusionary criteria, a total of 178 MD patients and 115 C patients underwent a colonoscopy during the designated study period. A total of 67 colonoscopy reports for MD patients and 72 for C were included for data analysis. Age and gender matched controls were randomly selected from this population to serve as controls in a numerically comparable group. The average age for MD patients was 52.2 ± 9.2 years (range: 32-72 years) compared to 54.6 ± 15.5 years (range: 20-81 years) for C ($P = 0.27$). Sixty nine percent of patients in MD and 65% in C group were males ($P = 0.67$). When evaluating colonoscopy reports for bowel visualization, MD patients had significantly greater percentage of solid stool (i.e., poor visualization) compared to C (40.3% vs 6.9%, $P < 0.001$). Poor bowel preparation (35.8% vs 9.7%, $P < 0.001$) and need for repeat colonoscopy (32.8% vs 12.5%, $P = 0.004$) were significantly higher in MD group compared to C, respectively. Under univariate analysis, factors significantly associated with MD group were presence of fecal particulate [odds ratio (OR), 3.89, 95% CI: 1.33-11.36, $P = 0.01$] and solid stool (OR, 13.5, 95% CI: 4.21-43.31, $P < 0.001$). Fair (OR, 3.82, 95% CI: 1.63-8.96, $P = 0.002$) and poor (OR, 8.10, 95% CI: 3.05-21.56, $P < 0.001$) assessment of bowel preparation were more likely to be associated with MD patients. Requirement for repeat colonoscopy was also significant higher in MD group (OR, 3.42, 95%

CI: 1.44-8.13, $P = 0.01$). In the multivariate analyses, the only variable independently associated with MD group was presence of solid stool (OR, 7.77, 95% CI: 1.66-36.47, $P = 0.01$). Subgroup analysis demonstrated a general trend towards poorer bowel visualization with higher methadone dosage. ANOVA analysis demonstrated that mean methadone dose associated with presence of solid stool (poor visualization) was significantly higher compared to mean dosage for clean colon (excellent visualization, $P = 0.02$) or for those with liquid stool only (good visualization, $P = 0.01$).

CONCLUSION: Methadone dependence is a risk factor for poor bowel visualization and leads to more repeat colonoscopies. More aggressive bowel preparation may be needed in MD patients.

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Key words: Colonoscopy; Methadone; Opioid; Inadequate bowel preparation; Colonoscopy preparation; Methadone dose

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INTRODUCTION

Approximately 7 million colonoscopies are performed annually in the United States^[1,2]. It is the preferred modality for colorectal cancer screening^[3,4]. A successful colonoscopy requires adequate pre-colonoscopy bowel preparation^[5]. The adequacy of the preparation depends on its ability to reliably empty the colon of fecal material without altering the colonic mucosa^[2,6]. Inability to properly visualize the lumen due to retained fecal material may result in missed pathologies, diagnostic delays and repeat procedures, with potentially adverse medical, legal, and economic implications. It has been reported that over 20% of all colonoscopies performed in the United States fail due to suboptimal bowel preparation or poor colonic visualization^[7,8], leading to an estimated 12%-22% increase in long term costs related to the procedure^[9].

Concurrently, opioid use in the United States has increased sharply over the last decade^[10-13]. An estimated 3% of all adult population in the United States is on long term opioid treatment for chronic pain management^[10,14]. Methadone, a synthetic opioid, is commonly used for analgesia in patients with malignancy^[15], and intractable neuropathy^[16]. It is also standard therapy for the treatment

of opioid addiction^[17]. However, methadone can cause constipation due to its anti-peristaltic effects on the entire gastrointestinal (GI) tract^[18]. Therefore, it is reasonable to postulate that adequate colonic visualization may be difficult to accomplish in patients on chronic methadone therapy due to excessive fecal retention.

Although studies have demonstrated a variety of risk factors for suboptimal preparation and poor bowel visualization^[19,20], the effects of opioids on the quality of bowel visualization has not been evaluated. Our aim was to examine the quality of colonoscopic bowel visualization in methadone dependent patients.

MATERIALS AND METHODS

Patient selection

This was a retrospective cohort study of patients selected from Beth Israel Medical Center (BIMC) at Albert Einstein College of Medicine endoscopy database who had an in-patient colonoscopy performed between October, 2004 and May, 2009. The study was approved by BIMC Institutional Review Board. Patients were cross referenced using BIMC's computerized medical records, out-patient records, and discharge summaries to obtain demographic, clinical, medications and laboratory data. Search filters for "methadone" were applied to select for patients. Patients with risk factors known to cause constipation or gastric dysmotility were excluded (Table 1). Eligible patients were divided into two groups: (1) methadone dependent (MD); and (2) those without any history of opioid usage (C). MD patients and their dosages were verified from their respective institutional methadone maintenance treatment program. Patients whose methadone dose was not verified or those who received methadone on an as needed basis were also excluded. The colonoscopy reports of all eligible patients were analyzed for degree of bowel visualization, assessment of the bowel preparation (good, fair, or poor), and whether a repeat colonoscopy was required. Age and gender matched controls who were free of any opioid exposure for at least 4 wk before colonoscopy were selected from our database as controls.

Evaluation of bowel visualization

Bowel visualization was scored on a 4 point scale utilized in multiple prior studies examining quality of bowel visualization^[20-22]. Points were assigned as following: excellent = 1, good = 2, fair = 3, or poor = 4^[20-22]. Excellent visualization was defined as a clean bowel without presence of any stool and small to moderate amount of clear liquid. Good was defined as presence of small amount of liquid fecal material able to be easily suctioned. Fair was defined as semisolid stool (fecal particulate), able to be washed out with more than 90% of the colon visualized. Poor was defined as presence of large amounts of solid stool obscuring more than 90% of the colonic mucosa.

Statistical analysis

Descriptive statistics of mean and standard deviation

Table 1 Exclusion criteria for patient enrollment

Patient with history of
Diabetes, neurological or any thyroid dysfunction
Active inflammatory bowel disease
Scleroderma
Chronic constipation
Chronic diarrhea
Any acute or chronic renal insufficient or on hemodialysis
Radiation to colon
Colectomy, or hemi-colectomy
Positive stool studies for any pathogen
Iron replacement therapy
Fentanyl, dilaudid, or any other opioids
Pancreatic insufficiency or pancreatic enzyme replacement therapy
Tricyclic antidepressants
Colonoscopy performed on emergent basis or on unstable patients
Colonoscopy report without comment on adequacy of bowel preparation or bowel images

Table 2 Clinical characteristics of the enrolled patients *n* (%)

	Methadone patients	Controls	<i>P</i> value
Number of patients	56	68	
Number of colonoscopies	67	72	
Average age (yr)	52.2 ± 9.2	54.6 ± 15.5	0.27
Gender			0.67
Male	46 (68.7)	47 (65.3)	
Female	21 (31.3)	25 (34.7)	
Indication			
Bleeding	22 (32.4)	47 (40.9)	
Anemia	3 (4.4)	25 (21.7)	
Screening	31 (45.6)	11 (9.6)	
Abdominal pain	5 (7.4)	8 (7)	
Other	7 (10.3)	24 (20.9)	

were used to describe the continuous variables. Percent-age and frequency were used to describe the categorical variables. Analysis of variance (ANOVA) compared for differences in the continuous variables. Pearson χ^2 test compared for differences in the categorical variables. *P* values were two sided, and < 0.05 were considered statistically significant. All variables significantly differing between MD and C groups were included in both univariate and multivariate logistic regression analyses. ANOVA was used to compare methadone dose to bowel visualization level. Least significant difference post-hoc comparisons were performed. All analysis were performed with IBM SPSS Statistics Version 19.

RESULTS

Demographics and clinical characteristics

A total of 178 MD patients underwent colonoscopy during the designated study period, out of which 57 were excluded for concomitant use of other opioids or prescribed methadone on an as needed basis, 48 were excluded for unverifiable methadone dosage and 17 for either incomplete colonoscopy reports, colonoscopies performed urgently, or for prematurely terminated examinations for unknown reasons. A total of 115 colo-

Table 3 Comparisons of bowel visualization, preparation assessment, and rate of repeat colonoscopy between methadone and control patients *n* (%)

Variables	Methadone patients (<i>n</i> = 67)	Control (<i>n</i> = 72)	<i>P</i> value
Bowel visualization			< 0.001
Excellent	12 (17.9)	30 (41.7)	
Good	14 (20.9)	28 (38.9)	
Fair	14 (20.9)	9 (12.5)	
Poor	27 (40.3)	5 (6.9)	
Bowel preparation assessment			< 0.001
Good	22 (32.8)	52 (72.2)	
Fair	21 (31.3)	13 (18.1)	
Poor	24 (35.8)	7 (9.7)	
Repeat colonoscopy	22 (32.8)	9 (12.5)	0.004

noscopies were performed during the same study period on opioid naïve patients who passed the exclusion criteria for the study. Age and gender matched patients were randomly selected from this population to serve as controls in a numerically comparable group. In the final analysis, 56 patients were included in the MD group and 68 in control group. This yielded a total of 67 colonoscopy reports for MD patients and 72 for C, as some patients underwent repeat examinations. Baseline characteristics of all patients are listed in Table 2. The average age for MD patients was 52.2 ± 9.2 years (range: 32-72 years) years compared to 54.6 ± 15.5 years (range: 20-81 years) for C ($P = 0.27$). Sixty nine percent of patients in MD and 65% in C group were males ($P = 0.67$).

Colonoscopy evaluation

Table 3 compares bowel visualization, overall assessment of bowel preparation, and need for repeat colonoscopies between MD and C groups. Bowel visualization quality significantly differed between the two groups, with data suggesting greater percentages of solid stool (poor visualization) for MD compared to C (40.3% *vs* 6.9%, $P < 0.001$). Poor bowel preparation (35.8% *vs* 9.7%, $P < 0.001$) and need for repeat colonoscopy (32.8% *vs* 12.5%, $P = 0.004$) were significantly higher in MD group compared to C.

Results for univariate and multivariate analysis are listed in Table 4. In the univariate analysis, factors significantly associated with MD group were presence of fecal particulate [odds ratio (OR), 3.89, 95% CI: 1.33-11.36, $P = 0.01$] and solid stool (OR, 13.5, 95% CI: 4.21-43.31, $P < 0.001$). Bowel preparation assessment had significantly greater odds ratios for fair and poor status in the MD group (Table 4). Requirement for repeat colonoscopy was also significant in the MD group (OR, 3.42, 95% CI: 1.44-8.13, $P = 0.01$). However, in the multivariate analyses, the only significant variable independently associated with MD group was presence of solid stool (OR, 7.77, 95% CI: 1.66-36.47, $P = 0.01$).

Methadone dosage and bowel visualization

A subgroup analysis limited to MD patients was per-

Table 4 Univariate and multivariate analysis in methadone dependent patients

Variables	Univariate OR (95% CI)	P value	Multivariate OR (95% CI)	P value
Bowel visualization				
Excellent	1.00		1.00	
Good	1.25 (0.50, 3.16)	0.64	1.07 (0.41, 2.80)	0.89
Fair	3.89 (1.33, 11.36)	0.01	2.51 (0.74, 8.50)	0.14
Poor	13.50 (4.21, 43.31)	< 0.001	7.77 (1.66, 36.47)	0.01
Bowel preparation assessment				
Good	1.00		1.00	
Fair	3.82 (1.63, 8.96)	0.002	2.29 (0.86, 6.10)	0.10
Poor	8.10 (3.05, 21.56)	< 0.001	2.61 (0.56, 12.21)	0.22
Repeat colonoscopy	3.42 (1.44, 8.13)	0.01	0.73 (0.19, 2.77)	0.64

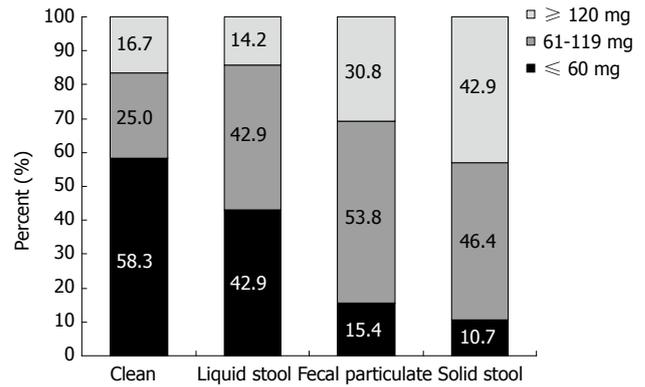
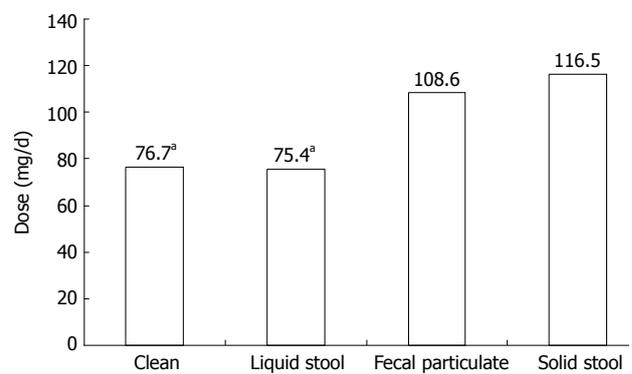
OR: Odds ratio.

formed to evaluate the relationship between methadone dosage and quality of bowel visualization. There was a general trend towards poorer visualization with higher dosage (Figure 1). Mean methadone doses for quality of bowel visualization levels were: 76.7 ± 54.3 mg for clean bowel, 75.4 ± 45.8 mg for liquid stool, 108.6 ± 46.1 mg for fecal particulate, and 116.5 ± 42.3 mg for presence of solid stool (Figure 2). ANOVA analysis demonstrated statistically significant differences in mean methadone dosage between the quality of bowel visualization ($P = 0.02$). Least significant difference post-hoc comparisons showed that mean methadone dose associated with the presence of solid stool (poor visualization) was significantly higher compared to mean dosage for clean colon (excellent visualization, $P = 0.02$) or for those with liquid stool only (good visualization, $P = 0.01$).

DISCUSSION

To our knowledge, this is the first study to examine the effects of methadone on bowel visualization. After applying strict exclusionary criteria eliminating many known causes of GI dysmotility, our results show that chronic methadone use is an independent risk factor for poor bowel visualization due to higher incidence of retained solid stool. Bowel visualization may also be dose dependent since mean methadone dose for colonoscopies containing solid stool was significantly higher compared to the clean colonoscopies or those with only liquid stool.

Prior studies on bowel visualization have used a scoring system to evaluate for the degree of colonic stool retention^[20-22]. We used the identical scoring system to examine bowel visualization in methadone dependent patients. Compared to opioid naïve patients, methadone dependent patients had a significantly higher rate of retained solid stool (poor visualization) and fecal particulate (fair visualization). Even under multivariate analysis, methadone patients had approximately an 8-fold higher occurrence for presence of solid stool compared to controls. Methadone, as with other opioids, binds to μ receptors in the central nervous system^[23], and periph-

**Figure 1** Bowel visualization according to methadone dose.**Figure 2** Mean methadone dose for quality of bowel visualization. ^a $P < 0.05$ vs solid stool.

eral sensory nerve fibers terminals^[24], including enteric neurons^[10,25]. Opioids inhibit both gastric emptying and intestinal propulsive motor activity^[17] throughout the entire GI tract while stimulating contraction of the pyloric and ileo-cecal sphincters^[10,26]. The densest concentrations of mu receptors are found in the stomach and colon^[27], therefore decreased colonic propulsion is also likely. Furthermore, it is well known that one of the major functions of a normal colon includes water absorption^[28]. Opioids stimulate fluid and water absorption mainly due to delayed transit time and increased luminal contact time^[10]. The combination of multiple effects of methadone throughout the GI tract in conjunction with increased colonic fluid resorption from decreased propulsion most likely explains the significantly higher presence of solid stool in methadone dependent patients.

We further examined whether there was a relationship between methadone dosage and bowel visualization. Our results show that methadone patients whose colonoscopies contained solid stool were consuming a significantly higher dose compared to methadone patients with clean bowel or liquid stool. Methadone pharmacokinetics has demonstrated a strong linear relationship between plasma concentration and methadone dose^[29,30]. Therefore, it might be possible that consuming higher methadone dose led to higher plasma concentration and subsequently more side effects, including

decreased transit time and increased water absorption, leading to higher presence of solid stool. Unfortunately, studies on the relationship between opioid dosage and GI motor function are limited. A study by Delgado-Aros *et al*^[31] examined the effects of asimadoline, a κ receptor opioid agonist, in patients with irritable bowel syndrome and found no difference in GI motor function with increasing dosage. However, κ receptors are involved in visceral pain perception^[32,33] and do not affect GI motility. A study by Ness *et al*^[19] on factors affecting colonic preparation demonstrated no association between narcotics use and inadequate bowel preparation. However, it is unclear whether their study population had other comorbidities which may have confounded the results^[19]. Furthermore, information on the type of narcotics (opioid *vs* non-opioid), dosage, and duration of usage were lacking, and the number of patients on narcotics were small ($n = 14$)^[19].

The presence of solid stool may also have factored into the requirement for repeat colonoscopies. Methadone dependent patients were significantly more likely to require repeat colonoscopies compared to non-opioid patients under univariate analysis. However, results were non-significant when controlling for other variables under multivariate analysis. Therefore, the requirement for a repeat colonoscopy in methadone patients is dependent on other factors, most likely the presence of solid stool obscuring proper luminal visualization. Another explanation for the lack of significant results under multivariate analysis may be due to the relatively small sample size, since the range of the odds ratio was large under univariate analysis. Further studies using a larger sample size are needed to validate these results. Nonetheless, our study underscores the importance of proper bowel visualization and the substantial costs associated with repeat colonoscopies. According to a cost analysis study by Rex *et al*^[9], the economic impact of repeat colonoscopies resulted in a 12%-22% increase in potential long term costs due to decreased time interval for subsequent colonoscopies and associated charges for polypectomy and histological examinations.

Lastly, we evaluated the overall assessment of the adequacy of bowel preparation. The American Society of Gastrointestinal Endoscopy and the American College of Gastroenterology Taskforce on Quality in Endoscopy suggest that every colonoscopy report should include an overall assessment, although acknowledge that it lacks standardized definitions due to endoscopist subjectivity^[54]. Multiple prior studies have evaluated the adequacy of bowel preparation based on the endoscopist's overall assessment of the colonoscopy^[8,19,35]. Harewood *et al*^[8] demonstrated that approximately 25% of colonoscopies examining bowel preparation and detection of colonic neoplasia were considered "inadequate," defined as "fair" or "poor". Standardized definitions for preparation quality were not provided to the endoscopists^[8]. Similar criteria for bowel assessment were applied in the study by Kazarian *et al*^[35] examining adequacy of bowel

preparation in urban populations. In our study, a significantly higher percentage of colonoscopies performed on methadone patients were assessed as "poor" compared to opioid naive patients. While the assessment of poor preparation was not independently associated with methadone patients, the results were significant under univariate analysis. This suggests that the significantly higher presence of solid stool in the methadone patients most likely contributed to the overall poor bowel assessment. Inter-observer variability in the assessment of the bowel preparation quality between endoscopists may also have contributed to the lack of significant results under multivariate analysis. Colonoscopy reports from multiple endoscopists were evaluated, which represents a limiting factor in our study. However, other studies on the quality of bowel preparations have also been limited by similar inter-observer bias due to multiple endoscopists^[7,8]. Further studies on methadone's effect on quality of bowel assessment based on a single endoscopist are needed to validate our results.

Due to the retrospective nature of our study, there were several other limitations. Information on the time elapsed between bowel preparation to procedure, and type of purgative ingested were not available. Therefore, bowel preparation may not have been standardized. Our study was also limited due to potential inter-observer bias since there were multiple endoscopists whose colonoscopy evaluations were utilized. This raises the concern that one endoscopist may have a more stringent assessment of the quality of bowel preparation than others. However, these limitations applied to both methadone and control groups equally, thereby reducing their confounding effects. Data on colonoscopic findings and need for therapeutic interventions were not available since it was outside the scope of our investigation into bowel visualization and preparation. We were also unable to apply the Ottawa^[56] or Boston Bowel Preparation^[57] Scales. These scales utilize a cumulative scoring system based on a combination of fluid quality and bowel location to determinate the quality of bowel preparation. While it is a validated system, the aims of our study were to examine the need for repeat colonoscopies and the presence of retained fecal matter instead of its location. Another limitation is the small sample size, including the number of controls, in our study. Although this is a preliminary study, the most likely explanation for the limited number of patients, specifically the controls, is due to the extensive exclusionary criteria applied. We excluded all individuals with any pathology that may affect GI motility, including many common diseases, such as diabetes and thyroid dysfunction, surgical interventions, and medications. We also excluded all partial colonoscopy reports that did not contain information on bowel assessment or those without images. Lastly, our study was performed at a single institution, thereby limiting a broad generalization of the results. Nevertheless, these limitations underscore the importance of the effects of methadone, and other opioids, on bowel visualiza-

tion. Currently, studies in this area are lacking. Further prospective studies in methadone and other opioids on colonoscopic outcomes are needed to validate the results of our preliminary study.

In conclusion, methadone dependent patients may require more repeat colonoscopies due to poor bowel visualization and retained stool. Poor bowel visualization may also be dose dependent. Methadone and other opioid use has risen drastically over the last decade in the United States, and an estimated 3% of all adults are currently on chronic opioid treatment^[10,14]. Considering the high numbers of colonoscopies performed annually in the United States, it is reasonable to conclude that clinicians will encounter a significant number of patients on chronic opioid therapy awaiting a colonoscopy. In fact, the average age for methadone dependent patients in our study was in the range where a screening colonoscopy should be performed on all individuals. Our results also suggest that a more aggressive approach to bowel preparation may be needed in methadone, or other opioid dependent patients. Current bowel cleansing practices may need to be revisited in these patients to not only improve the purgative effects, but also decrease the need for repeat colonoscopies. Recent investigations on peripherally acting μ -opioid receptor antagonists, such as methylnaltrexone or alvimopan^[38], have shown benefit in treating opioid induced constipation, and may aid in improved bowel preparations in these patients. The utility of these antagonists in the setting of pre-colonoscopy bowel preparation for opioid dependent patients are lacking and is an area of potential future research.

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COMMENTS

Background

While approximately 7 million colonoscopies are performed in the United States annually, over 20% of all colonoscopies fail due to suboptimal bowel preparation or poor colonic visualization. This has led to an estimated 12%-22% increase in long term costs associated with colonoscopy. Concurrently, opioid use has increased sharply over the last decade in the United States. It is estimated that 3% of all adults are currently opioid dependent.

Research frontiers

Opioids, such as methadone, both inhibit intestinal propulsive motor activity throughout the entire gastrointestinal (GI) tract and stimulate fluid and water absorption in the colon. These effects lead to constipation and fecal retention. Fecal retention can obscure proper bowel visualization, thus potentially resulting in missed pathologies, diagnostic delays and repeat procedures. Studies on colonoscopy outcomes and bowel visualization in methadone dependent patients are lacking. In this study, the authors suggest that chronic methadone usage is a risk factor for poor bowel visualization and preparation, thereby leading to more repeat colonoscopies. These effects appear to be dose dependent.

Innovations and breakthroughs

Studies on the relationship between opioids and colonoscopy outcomes are limited. A prior study demonstrated no association between narcotics use and inadequate bowel preparation. However, there were several limitations, including small number of patients on unspecified narcotics without information on

dosage and duration of narcotic use. This is the first and largest study to examine the effects of methadone dependence on bowel visualization and need for repeat colonoscopies. Furthermore, they examined whether there was a relationship between methadone dosage and colonoscopy outcomes.

Applications

By demonstrating that methadone is another risk factor for failure of colonoscopies, pre-colonoscopy bowel preparation methods may need to be re-evaluated in methadone dependent patients to improve bowel visualization and reduce the need for repeat colonoscopies. Mu-receptor antagonists used to treat opioid induced constipation may serve a role in pre-colonoscopy bowel preparation to improve visualization and reduced rates of repeat colonoscopies in methadone dependent patients.

Terminology

Methadone, a synthetic mu receptor agonist, is a commonly used opioid for analgesia in patients with malignancy, and intractable neuropathy. It is also standard therapy for the treatment of opioid addiction. In addition to slowing GI motility and increased fluid resorption in the colon, methadone pharmacokinetics has demonstrated a strong linear relationship between methadone dose and plasma concentrations.

Peer review

In this novel study, the authors retrospectively examined bowel visualization and preparation assessment, and need for repeat colonoscopies in a small cohort of methadone dependent patients. The study revealed that chronic methadone usage may be an additional risk factor for failure of colonoscopy due to poorer visualization and bowel preparation, and leads to a higher need for repeat colonoscopies. This association may also be dose dependent. Larger, prospective studies are needed to affirm these findings.

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Predictive value of symptoms and demographics in diagnosing malignancy or peptic stricture

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Abstract

AIM: To determine which features of history and demographics predict a diagnosis of malignancy or peptic stricture in patients presenting with dysphagia.

METHODS: A prospective case-control study of 2000 consecutive referrals (1031 female, age range: 17-103 years) to a rapid access service for dysphagia, based in a teaching hospital within the United Kingdom, over 7 years. The service consists of a nurse-led telephone triage followed by investigation (barium swallow or gastroscopy), if appropriate, within 2 wk. Logistic regression analysis of demographic and clinical variables was performed. This includes age, sex, duration of dysphagia, whether to liquids or solids, and whether there are associated features (reflux, odynophagia, weight loss, regurgitation). We determined odds ratio (OR) for these variables for the diagnoses of malignancy and peptic stricture. We determined the value of the Edinburgh Dysphagia Score (EDS) in predicting cancer in our cohort. Multivariate logistic regression

was performed and $P < 0.05$ considered significant. The local ethics committee confirmed ethics approval was not required (audit).

RESULTS: The commonest diagnosis is gastro-esophageal reflux disease (41.3%). Malignancy (11.0%) and peptic stricture (10.0%) were also relatively common. Malignancies were diagnosed by histology (97%) or on radiological criteria, either sequential barium swallows showing progression of disease or unequivocal evidence of malignancy on computed tomography. The majority of malignancies were esophago-gastric in origin but ear, nose and throat tumors, pancreatic cancer and extrinsic compression from lung or mediastinal metastatic cancer were also found. Malignancy was statistically more frequent in older patients (aged >73 years, OR 1.1-3.3, age < 60 years 6.5%, 60-73 years 11.2%, > 73 years 11.8%, $P < 0.05$), males (OR 2.2-4.8, males 14.5%, females 5.6%, $P < 0.0005$), short duration of dysphagia (≤ 8 wk, OR 4.5-20.7, 16.6%, 8-26 wk 14.5%, > 26 wk 2.5%, $P < 0.0005$), progressive symptoms (OR 1.3-2.6: progressive 14.8%, intermittent 9.3%, $P < 0.001$), with weight loss of ≥ 2 kg (OR 2.5-5.1, weight loss 22.1%, without weight loss 6.4%, $P < 0.0005$) and without reflux (OR 1.2-2.5, reflux 7.2%, no reflux 15.5%, $P < 0.0005$). The likelihood of malignancy was greater in those who described true dysphagia (food or drink sticking within 5 s of swallowing than those who did not (15.1% vs 5.2% respectively, $P < 0.001$). The sensitivity, specificity, positive predictive value and negative predictive value of the EDS were 98.4%, 9.3%, 11.8% and 98.0% respectively. Three patients with an EDS of 3 (high risk EDS ≥ 3.5) had malignancy. Unlike the original validation cohort, there was no difference in likelihood of malignancy based on level of dysphagia (pharyngeal level dysphagia 11.9% vs mid sternal or lower sternal dysphagia 12.4%). Peptic stricture was statistically more frequent in those with longer duration of symptoms (> 6 mo, OR 1.2-2.9, ≤ 8 wk 9.8%, 8-26 wk 10.6%, > 26 wk 15.7%, $P < 0.05$) and over

60 s (OR 1.2-3.0, age < 60 years 6.2%, 60-73 years 10.2%, > 73 years 10.6%, $P < 0.05$).

CONCLUSION: Malignancy and peptic stricture are frequent findings in those referred with dysphagia. The predictive value for associated features could help determine need for fast track investigation whilst reducing service pressures.

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Key words: Dysphagia; Deglutition disorders; Esophageal neoplasms; Esophageal stenosis; Gastroscopy; Barium swallow; Predictive value of tests

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Murray IA, Palmer J, Waters C, Dalton HR. Predictive value of symptoms and demographics in diagnosing malignancy or peptic stricture. *World J Gastroenterol* 2012; 18(32): 4357-4362 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i32/4357.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i32.4357>

INTRODUCTION

Esophageal cancer accounts for 3% of all cancer diagnoses in the United Kingdom (an annual incidence of nearly 8000) and has poor prognosis^[1-4]. The Department of Health in the United Kingdom has produced guidelines to identify patients with dyspepsia at higher risk of upper gastro-intestinal malignancy and requiring rapid referral and investigation^[5]. Dysphagia is an alarm symptom with a high predictive value for finding significant pathology [odds ratio (OR) 2.0-3.1 for malignancy in 3600 referrals to a rapid access upper gastro-intestinal cancer service]^[6].

However, dysphagia is common, occurring in 5%-8% of those over 50^[7]. It can be due to many different underlying conditions, including malignancy.

Many patients referred to secondary care with “dysphagia” do not actually have any swallowing difficulties^[8]. Despite it being a relatively good predictive symptom for cancer diagnosis, even in those with true dysphagia, less than 10% have cancer^[8]. Patients presenting with dysphagia require rapid assessment, diagnosis and treatment. An accurate diagnosis is dependent upon history and appropriate investigations, which may include barium swallow, gastroscopy or esophageal manometry^[9-13].

If it were possible to predict which patient demographics and symptoms were most highly predictive of serious pathology, especially malignancy or peptic stricture and which predicted a non-serious problem, it would allow health resources to be targeted towards rapid investigation in the high risk group. A scoring system, the Edinburgh Dysphagia Score (EDS) has been devised to

predict which patients require fast track investigations^[14].

The aim of our study is to identify which factors are strongest predictors of malignancy or peptic stricture in patients referred to a rapid access service with dysphagia. We use our data to validate the EDS on a larger patient cohort.

MATERIALS AND METHODS

The Royal Cornwall Hospital serves a largely rural population of 450 000, more than 99% of whom are white. The county is one of the poorest in the United Kingdom.

The dysphagia hotline (DHL) service consists of an initial telephone triage by our nurse endoscopist with barium swallow or endoscopy within one week. The radiology department hot report DHL barium swallow examinations and if the examination is abnormal, the patient is given diet Cola and metoclopramide and undergoes gastroscopy after 2 h^[15].

We prospectively collect data on patient demographics and final diagnosis following gastroscopy or barium swallow based on test results and clinical opinion. Duration of dysphagia, whether for both liquids and solids, and whether there are associated features (reflux, odynophagia, weight loss, regurgitation) are all prospectively recorded. Review of demographics, patient presentation and final diagnosis showed highly predictive variables for each major diagnosis, so these were formally analysed.

The EDS was determined for each patient (determined by scores for age, weight loss (> 3 kg), duration of symptoms, sex, location of dysphagia and presence of acid reflux)^[14].

Statistical analysis

Statistical analysis of the data was performed using IBM SPSS 19 software. The relationship between the variables and the diagnosis was explored using Pearson's χ^2 Independence test. The predictive value of each variable in diagnosing malignancy and in diagnosing peptic stricture was explored using logistic regression. In both analysis, a P value less than 0.05 was considered statistically significant.

The South West Regional Ethics committee determined that formal ethics approval was not required as the study fell within the category of audit.

RESULTS

From April 2004 to January 2011, 2000 patients were referred, 48.5% male, age range 17-103 years, mean 68.1 years (SD 14.1 years). Of these, 225 (11.2%) did not undergo investigation through the dysphagia hotline, mainly because they refused any investigations but also because we were unable to contact them by telephone, or they were admitted prior to test. Two patients' data could not be interpreted for clerical reasons. Three hundred and thirty-five patients (20% of those investigated) denied true dysphagia, defined as the feeling of food or

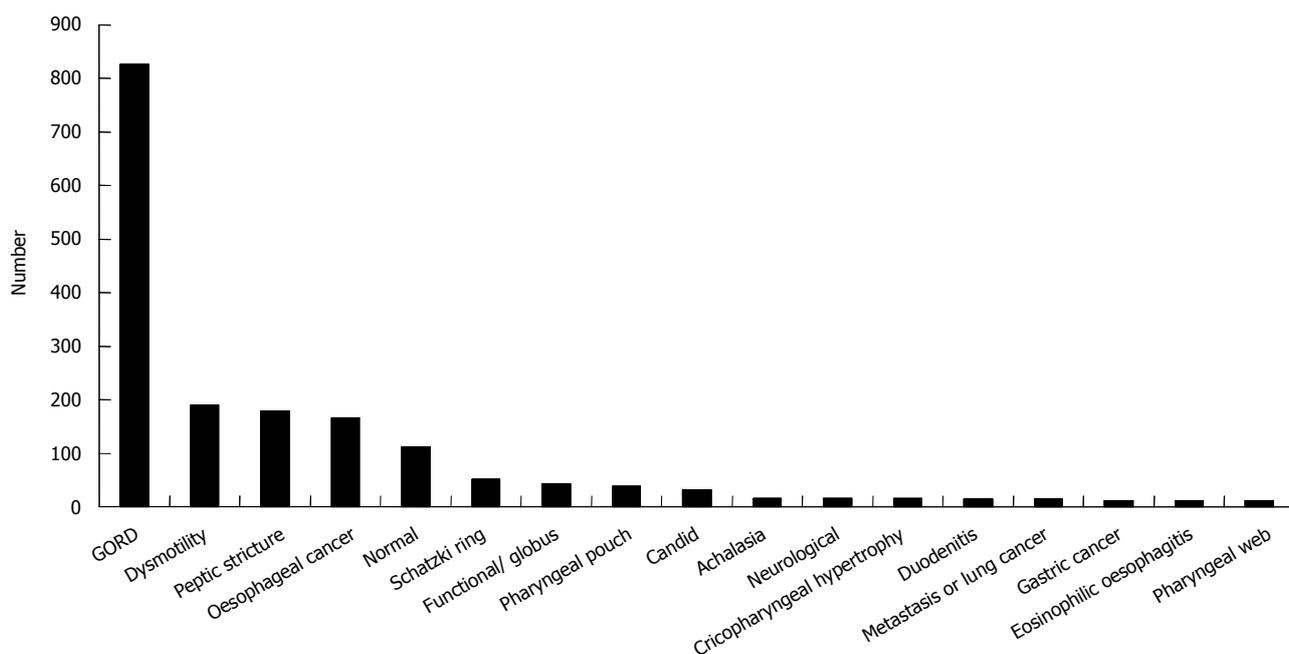


Figure 1 The outcome following investigation of 2000 consecutive patients referred with dysphagia. Some patients had more than one diagnosis and some had a diagnosis endoscopically which was unlikely to be the cause of the dysphagia. Only outcomes with an occurrence of 10 or more are shown. Other diagnoses with lower frequency were gastric (7), esophageal (5) and duodenal ulcers (4), compression from vascular structures including left atrium (4), pancreatic cancer (3), ear, nose and throat cancers (2), esophageal gastrointestinal stromal tumors (2), drug reaction (2), esophageal spasm (2) or diverticulum (2) and 1 each of respiratory infection, celiac disease, small intestinal stricture, goitre, post-operative stricture, pseudomembranous esophagitis, varices, pyloric ulcer, scleroderma, thyroglossal cyst and pharyngitis. Oesophageal cancer includes adenocarcinoma (98), squamous cancer (46), unspecified oesophageal cancers (4) and junctional cancers (18). GORD: Gastro-oesophageal reflux disease.

drink sticking within 5 s of swallowing, the majority describing globus although some presented with dyspepsia or weight loss.

Of those having investigations, 259 (14.6%) had barium swallow only, 1341 (75.5%) gastroscopy only and 175 (9.9%) had both procedures. Twenty patients failed to attend an appointment for procedures (7 barium swallows, 9 gastroscopies, the remainder unspecified) giving a DNA (did not attend) rate of 1.1% for patients referred for procedures.

The most common diagnosis was gastro-oesophageal reflux disease 826 (41.3%) with the more serious diagnoses of malignancy [199 (11.0%), 183 of gastrointestinal origin], peptic stricture 179 (10.0%), pharyngeal pouch 38 (2.1%) and achalasia 16 (0.9%). All outcomes are shown in Figure 1.

We investigated the likelihood of various significant pathologies based on patient demographics and presenting symptoms, including age (divided empirically into three similar sized groups of under 60 years, 60-73 years and over 73 years), sex, the type of dysphagia (to liquids, solids or both), whether the symptoms were progressive, whether “true dysphagia” or globus, and associated features including weight loss (defined as > 2 kg loss of weight in preceding 3 mo, the Department of Health criterion for a 2 wk wait referral), duration of dysphagia and presence or absence of reflux (percentages of each are shown on Table 1).

A diagnosis of malignancy or of peptic stricture was significantly associated with a history of true dysphagia

(feeling of food or drink sticking within 5 s of swallowing) than in those who did not ($P < 0.001$).

Logistic regression was performed using the Enter method to assess the impact of a number of factors on the likelihood of someone presenting with dysphagia having malignancy. The model contained seven independent variables (sex, age, duration of symptoms, nutrition, progressive symptoms, reflux and weight loss) however, the type of dysphagia was found to be highly non-significant ($P = 0.727$) and so the model was run again with removal of this variable.

The strongest predictor of a cancer diagnosis was the duration of symptoms (Table 2). If a patient reported symptom duration of less than eight weeks, the OR of having cancer was 9.6 higher than for symptoms more than twenty six weeks (95% CI: 4.5-20.7). The OR was 6 (95% CI: 2.8-12.8) for symptom duration between eight and twenty six weeks. The next strongest predictor was the presence of weight loss greater than 2 kg where the OR of 3.6 (95% CI: 2.5-5.1). Being male increased the likelihood of malignancy 3.3 fold (95% CI: 2.2-4.8) compared to females. Being less than sixty years old reduced the likelihood of a cancer diagnosis by 47.1% compared to being over 73 (OR 0.53, 95% CI: 0.3-0.9). If the patient had reflux, the OR of 0.54 (95% CI: 0.4-0.8) showed the likelihood of malignancy was significantly reduced and if symptoms were progressive then the OR of a cancer diagnosis was 1.8 (95% CI: 1.3-2.6). The sensitivity and specificity values are 97.6% and 31.1% respectively.

Table 1 Demographics and clinical features of the first 2000 patients referred to the dysphagia hotline (%)

Demographics			
Age	< 60 yr	60-73 yr	> 73 yr
	26.4	34.9	38.7
Sex	Male	Female	
	48.5	51.5	
Clinical features			
Nature of dysphagia	Solids	Liquids	Both
	79.6	0.7	19.7
Symptoms progressive	Yes	No	
	40	60	
"True" dysphagia	Dysphagia	Globus	
	81.3	18.7	
Weight loss \geq 2 kg in past 3 mo	Yes	No	
	28.5	71.5	
Presence of reflux	Yes	No	
	60.6	39.4	
Duration of dysphagia	< 8 wk	8-26 wk	> 26 wk
	34.5	42	23.5

Using an EDS $>$ 3.5 (10) to predict likelihood of cancer gave sensitivity, specificity, positive and negative predictive values of 98.4%, 9.3%, 11.8% and 98.0%. The three cancer cases that did not fall into the high risk group as defined in the original paper, had an EDS of 3.

Of patients with pharyngeal level dysphagia, 11.9% had malignancy, compared to 12.4% of patients with dysphagia affecting mid or lower chest ($P = \text{NS}$), i.e., in our cohort, the level of dysphagia was not significant for a diagnosis of malignancy.

Logistic regression was also used to assess the impact of a number of factors on the likelihood of someone presenting with dysphagia of having peptic stricture. The model originally contained seven independent variables. However five of these variables were not statistically significant and therefore were removed from the model [reflux ($P = 0.703$), sex ($P = 0.247$), progressive symptoms ($P = 0.196$), type of dysphagia ($P = 0.156$) and weight loss ($P = 0.069$). The only significant factors were age ($P = 0.012$) and duration of symptoms ($P = 0.008$)].

The OR of having a peptic stricture diagnosis is reduced by 48.9% for patients under 60 years old (OR 0.51, 95% CI: 0.33-0.80), and by 24.5% for patients between 60-73 years old (OR 0.76, 95% CI: 0.52-1.1) compared to those older than 73 years. The OR of having a peptic stricture diagnosis is reduced by 47.9% in patients presenting with symptoms of duration less than 8 wk (0.52, 95% CI: 0.34-0.81) and 37% in patients with symptoms of duration of between 8-26 wk (0.63, 95% CI: 0.43-0.92) compared to patients with symptom duration greater than 26 wk. The sensitivity and specificity values were 95.2% and 8.1% respectively.

In summary, in patients presenting with dysphagia, the likelihood of a diagnosis of cancer is increased by being male, over the age of 60 years, experiencing weight loss of $>$ 2 kg with progressive symptoms but without reflux and a symptom duration of less than eight weeks. Patients who have had their symptoms for greater than twenty six weeks and are over the age of 73

Table 2 Demographics and clinical features suggestive of malignancy in a dysphagic population

	Odds ratio	95% CI		Significance
		Lower	Upper	
Sex (male)	3.358	2.273	4.961	0.000
Progressive	1.807	1.259	2.593	0.001
Weight loss \geq 2 kg	3.572	2.501	5.104	0.000
Reflux	0.528	0.369	0.754	0.000
Age				
< 60 yr	0.529	0.320	0.872	0.013
60-73 yr	1.170	0.788	1.737	0.437
Duration				
< 8 wk	11.019	4.897	24.794	0.000
8-26 wk	6.936	3.124	15.398	0.000

Logistic regression analysis by the Enter method of 1400 patients investigated for dysphagia showing odds ratio with 95% CI and significance level compared to female patients, without progressive symptoms, without 2 kg or more weight loss, without reflux symptoms, aged over 73 years and with symptom duration of greater than 26 wk. The level of dysphagia (pharyngeal, mid chest or lower retrosternal) and the type of dysphagia (to liquids, solids or both) were removed as these were not statistically significant.

years are more likely to have a peptic stricture diagnosis than those presenting with the same symptoms that are younger and have symptoms for less than 26 wk.

DISCUSSION

For the past 7 years we have offered a telephone triage and one stop procedure service for dysphagia and have been referred 2000 patients. The commonest underlying cause for dysphagia is reflux disease but we have found malignancy in 10% of those referred and peptic stricture in 9%. We have prospectively collected data on patient demographics and symptoms at time of referral. Logistic regression analysis has enabled us to determine which symptoms and features make the diagnosis of malignancy or peptic stricture more likely.

Previous studies have found an incidence of cancer in 4%-15% of those referred with dysphagia^[6,16-19] making this an alarm symptom with a relatively high positive predictive value. We have confirmed this and also shown that in those referred to the DHL without dysphagia, the likelihood of malignancy is considerably lower.

Malignancy is more common in older men with a shorter duration of symptoms (less than 8 wk), with weight loss and without associated reflux. The negative association with reflux and positive association with weight loss has been noted previously^[8,14] as has the negative association with long duration of symptoms (greater than either 6 mo^[14] or 1 year^[8]). Because of the size of our cohort we have been able to demonstrate that those with particularly short duration of symptoms (less than 8 wk) have a markedly increased likelihood of malignancy (increased 11 fold) compared to those with symptoms from 8 wk to 6 mo (nearly 7 fold increase from those with symptoms of more than 6 mo).

Our findings are similar to those of Rhatigan *et al*^[14]

who have produced the “Edinburgh dysphagia score” to triage patients referred with dysphagia into low or high risk for malignancy. They have also found an increased likelihood of malignancy in patients who were male, older, with shorter duration of symptoms and have noted a negative association with symptoms of reflux. They found a significant relationship between type of dysphagia and the likelihood of cancer in univariate but not in multivariate analysis and hence they do not include it in their final calculation. In our study, the relationship was not statistically significant. Conventionally it is thought that dysphagia to solids is most likely due to organic obstruction whilst that to liquids is due to neuromuscular incoordination so it is interesting that both studies failed to demonstrate a significant relationship. It is possible in our own study that this was due to few patients having dysphagia to liquids alone.

There are several differences between their study and this one however. We failed to confirm a positive association of a malignant diagnosis with localisation of disease (in study of Rhatigan *et al.*^[14], dysphagia below the pharyngeal level was more likely to be associated with malignancy) or with progressive nature of symptoms (found to be an independent risk for malignancy but not included in their final scoring system). The reasons for this are not clear, although we did have 10 fold more patients with pharyngeal level dysphagia making it possible that the difference found previously had been a type 1 error due to a relatively small sample size. Alternatively there may be some unrecognised difference in referral patterns between the 2 hospitals.

We chose to group patients into three age ranges rather than investigating age as a continual variable but confirmed a strong association with older patients more likely to have malignancy and we chose 2 kg weight loss cut-off for weight loss as this is the weight chosen by the Department of Health in their guidelines for referral under the suspected cancer pathway. Only 8.4% of our patients fell into the low-risk group compared to 30.0% in study of Rhatigan *et al.*^[14]. This may have been because of the older age of our patients (mean age 68.1 years against 61.4 years in the original development cohort).

The high specificity and positive predictive value of the EDS was confirmed although again concerningly there were 3 patients who had malignancy but an EDS of less than 3.5. This figure is comparable to the study of Rhatigan *et al.*^[14] where the EDS failed to detect one malignancy in a cohort of 574 patients investigated, compared to 3 patients with malignancy in 1775 investigated. Clearly, whilst high risk patients with scores of 3.5 and above require urgent investigation, those with scores below this also require still to be investigated, albeit with a lower incidence of malignancy.

Further studies are required to determine whether the ORs are generalizable in other populations and in particular in non-whites. It would also be useful to record the effect of smoking and alcohol consumption on likelihood of both diagnoses.

As with malignancy, the likelihood of peptic stricture is greater in older patients but in contrast to a diagnosis of malignancy which is associated with a shorter duration of symptoms, a longer duration of symptoms (greater than 26 wk) is considered a feature of a peptic stricture diagnosis. No other clinical features were significantly associated with a diagnosis of peptic stricture. The associated with long duration of symptoms in older patients was recognized nearly 20 years ago but is worth re-iterating^[20-22].

Interestingly type of dysphagia (to solids rather than liquids), was not significant for neither malignancy nor peptic stricture. It is recognized that a history of dysphagia to solids progressing to both solids and liquids is indicative of mechanical obstruction whereas dysphagia to both at the onset is likely to be functional in origin^[23]. Relatively few of our patients had dysphagia to liquids only or both and we did not ask about the nature of the dysphagia at the onset which might explain this.

Likewise a history of reflux did not predict peptic stricture and appeared protective against a diagnosis of malignancy. Reflux is a known risk factor for esophageal adenocarcinoma and cardiac tumors (OR 7.7 and 2.0 respectively^[24]. In this and study of Rhatigan *et al.*^[14] it may simply have been more strongly associated with a final alternative diagnosis, namely reflux esophagitis^[21].

Future studies could focus on other factors which are recognized as risk factors for esophageal malignancy such as alcohol intake and smoking^[25-29] and this could improve the model of malignancy prediction.

We have prospectively obtained history from patients undergoing investigation for dysphagia and have demonstrated which factors are most likely to be indicative of malignancy or peptic stricture disease and hence which necessitate urgent investigation. We have confirmed the value of the EDS in recognising a smaller group of patients with dysphagia who require less urgent investigation.

COMMENTS

Background

Dysphagia can be the presenting symptom of a serious pathology, namely malignancy or peptic stricture. Determining which patients are more likely to have malignancy or stricture could help determine which patients need urgent investigation.

Research frontiers

A previous study from Scotland has shown malignancy to be more common in older males, with short duration of progressive symptoms, no reflux, weight loss and dysphagia not at the pharyngeal level and produced the Edinburgh Dysphagia Score (EDS).

Innovations and breakthroughs

The authors investigated 1775 patients with dysphagia and confirmed earlier findings malignancy to be more common in older men with progressive symptoms of less than 8 wk duration and weight loss of at least 2 kg. Malignancy was more common in those without reflux but level of dysphagia did not predict malignancy. An EDS of less than three predicted no malignancy. Peptic stricture was more common in older patients with longer duration of symptoms.

Applications

The authors confirmed the value of the EDS but caution that a score of 3 may still predict malignancy (contrary to the original article). Authors also defined

predictors of peptic stricture. Where resources are limited these predictors could be used to expedite investigations in high risk patients.

Peer review

It is larger scale cohort of validation study for the EDS than ever before. EDS is useful for primarily diagnosing esophageal malignancy associated with dysphagia.

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How many cases of laryngopharyngeal reflux suspected by laryngoscopy are gastroesophageal reflux disease-related?

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Abstract

AIM: To investigate the prevalence of gastroesophageal reflux disease (GERD) in patients with a laryngoscopic diagnosis of laryngopharyngeal reflux (LPR).

METHODS: Between May 2011 and October 2011, 41 consecutive patients with laryngopharyngeal symptoms (LPS) and laryngoscopic diagnosis of LPR were empirically treated with proton pump inhibitors (PPIs) for at least 8 wk, and the therapeutic outcome was assessed through validated questionnaires (GERD impact scale,

GIS; visual analogue scale, VAS). LPR diagnosis was performed by ear, nose and throat specialists using the reflux finding score (RFS) and reflux symptom index (RSI). After a 16-d wash-out from PPIs, all patients underwent an upper endoscopy, stationary esophageal manometry, 24-h multichannel intraluminal impedance and pH (MII-pH) esophageal monitoring. A positive correlation between LPR diagnosis and GERD was supposed based on the presence of esophagitis (ERD), pathological acid exposure time (AET) in the absence of esophageal erosions (NERD), and a positive correlation between symptoms and refluxes (hypersensitive esophagus, HE).

RESULTS: The male/female ratio was 0.52 (14/27), the mean age \pm SD was 51.5 ± 12.7 years, and the mean body mass index was 25.7 ± 3.4 kg/m². All subjects reported one or more LPS. Twenty-five out of 41 patients also had typical GERD symptoms (heartburn and/or regurgitation). The most frequent laryngoscopic findings were posterior laryngeal hyperemia (38/41), linear indentation in the medial edge of the vocal fold (31/41), vocal fold nodules (6/41) and diffuse infraglottic oedema (25/41). The GIS analysis showed that 10/41 patients reported symptom relief with PPI therapy ($P < 0.05$); conversely, 23/41 did not report any clinical improvement. At the same time, the VAS analysis showed a significant reduction in typical GERD symptoms after PPI therapy ($P < 0.001$). A significant reduction in LPS symptoms. On the other hand, such result was not recorded for LPS. Esophagitis was detected in 2/41 patients, and ineffective esophageal motility was found in 3/41 patients. The MII-pH analysis showed an abnormal AET in 5/41 patients (2 ERD and 3 NERD); 11/41 patients had a normal AET and a positive association between symptoms and refluxes (HE), and 25/41 patients had a normal AET and a negative association between symptoms and refluxes (no GERD patients). It is noteworthy that HE patients had a posi-

tive association with typical GERD-related symptoms. Gas refluxes were found more frequently in patients with globus (29.7 ± 3.6) and hoarseness (21.5 ± 7.4) than in patients with heartburn or regurgitation (7.8 ± 6.2). Gas refluxes were positively associated with extra-esophageal symptoms ($P < 0.05$). Overall, no differences were found among the three groups of patients in terms of the frequency of laryngeal signs. The proximal reflux was abnormal in patients with ERD/NERD only. The differences observed by means of MII-pH analysis among the three subgroups of patients (ERD/NERD, HE, no GERD) were not demonstrated with the RSI and RFS. Moreover, only the number of gas refluxes was found to have a significant association with the RFS ($P = 0.028$ and $P = 0.026$, nominal and numerical correlation, respectively).

CONCLUSION: MII-pH analysis confirmed GERD diagnosis in less than 40% of patients with previous diagnosis of LPR, most likely because of the low specificity of the laryngoscopic findings.

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Key words: Laryngopharyngeal reflux; Gastroesophageal reflux; Multichannel impedance and pH monitoring; Extra-esophageal reflux syndromes; Chronic laryngitis

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is one of the most common gastrointestinal disorders in Western countries^[1]. The manifestations of GERD have been recently classified into either esophageal or extra-esophageal syndromes (EES)^[1]. Among the latter, Vakil *et al*^[1] have included the atypical manifestations of GERD such as chronic cough and laryngopharyngeal symptoms (LPS) (i.e., laryngitis, globus, throat discomfort), which are increasingly recognised by general physicians, lung specialists and ear, nose and throat (ENT) surgeons^[2,3]. In particular, there is a large number of data on the growing prevalence of LPS in GERD patients^[4-6].

Despite the recognition that GERD can provoke laryngeal symptoms, the diagnosis of laryngopharyngeal reflux (LPR) remains a very difficult task. Initially, pa-

tients with laryngeal symptoms undergo a laryngoscopy and a chest X-ray to rule out malignancies. Once cancer is excluded, a diagnosis of LPR is suspected. The diagnosis of LPR is usually performed by ENT surgeons in case of detection of the following laryngoscopic findings: erythema, oedema, ventricular obliteration, post-cricoid hyperplasia and pseudosulcus^[7]. However, these laryngoscopic findings are also common in healthy volunteers, and this largely limits their diagnostic value^[7]. Moreover, there are several controversies regarding how to confirm LPR diagnosis and, more generally, EES diagnosis. Upper gastrointestinal endoscopy has been demonstrated to have low sensitivity^[8,9], the proton pump inhibitor test has been shown to have low specificity^[10], and radiologic studies have limited sensitivity and specificity^[8]. Moreover, the sensitivity and specificity of ambulatory pH monitoring as a means for diagnosing reflux in patients with extra-esophageal GERD symptoms have been challenged^[1]. Recently, the availability of multichannel intraluminal impedance and pH monitoring (MII-pH) has modified the diagnostic approach towards atypical manifestations of GERD^[11-14]. MII-pH is able to detect not only acid but also non-acid reflux and proximal migration of the refluxate and can correlate symptoms with both types of reflux^[15-17]; additionally, there is a rising consensus that this technique should be considered as the gold standard for GERD diagnosis^[18]. At present, few data are available on the prevalence of LPR in patients with or without GERD symptoms and on the characteristics of overall reflux episodes in those patients.

The aim of this study was to evaluate the prevalence of GERD in patients with a recent laryngoscopic diagnosis of LPR by means of MII-pH. The second endpoint was to assess the effectiveness of an empirical treatment with proton pump inhibitors (PPIs) in patients with both GERD-related and non-GERD-related LPR.

MATERIALS AND METHODS

Study subjects

Between May 2011 and October 2011, 41 consecutive patients with LPS and an ENT diagnosis of LPR were prospectively enrolled in the study. During the first visit, a distinct investigator completed a structured interview on the patients, recording a careful medical history (with recording of height and weight), current medications, tobacco use and alcohol consumption. All patients signed a written informed consent form before entering into the study. The study was designed and carried out in accordance with the Helsinki Declaration (Sixth revision, Seul 2008).

Inclusion criteria were as follows: LPS for at least three consecutive months during the last year, previous history of dysphonia, cough, hoarseness, throat globe and/or dysphagia and an ENT diagnosis of LPR. In particular, such a diagnosis was performed after an accurate phoniatric and otorhinolaryngoiatric anamnesis and a general ENT examination with a flexible rhino-pharyn-

go-laryngoscope with an optical fibre. The reflux findings score (RFS) was completed by the otolaryngologist (RFS > 7), suggestive value for LPR), and all patients were asked to complete the reflux symptom index (RSI) (RSI > 13, suggestive value for LPR)^[19]. Patients with a RFS > 7 and a RSI > 13 were considered affected by LPR.

Exclusion criteria were as follows: Previous surgery in the upper digestive tract, pregnancy and/or breastfeeding, eating disorders with vomiting, underlying psychiatric illness, use of non-steroidal anti-inflammatory drugs and aspirin, and peptic ulcer at a previous endoscopy.

All the enrolled patients were allowed an empirical treatment with PPIs for at least 8 wk, and the therapeutic outcome was recorded through a validated questionnaire (GERD impact scale, GIS), which was completed before and after therapy^[20]. The GIS comprises eight questions about the frequency, over the previous 2 wk, of the following items: acid-related symptoms; chest pain; extra-esophageal symptoms; impact of symptoms on sleep, work, meals and social occasions; and the use of additional non-prescription medications. A 4-point rating Likert scale was used to describe the frequency of the symptoms over the previous 2 wk: 0 = none (absence of symptoms), 1 = mild (symptoms present for a little of the time), 2 = moderate (symptoms present for some of the time), and 3 = severe (symptoms present all of the time). Patients who responded with a score of 2 or 3 were considered as non-responders to PPI therapy.

The patients were also asked to rate their satisfaction with the symptom control on a global visual analogue scale (VAS) from 0 (no relief at all) to 10 (complete symptom relief). The VAS score has been used as a self-assessment tool for symptom measurement, which has been used in many other trials for evaluation of ENT symptoms and typical and atypical GERD symptoms^[21,22].

After 8 wk of PPI therapy, all patients underwent upper endoscopy, stationary esophageal manometry and 24-h MII-pH esophageal monitoring. All patients discontinued PPI therapy at least 16 d before undergoing the planned esophageal investigations. The patients were only allowed to take alginates, on an as-needed basis, as rescue therapy. During upper gastrointestinal endoscopy, biopsies were taken from the gastric antrum and corpus to assess the presence of *Helicobacter pylori* and atrophic gastritis. Stationary manometry and MII-pH were performed after an overnight fast.

Stationary esophageal manometry

All subjects underwent stationary esophageal manometry to determine the distance of the proximal border of the lower esophageal sphincter (LES) from the nostrils and to evaluate the esophageal peristaltic wave. This study was performed by means of an eight-channel water-perfused manometric catheter with an external diameter of 4.5 mm (Dyno 2000[®] Menfis, BioMedica, Bologna Italy), equipped with computer-based data recording and storage. Esophageal body motility and LES relaxation

were tested by at least 10 wet swallows of 5 mL of water. Wave amplitude and duration were measured by means of four openings located at 5, 10, 15 and 20 cm above the LES. A stationary pull-through technique was then used to accurately locate the position of the LES.

Esophageal MII-pH

MII-pH was performed using a polyvinyl catheter (diameter: 2.3 mm), equipped with an antimony pH electrode and several cylindrical electrodes, with a length of 4 mm, placed at intervals of approximately 2 cm (Sandhill Scientific Inc., Highland Ranch, CO). Each pair of adjacent electrodes represented an impedance-measuring segment corresponding to one recording channel. The single-use MII-pH catheter was positioned with the pH electrode 5 cm above the LES and the six impedance recording channels positioned at 3, 5, 7, 9, 15 and 17 cm above the LES.

The methodology of probe calibration, catheter placement, patient instruction and performance has been previously described^[23].

MII-pH data analysis

At the end of the recording period, MII-pH tracings were reviewed manually to ensure accurate detection and classification of reflux episodes. Meal periods were excluded from the analysis. Impedance and pH data were used to determine the number and type of reflux episodes as well as the acid exposure time (AET) (reflux percent time) in each patient. In particular, the distal esophageal AET was defined as the total time with a pH measurement below 4 divided by the total time of monitoring. A percent time lower than 4.2% with pH < 4, over 24-h, was considered normal^[23,24]. Reflux events were characterised according to previously reported criteria^[25]. Total reflux number, esophageal AET and correlation between symptoms and reflux using the symptom index (SI) and symptom association probability (SAP) were evaluated for each patient as previously described^[26]. The symptoms were considered to be related to reflux if they occurred within a 2-min time window after the onset of the reflux episode^[27]. For symptom analysis, weakly acidic and weakly alkaline refluxes were pooled as non-acid reflux episodes (nadir pH > 4).

Statistical analysis

MII-pH data were matched with the ENT diagnosis. Statistical analysis was performed with the Chi-squared test and the Fisher exact test to evaluate nominal values, and Pearson's correlation was performed to explore numerical values. The results were considered statistically significant for *P* values < 0.05.

RESULTS

Demographic and clinical characteristics

The study evaluated 14 males and 27 females (M/F ratio 0.52), with a mean age \pm SD of 51.5 \pm 12.7 years and a

Table 1 Results of the gastroesophageal reflux disease impact scale questionnaire before and after proton pump inhibitor therapy

How often have you had the following symptoms: (GIS questionnaire)	Before PPI therapy				After PPI therapy				P value
	Always	Often	Sometimes	Never	Always	Often	Sometimes	Never	
Pain in your chest or behind the breastbone?	1	0	0	0	0	0	1	0	NS
Burning sensation in your chest or behind the breastbone?	10	4	1	0	3	1	1	10	< 0.05
Regurgitation or acid taste in your mouth?	2	5	2	0	1	1	3	4	< 0.05
Pain or burning in your upper stomach?	1	2	2	0	0	1	1	3	< 0.05
Sore throat or hoarseness that is related to your heartburn or acid reflux?	27	5	9	0	23	8	6	4	NS

NS: Not statistically significant; GIS: Gastroesophageal reflux disease impact scale; PPI: Proton pump inhibitor.

Table 2 Results of the visual analytic scale

Symptoms	Pre-PPI	Post-PPI	P value
Chest pain	7.1 ± 2.4	3.3 ± 0.9	0.0001 ¹
Heartburn	8.5 ± 3.2	2.3 ± 1.1	0.0001 ¹
Regurgitation	6.8 ± 1.5	4.1 ± 1.9	0.0001 ¹
Epigastric pain	5.9 ± 3.6	3.7 ± 2.4	0.0021
Hoarseness	7.4 ± 2.2	6.8 ± 2.7	0.273
Globus	9.3 ± 3.8	7.9 ± 3.5	0.087
Cough	7.9 ± 2.6	6.8 ± 2.8	0.069
Throat discomfort	8.1 ± 3.4	6.9 ± 2.1	0.058
Dysphonia	6.5 ± 2.1	5.5 ± 3.5	0.121

¹Statistically significant differences. PPI: Proton pump inhibitor.

mean body mass index of 25.7 ± 3.4 kg/m². Eight patients out of 41 (19.5%) were current smokers (5-10 cigarettes/d); 11/41 (28.8%) reported 2 to 3 units of alcohol consumption per day, and 33/41 (73.3%) drank two cups of coffee daily.

Symptoms

All subjects reported one or more LPS, and 25/41 patients also had typical GERD symptoms (heartburn and/or regurgitation). In particular, they described the predominant symptom (the most troublesome/frequent symptom during the day) and the overall most frequent symptoms in the last 6 mo. The predominant symptoms were globus 13 (31.7%), heartburn 10 (24.4%), hoarseness 9 (22%), sore throat 6 (14.6%), regurgitation 2 (4.9%) and epigastric pain 1 (2.4%). The overall most frequent symptoms were globus 21 (51.2%), heartburn 15 (36.6%), hoarseness 14 (34.1%), sore throat 13 (31.7%), regurgitation 9 (22%), dysphonia 9 (22%), belch 7 (17.1%), epigastric pain 5 (12.2%), and chronic cough 3 (7.3%).

The prevalence of symptom relief after PPI therapy, evaluated with the GIS questionnaire, showed that 10/41 (24.4%) patients reported at least one typical GERD symptom with “well controlled symptoms” (0) and that 23/41 (56.1%) patients reported only LPS without any symptom relief. All the details regarding the prevalence of symptom relief are described in Table 1.

The VAS analysis showed a significant reduction in typical GERD symptoms after PPI therapy. This reduction was not recorded for LPS. All details are presented in Table 2.

Upper gastrointestinal endoscopy

Upper endoscopy showed esophagitis (ERD) in 2/41 (4.9%) patients. None of the patients were diagnosed with complications of GERD (i.e., Barrett's esophagus, stenosis, adenocarcinoma). No other lesion or mucosal abnormality was detected during the examination.

Endoscopic hiatal hernia was found in 17/41 (41.5%) patients. With regard to the histological findings of the corpus and antrum biopsies, 4 out of 41 (9.75%) patients had *Helicobacter pylori* infection, and no one had atrophic gastritis or intestinal metaplasia.

Pathophysiological esophageal investigations

Two out of 41 (4.9%) patients presented with ineffective esophageal motility at the stationary manometry. Thirty-nine out of 41 (95.1%) patients did not present with abnormal esophageal motility.

The MII-pH analysis showed an abnormal AET in 5/41 (12.2%) patients [2 ERD and 3 non erosive esophagitis (NERD)]; 11/41 (26.8%) patients had a normal AET and a positive SAP (hypersensitive esophagus, HE), and; 25/41 patients had a normal AET and a negative association between symptoms and refluxes (no GERD patients). HE patients presented with a positive SAP for typical GERD-related symptoms (7 heartburn and 4 regurgitation).

The percentage of proximal reflux was abnormal (up more than 33%) in 4 cases with ERD/NERD (9.8%).

Gas refluxes were found more frequently in patients with globus (29.7 ± 3.6) and hoarseness (21.5 ± 7.4) than in patients with heartburn or regurgitation (7.8 ± 6.2). The SAP analysis for gas refluxes was positive for extra-esophageal symptoms.

Laryngoscopic examination

The most frequent laryngoscopic findings in our selected patients, classified by our MII-pH results, are shown in Table 3. Overall, no differences were found among the three groups of patients in terms of the frequency of the laryngeal signs. In particular, both ERD and NERD patients did not show severe findings of laryngeal disease.

The differences observed among the three subgroups of patients (ERD/NERD, HE, no GERD) with esophageal pathophysiological analysis (MII-pH) were not demonstrated with the ENT symptom questionnaire (RSI) or with the laryngoscopic findings (RFS), as shown in Table 4.

Table 3 Laryngoscopic findings with the reflux finding score in 41 patients with suspected laryngopharyngeal reflux, classified using multichannel intraluminal impedance and pH monitoring

Laryngoscopic findings	Ordinal scale	ERD/NERD (5)	HE (11)	No GERD ¹ (25)	P value
Infraglottic oedema (pseudosulcus)	0 = Absent	4	10	23	0.592
	2 = Present	1	1	2	
Ventricular obliteration	0 = None	4	8	21	0.553
	2 = Partial	1	3	4	
	4 = Complete	0	0	0	
Erythema/hyperemia	0 = None	0	0	1	0.474
	2 = Arytenoids only	2	6	15	
	4 = Diffuse	3	5	9	
Vocal fold oedema	0 = None	3	7	19	0.375
	1 = Mild	2	1	4	
	2 = Moderate	0	3	2	
	3 = Severe	0	0	0	
Diffuse laryngeal oedema	0 = None	0	4	10	0.271
	1 = Mild	1	4	7	
	2 = Moderate	2	3	5	
	3 = Severe	2	0	3	
Posterior commissure hypertrophy	0 = None	0	0	0	0.763
	1 = Mild	2	5	12	
	2 = Moderate	1	5	10	
	3 = Severe	2	1	3	
Granuloma/granulation	0 = Absent	5	10	24	0.876
	2 = Present	0	1	1	
	4 = Obstructing	0	0	0	
Thick endolaryngeal mucus	0 = Absent	2	7	12	0.909
	2 = Present	3	4	10	

¹Patients with normal acid exposure time and without correlation between symptoms and refluxes. ERD: Erosive esophagitis; NERD: Non erosive esophagitis; HE: Hypersensitive esophagus.

Table 4 Correlation between multichannel intraluminal impedance and pH analysis and the reflux finding score/ reflux symptom index analysis

	ERD/NERD	HE	No GERD ¹	P value
AET (%)	7.4 ± 3.2	3.5 ± 1.7	1.9 ± 0.8	< 0.05
Reflux number (n)	103.2 ± 12.1	44.7 ± 6.2	35.1 ± 7.4	< 0.05
Proximal refluxes (mean %)	31	29	18	< 0.05
Acid refluxes (n)	62.5 ± 15.4	32.9 ± 5.1	19.7 ± 6.2	< 0.05
Non-acid refluxes (n)	40.1 ± 7.6	13.1 ± 4.4	15.8 ± 4.9	< 0.05
Gas refluxes (n)	11.6 ± 9.7	13.1 ± 8.1	21.7 ± 15.3	< 0.05
SAP/SI	Positive	Positive	Negative	-
RFS	10.9 ± 3.3	9.1 ± 2.7	7.6 ± 3.1	NS
RSI	14.3 ± 5.2	16.3 ± 4.7	15.8 ± 4.9	NS

¹Patients with normal acid exposure time and without correlation between symptoms and refluxes. AET: Acid exposure time; ERD: Erosive esophagitis; NERD: Non erosive esophagitis; HE: Hypersensitive esophagus. SAP/SI: Symptom association probability/symptom index; RFS: Reflux finding score; RSI: Reflux symptom index; NS: Not statistically significant.

A nominal (categorical) correlation (pathological vs non pathological) was performed considering endoscopic and esophageal pathophysiological examinations (results of endoscopy, MII-pH, AET value, total number of reflux events, number of proximal refluxes, gas refluxes, SAP). No match results were statistically significant. Only the number of gas refluxes was associated with the RFS ($P = 0.028$). The numerical correlation showed the same results: the correlation between the RFS and gas refluxes

Table 5 Results of nominal and numerical correlation

	Nominal correlation			Numerical correlation	
	RFS	RSI	RGE	RFS	RSI
AET (%)	NS	NS	$P < 0.001$	NS	NS
Total reflux number	NS	NS	$P < 0.001$	NS	NS
Acid reflux number	NS	NS	$P < 0.05$	NS	NS
Non-acid reflux number	NS	NS	$P < 0.05$	NS	NS
Proximal reflux number	NS	NS	$P < 0.04$	NS	NS
SAP	NS	NS	$P < 0.009$	NS	NS
Gas refluxes	$P = 0.028$	NS	NS	$P = 0.026$	NS
Upper endoscopy	NS	$P < 0.001$	$P = 0.009$	NS	NS
MII-pH (diagnosis)	NS	NS	$P < 0.001$	NS	NS

RFS: Reflux finding score; RSI: Reflux symptom index; RGE: Gastroesophageal reflux diagnosis; AET: Acid exposure time; NS: Not statistically significant; MII-pH: Multichannel intraluminal impedance and pH; SAP: Symptom association probability.

was confirmed ($P = 0.026$). All detailed results are shown in Table 5.

DISCUSSION

GERD is considered an important cause of laryngeal inflammation^[28]. The most common symptoms of this condition, termed LPS by ENT physicians, include hoarseness, throat pain, sensation of a lump in the throat, cough and repetitive throat clearing. However, these symptoms

are nonspecific and can also be seen in other diseases such as post-nasal drip syndrome or environmental exposure to allergens and other irritants^[29]. Lundell *et al*^[30] showed that acid is an uncommon cause of LPS in the absence of typical reflux symptoms or endoscopic features of reflux esophagitis. A similar finding was demonstrated in a more recent study by Ang *et al*^[31] where 14% of patients investigated for suspected EES showed an abnormal AET, suggesting that acid and non-acid refluxes do not play different roles in the genesis of extra-esophageal symptoms. Likewise, signs of laryngeal inflammation (i.e., hyperemia, oedema) are not specific to GERD. In 2007, Vavricka *et al*^[32] evaluated the prevalence of specific laryngopharyngeal changes thought to be GERD-related in patients with known reflux disease ($n = 132$) *vs* normal subjects ($n = 132$). Ten specific hypopharyngeal and laryngeal sites were evaluated: the posterior pharyngeal wall, the interarytenoid bar, the posterior commissure, the posterior cricoid wall, the arytenoids complex, the true vocal folds, the false vocal folds, the anterior commissure, the epiglottis and the aryepiglottic fold. Investigators found that the prevalence of laryngeal lesions was the same in both groups. Moreover, most signs identified in patients suspected of having LPR were also present in healthy subjects without any symptoms^[33]. Milstein *et al*^[34] performed a laryngoscopic evaluation of 52 non-smoking volunteers without any history of ENT disease or GERD-related symptoms and observed the presence of one or more signs of tissue irritation in 93% of the subjects. Laryngoscopic or laryngostroboscopic examinations are determinant for excluding laryngeal nodules or neoplastic lesions but are not specific for diagnosing LPR^[35]. Thus, in keeping with these considerations, the utility of laryngoscopy in detecting GERD-associated laryngitis remains uncertain^[33,34]. The use of MII-pH technology has provided new insights into the complex pathogenesis underlying atypical GERD symptoms. Based on our findings, LPS are not always due to GERD and RFS. Although RFS is a useful score for ENT, it is not able to accurately identify patients with LPR due to GERD. Nevertheless, in clinical practice, GERD is often considered as the underlying cause of laryngeal symptoms even in those patients who have a negative MII-pH or in those undergoing twice daily PPI therapy without any efficacy. At present, different causes that might be involved in the genesis of GERD-unrelated LPS are not known, highlighting the need for future studies in this field. We should focus our efforts on searching for these other causes; reflux might be the easy answer, but we must look for difficult answers when logic suggests that direction. Chronic laryngitis is a heterogeneous disease, and GERD may be just one of the causes or an aggravating factor. Patients with and without troublesome reflux symptoms may have different pathophysiological mechanisms and may therefore require different therapies.

Notably, one study demonstrated that gas refluxes with weak acidity were more common in patients with reflux-attributed laryngitis compared to GERD patients and controls^[36]. In keeping with this finding, our results

showed that the only characteristic of refluxes associated with LPR was the presence of gas refluxes. The mechanisms by which gas refluxes may develop into LPS are far from being clarified. It has been hypothesised that gas refluxes carry aerosolised droplets containing hydrogen and pepsin that are able to generate troublesome symptoms into the proximal esophagus and pharyngeal/laryngeal mucosa. Indeed, microaspiration of acid aerosolised droplets is considered one of the most important mechanisms for laryngeal inflammation. Hydrochloric acid vaporises easily and can result in a concentrated cloud of acidic vapour entering the airways^[37].

An increasing number of studies are using the presence of pepsin in clinical samples as a marker for gastroesophageal reflux because it is produced exclusively by the stomach. Indeed, reflux has been documented by detection of pepsin in the trachea, lung, sinus, middle ear, combined sputum and saliva, and breath condensate. Of note, pepsin is stable up to pH 7 and regains activity after reacidification^[38]. In this regard, two recent review articles have highlighted that an immunologic pepsin assay is a rapid, sensitive, and specific tool for correlation of reflux with airway disease and is a reliable diagnostic marker of EES^[39,40]. In particular, extra-esophageal reflux can now be detected by recognising pharyngeal acidification using a miniaturised pH probe and by the non-invasive identification of pepsin in saliva and in exhaled breath condensate using the pepsin immunoassay^[40].

Recently, a new technology able to detect aerosols of acid and gaseous clouds of acid has been described: the Dx-pH measurement system (Dx-pH) (Respiratory Technology Corp., San Diego, CA). Dx-pH is a highly sensitive and minimally invasive device for the detection of acid reflux in the posterior oropharynx. It uses a nasopharyngeal catheter with a sensor that is able to measure pH in either liquid or aerosolised droplets^[41]. A number of preliminary studies have suggested that this technique may have a role in identifying patients with extra-esophageal symptoms caused by reflux disease^[40].

PPI therapy is considered to be the standard of care in patients with LPS when GERD is the underlying suspected aetiology. In clinical practice, it is believed that patients with reflux-related laryngitis require more aggressive and prolonged PPI treatments to achieve an improvement of laryngeal symptoms than those with typical GERD symptoms^[42]. Conversely, several placebo-controlled trials and meta-analyses have failed to demonstrate any therapeutic benefit of PPIs^[43-47]. Some studies have shown that the proportion of patients with marked improvement in laryngeal symptoms after PPI therapy is higher in GERD patients than in those without GERD^[48,49]. On the other hand, the most recent multicenter study, with 145 patients suspected of having LPR, did not show any benefit in patients treated with esomeprazole 40 mg *bid* for 4 mo *vs* placebo^[43].

In the present study, patients with typical GERD symptoms and an abnormal AET had increased symptom relief after PPI therapy. Atypical/extra-esophageal

GERD-suspected symptoms are less responsive to antisecretive therapy.

In conclusion, current knowledge on LPR diagnosis and management needs to be expanded with new diagnostic techniques to better understand the underlying pathophysiological mechanisms. In this respect, the present study underscores the importance of MII-pH monitoring to assess the presence of an established association between GERD and suspected LPR.

COMMENTS

Background

Laryngopharyngeal reflux is defined as the reflux of gastric contents into the larynx and pharynx, and it is the most extensively investigated extra-esophageal syndrome with an established association with gastroesophageal reflux disease (GERD). It may be manifested as laryngeal symptoms as well as laryngoscopic findings. However, laryngoscopic findings are not specific, and this largely limits their diagnostic value. Moreover, there are currently several controversies regarding accurate confirmation of such a diagnosis.

Research frontiers

In the area of chronic laryngitis, the research hotspot is how to diagnose and manage laryngopharyngeal reflux (LPR). In particular, new diagnostic techniques to better understand the underlying pathophysiological mechanisms are necessary. Indeed, GERD may represent just one of the causes or an aggravating factor of laryngopharyngeal symptoms (LPS).

Innovations and breakthroughs

The use of esophageal multichannel impedance and pH technology has provided new insights into the complex pathogenesis underlying atypical reflux symptoms. In clinical practice, GERD is often considered to be the underlying cause of laryngeal symptoms, even in those patients who have a negative impedance and pH study or in those undergoing twice daily proton pump inhibitor therapy without any efficacy. In the present study, LPS were not always due to GERD, and laryngoscopic findings were not able to accurately identify patients with LPR due to GERD. Based on the findings, the only characteristic of refluxes associated with LPR was the presence of gas refluxes, although the mechanisms by which gas refluxes may contribute to LPR are far from being clarified. Overall, patients with typical reflux symptoms and abnormal acid exposure time had increased symptom relief after proton pump inhibitor therapy. Conversely, extra-esophageal reflux-suspected symptoms were less responsive to antisecretive therapy.

Applications

The present study underscores the importance of impedance and pH monitoring to assess the presence of an established association between GERD and suspected LPR.

Terminology

Extra-esophageal syndromes: The manifestations of GERD have been recently classified into either esophageal or extra-esophageal syndromes. Among the latter, the atypical manifestations of GERD such as chronic cough and LPS (i.e., laryngitis, globus, throat discomfort) have been included; Laryngopharyngeal reflux: Laryngopharyngeal reflux is a condition with an established association with GERD and is defined as the reflux of gastric contents into the larynx and pharynx; Multichannel intraluminal impedance and pH monitoring: This is a technique that is able to detect both acid and non-acid reflux and proximal migration of the refluxate, to physically characterise the refluxate (i.e., liquid, gas, mixed), and to correlate symptoms with each type of reflux.

Peer review

This is an interesting and well-structured study aimed to evaluate the diagnostic capacity of laryngoscopic findings suspected to be related to GERD, as performed by ear, nose and throat physicians. The LPR definition is based on the symptoms, although the criteria for LPR symptoms have not been established by many papers. The entry number is relatively small. The authors use multichannel intraluminal impedance and pH monitoring and many questionnaires as the diagnostic gold standard. Of note, they found that laryngoscopic findings had a poor sensitivity and were not related to the multichannel intraluminal impedance and pH results. From their data, the authors suggested that another reason for LPR besides acid reflux was gas reflux.

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Alginate controls heartburn in patients with erosive and nonerosive reflux disease

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Abstract

AIM: To evaluate the effect of a novel alginate-based compound, Faringel, in modifying reflux characteristics and controlling symptoms.

METHODS: In this prospective, open-label study, 40 patients reporting heartburn and regurgitation with proven reflux disease (i.e., positive impedance-pH test/evidence of erosive esophagitis at upper endoscopy) underwent 2 h impedance-pH testing after eating a refluxogenic meal. They were studied for 1 h under basal conditions and 1 h after taking 10 mL Faringel. In both sessions, measurements were obtained in right lateral and supine decubitus positions. Patients also com-

pleted a validated questionnaire consisting of a 2-item 5-point (0-4) Likert scale and a 10-cm visual analogue scale (VAS) in order to evaluate the efficacy of Faringel in symptom relief. Tolerability of the treatment was assessed using a 6-point Likert scale ranging from very good (1) to very poor (6).

RESULTS: Faringel decreased significantly ($P < 0.001$), in both the right lateral and supine decubitus positions, esophageal acid exposure time [median 10 (25th-75th percentil 6-16) *vs* 5.8 (4-10) and 16 (11-19) *vs* 7.5 (5-11), respectively] and acid refluxes [5 (3-8) *vs* 1 (1-1) and 6 (4-8) *vs* 2 (1-2), respectively], but increased significantly ($P < 0.01$) the number of nonacid reflux events compared with baseline [2 (1-3) *vs* 3 (2-5) and 3 (2-4) *vs* 6 (3-8), respectively]. Percentage of proximal migration decreased in both decubitus positions (60% *vs* 32% and 64% *vs* 35%, respectively; $P < 0.001$). Faringel was significantly effective in controlling heartburn, based on both the Likert scale [3.1 (range 1-4) *vs* 0.9 (0-2); $P < 0.001$] and VAS score [7.1 (3-9.8) *vs* 2 (0.1-4.8); $P < 0.001$], but it had less success against regurgitation, based on both the Likert scale [2.6 (1-4) *vs* 2.2 (1-4); $P =$ not significant (NS)] and VAS score [5.6 (2-9.6) *vs* 3.9 (1-8.8); $P =$ NS]. Overall, the tolerability of Faringel was very good 5 (2-6), with only two patients reporting modest adverse events (i.e., nausea and bloating).

CONCLUSION: Our findings demonstrate that Faringel is well-tolerated and effective in reducing heartburn by modifying esophageal acid exposure time, number of acid refluxes and their proximal migration.

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Key words: Impedance pH-metry; Nonerosive reflux disease; Erosive esophagitis; Nonacid reflux; Proximal reflux

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is a common problem affecting about 20% of the population in western countries^[1]. Nonerosive reflux disease (NERD) and erosive reflux disease (ERD) represent the most common phenotypic presentations of GERD, accounting for 90%-95% of the overall GERD patients^[2]. Previous studies have documented that patients with ERD and NERD present the same clinical picture in terms of frequency and severity of reflux symptoms^[2,3].

To date, the use of proton pump inhibitors (PPIs) has been considered the best therapeutic option for GERD patients, given their high efficacy in determining symptom relief and in inducing esophageal mucosal healing^[4,5]. On the other hand, there is increasing evidence that not all patients respond satisfactorily to this kind of treatment and that about 30%-35% of the patients require additional intervention to control symptoms^[6-8]. Thus, traditional antacids are frequently used as add-on therapy in order to neutralize gastric acidity and to help control of heartburn^[7,9,10]. However, there are limited data regarding the mechanisms by which they are able to modify the determinants of reflux symptom perception^[11]. Moreover, despite their utility, the majority of current antacid formulations are not well tolerated by patients and this limits their widespread use and efficacy.

Recently, a novel compound, Faringel (CADIGroup, Rome, Italy), containing sodium bicarbonate and alginate with the addition of herbal components (i.e., honey, chamomille or *Matricaria recutita* L., *Calendula officinalis*, *Aloe vera*, Propolis gel) has been introduced to the market. The first two elements are well known to have an antireflux effect due to their ability to neutralize gastric acidity and to create an alginate-based raft that remains in the upper part of the stomach as a physical barrier capable of preventing reflux episodes^[12-15], while the latter components have been recently associated with mild anti-inflammatory and analgesic effects, and it has been suggested that they may favor the healing of human mucosa^[16-22].

In recent years, multichannel intraluminal impedance combined with pH-metry (MII-pH) has been applied to assess the effectiveness of drugs or endoscopic devices proposed for the therapy of GERD, particularly if we want to know whether they can affect both acid and

nonacid reflux or are able to reduce the proximal migration of the refluxate^[23-26].

The aim of the present study was to evaluate the anti-reflux properties of an alginate antacid formulation (Faringel) on both acid and nonacid reflux episodes, and the height of the proximal extent of reflux events by means of MII-pH monitoring in patients with documented mild to moderate GERD. As a secondary aim, we assessed the therapeutic efficacy of this novel compound as well as its tolerability using validated questionnaires.

MATERIALS AND METHODS

Subjects

This was a prospective, open-label study, enrolling consecutive patients with typical reflux symptoms (i.e., heartburn and regurgitation) lasting for > 6 mo and occurring at least three times weekly, presenting to the University Hospital of Genova and to the University Hospital of Pisa, Italy. They were referred to our units because they were undergoing upper endoscopy, preoperative surgical evaluation, or being investigated for PPI refractoriness. Exclusion criteria were: history of thoracic, esophageal or gastric surgery; primary or secondary severe esophageal motility disorders (e.g., achalasia, scleroderma, diabetes mellitus, autonomic or peripheral neuropathy, myopathy); or history of alcohol or drug abuse. In women of childbearing age, pregnancy was excluded by urine analysis.

For comparison, normal values were obtained from a group of 48 healthy volunteers [HV; 22 male; mean age 44 years, range 22-77 years; mean body mass index (BMI) 23 kg/m², range 16-34 kg/m²] without any type of digestive and systemic symptoms, and previously studied in our laboratory^[27].

The study protocol was approved by the local Ethics Committee and performed according to the Declaration of Helsinki. All patients provided written informed consent to take part.

Study protocol

All subjects who agreed to undergo both upper gastrointestinal (GI) endoscopy and 24-h esophageal impedance pH, underwent physical and clinical examination and a detailed medical history was recorded. The medical history included information on their symptomatic response to previous PPI therapy taken for at least 8 wk at double dose. Patients reporting < 50% heartburn improvement were considered nonresponders to PPIs (i.e., heartburn more than twice weekly for at least 2 mo). Patients taking antisecretory or prokinetic drugs were asked to stop any medication at least 30 and 15 d before endoscopy, respectively. Antacids or alginate preparations were suggested in case of frequent symptoms. The frequency and intensity of symptoms and impact on quality of life were registered using a structured and validated questionnaire for the diagnosis of GERD^[28].

Thereafter, within 1-5 d (median 3 d) from the up-

per GI endoscopy, every patient underwent esophageal impedance-pH testing off-therapy using an ambulatory multichannel intraluminal impedance and pH (MII-pH) monitoring system (Sleuth, Sandhill Scientific, Highland Ranch, CO, United States), according to our methodology^[27]. During the test day, meal time and composition were standardized^[29]. Stationary esophageal manometry was performed before MII-pH in order to locate with accuracy the lower esophageal sphincter (LES). Other features regarding the variables of reflux measurement by MII-pH and data analysis have been previously reported^[30,31].

After the 24-h monitoring period, patients returned to our hospital service. Based on the results of endoscopy and impedance-pH testing, patients were classified as NERD, in case of absence of esophageal mucosal breaks in combination with an abnormal esophageal acid exposure time and/or a positive symptom association probability (> 95%) to acid and/or nonacid reflux during impedance-pH monitoring^[3,27], and as ERD, in case of presence of esophageal mucosal injury according to international criteria^[32]. Furthermore, patients with hypersensitive esophagus (i.e., normal upper endoscopy, normal MII-pH testing, and positivity for symptom association analysis) were ruled out from the whole group of NERD in order to include patients with well-documented GERD. Then, they were asked to ingest a refluxogenic meal consisting of a continental breakfast [one cappuccino, two brioches containing chocolate cream (450 kcal, 60%) fat and orange juice], at least 4 h after the breakfast, and completed two questionnaires including questions on the presence and intensity of heartburn and regurgitation after the meal, as well as a 10-cm visual analogue scale (VAS) scale for each one of the two symptoms (see below). Patients underwent an additional 2-h period of recording; 1 h under basal conditions and another 1 h after a single dose (10 mL) of Faringel. Studies were performed while patients laid in the right lateral decubitus position for 30 min and in the supine decubitus position for another 30 min; both under basal conditions and after Faringel treatment. In particular, the right lateral decubitus position was chosen because it has been shown to be associated with an increased esophageal acid exposure^[33]. Afterward, data recording was concluded. Between the two sections and at the end of the test, patients filled out both symptomatic questionnaires.

Symptom assessment

The primary efficacy parameter was the change in the sum score of the validated Likert scale^[34,35] filled in by the investigator. Intensity of heartburn (defined as a retrosternal burning sensation occurring in waves and tending to rise upward toward the neck) and regurgitation (return of partially digested food from the stomach to the mouth) during the test was recorded by interviewing the patient, using a 5-point Likert rating scale as follows: 0 = none (absence of symptoms); 1 = mild (minimal awareness of symptoms, which is easily tolerated); 2 = moderate (awareness of symptoms, which is bothersome

but tolerable); 3 = severe (symptoms hard to tolerate); 4 = very severe (symptom impossible to tolerate). The score was used for the outcome measurement as a sum score, with its highest value of 8 points representing the most severe symptom intensity.

Patients were also asked to rate their satisfaction with symptom control on a global VAS of 0 (no relief at all) to 100 (complete symptom relief). The VAS score has been used as a self-assessment tool for symptom measure, which has been adopted in many other trials for evaluation of visceral symptoms^[36,37].

The secondary target variable comprised the overall tolerability of the treatment, assessed by investigator and patient using a 6-point Likert scale ranging from very poor (1) to very good (6). Together with the exploratory target variables, the number of responders and patients free of symptoms were also studied. Responders were defined as patients for whom a 40% improvement in the Likert scale was achieved, whereas patients free of symptoms were defined as subjects showing an overall sum score of 0 or 1 point when treatment stopped.

Statistical analysis

Differences in proportions were compared using the χ^2 or Fisher's exact test, depending on the sample size. Unless otherwise specified, data were presented as median and percentile values (25th, 75th, 95th percentile). In case of non-normally distributed data, differences between patients were compared using the Kruskal-Wallis and/or Mann-Whitney tests. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Patients

Forty patients with heartburn (20 female/20 male, mean age 48 years, range 18-76 years) reporting at least one symptom during the testing day were included in the study. Detailed demographic and clinical features of GERD patients and HVs are shown in Table 1. There was no difference among them and HVs in terms of sex and age. The prevalence of hiatal hernia as well as mean BMI was significantly higher in patients with GERD compared to the HVs ($P < 0.01$). All subjects tolerated well the examination and the test meal. No important technical failure occurred.

24-h impedance-pH data

Detailed impedance-pH characteristics of our patients and HVs are reported in Table 2. Patients with GERD had significantly greater distal esophageal acid exposure time compared to HVs (5.5 *vs* 0.7, $P < 0.0001$). The median total number of reflux episodes was 65 and the median number of acid reflux events was 53, and both were significantly higher in GERD patients compared to HVs (32 and 17, respectively; $P < 0.0001$). Patients with GERD and HVs had a similar number of nonacid reflux episodes (20 *vs* 18, $P < 0.0615$). The percentage of total

Table 1 Demographic and clinical characteristics of gastro-esophageal reflux disease patients and healthy volunteers *n* (%)

Demographic and clinical parameters	GERD patients	HVs	<i>P</i> value
Patients	40	48	
Female/male	20/20	27/21	NS
Mean age, yr (range)	48 (18-76)	44 (22-77)	NS
Mean BMI, kg/m ² (range)	26 (20-32)	23 (16-34)	< 0.05
NERD/ERD	25 (63)/15 (38)	NA	
Patients with hiatal hernia	22 (55)	4 (10)	< 0.01
Patients responding to PPI therapy	34 (85)	NA	

NERD: Nonerosive reflux disease; ERD: Erosive reflux disease; GERD: Gastroesophageal reflux disease; PPI: Proton pump inhibitor; BMI: Body mass index; HV: Healthy volunteer.

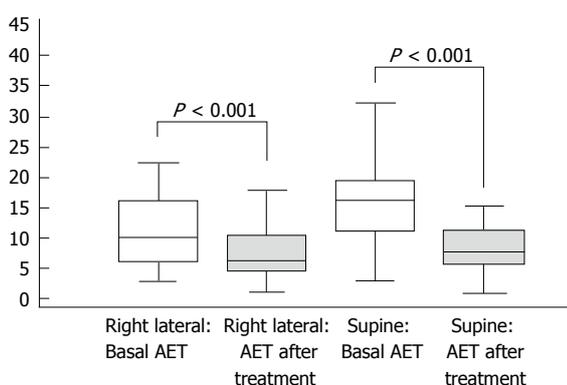


Figure 1 Median esophageal acid exposure under basal conditions and after Faringel intake in the two decubitus positions.

reflux episodes reaching the proximal measuring site (15 cm above the LES) was higher in GERD patients than in HVs (46% *vs* 33%, *P* < 0.0001).

2-h impedance-pH data before and after treatment

As shown in Figures 1 and 2A, Faringel decreased significantly (*P* < 0.001), in both the right lateral and supine decubitus positions, esophageal acid exposure time [10 (6-16; 23) *vs* 5.8 (4-10; 16) and 16 (11-19; 32) *vs* 7.5 (5-11; 15), respectively], acid reflux events [5 (3-8; 11) *vs* 1 (1-1; 2) and 6 (4-8; 11) *vs* 2 (1-2; 5), respectively], and proximal reflux episodes [4 (3-6; 11) *vs* 1 (1-2; 3) and 5 (4-7; 11) *vs* 3 (2-3; 4), respectively]. Also, the percentage of proximal migration of reflux events decreased significantly in both the right lateral and supine decubitus positions (60% *vs* 32% and 64% *vs* 35%) compared with baseline. In contrast, Faringel increased significantly (*P* < 0.01) the number of nonacid reflux events compared with baseline [2 (1-3; 5) *vs* 3 (2-5; 7) and 3 (2-4; 7) *vs* 6 (3-8; 13); Figure 2B]. The number of total reflux episodes slightly significantly decreased in the right lateral decubitus position, before and after Faringel treatment, while no difference was found in the supine decubitus position [7 (5-10; 15) *vs* 4 (3-5; 10) and 8.5 (7-11; 16) *vs* 7 (5-10; 17), *P* = 0.0001 and *P* = 0.1321, respectively].

Table 2 Impedance-pH features in gastro-esophageal reflux disease patients and healthy volunteers

Impedance-pH features	GERD patients	HVs	<i>P</i> value
% pH < 4 upright	6.9 (5.5-12; 28)	1 (0.2-1.9; 5)	< 0.0001
% pH < 4 recumbent	3.5 (1.2-7; 18)	0 (0-0.1; 2.1)	< 0.0001
% pH < 4 total	5.5 (4.3-9; 22)	0.7 (0.2-1.4; 4.2)	< 0.0001
GER total	65 (54-108; 177)	32 (18-43; 54)	< 0.0001
GER acid	53 (34-72; 95)	17 (8-31; 45)	< 0.0001
GER nonacid	20 (15-37; 116)	18 (14-26; 45)	0.0615
Prox. extension	31 (20-48; 86)	9 (4-17; 30)	< 0.0001

GER: Gastroesophageal reflux; GERD: Gastroesophageal reflux disease; HV: Healthy volunteer.

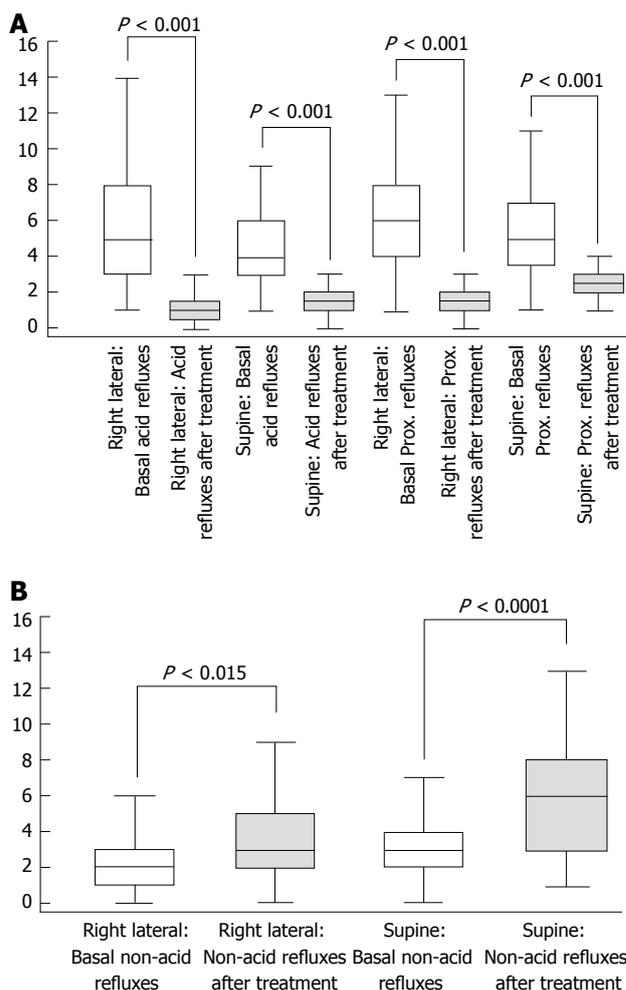


Figure 2 Number of acid reflux (A) and non-acid reflux (B) episodes under basal conditions and after Faringel intake in the two decubitus positions.

Symptom relief and drug tolerability

Patients reported a greater mean (range) number of symptoms before than after treatment and this included both heartburn [2.5 (1-9) *vs* 1 (0-2)] and regurgitation [2 (1-5) *vs* 1 (0-2)]. Faringel was found to be significantly effective in controlling heartburn, based on the both Likert scale [3.1 (range 1-4) *vs* 0.9 (0-2); *P* < 0.001] and

VAS score [7.1 (3-9.8) *vs* 2 (0.1-4.8); $P < 0.001$], while it had less success against the symptom regurgitation based on both the Likert scale [2.6 (1-4) *vs* 2.2 (1-4); $P =$ not significant (NS)] and VAS score [5.6 (2-9.6) *vs* 3.9 (1-8.8); $P =$ NS]. Overall, the tolerability of Faringel was very good [5 (2-6)], with only two patients reporting modest adverse events (i.e., nausea and bloating).

DISCUSSION

Alginates are neutral polysaccharide polymers isolated from brown seaweed (*Phacophyceae*) and are classified as dietary fiber. They are constituted by a proportion of D-mannuroic and L-glucuronic acids. In the presence of gastric acid, alginates precipitate and form a gel. One of the most interesting characteristics is due to the presence of sodium or potassium bicarbonate that, in the presence of gastric acid is converted to a dioxide which, when entrapped in the gel, converts it into a foam that floats on the surface of the gastric contents^[12]. Thus, thanks to their unique mechanism, alginate-based raft-forming formulations have been marketed worldwide for > 40 years under various brand names for the symptomatic treatment of GERD, and many studies have reported their efficacy^[12,38,39]. However, the majority of these studies have assessed only the control of symptoms without objective evaluation of the effect of these drugs on abnormal reflux by means of pH-monitoring, and even less using impedance-pH testing that is available in the clinical setting since few years.

Therefore, in our prospective study we evaluated the effect of a new alginate raft-forming formulation, Faringel, in a group of 40 patients with GERD who underwent 24 h MII-pH testing after a reflux-provocative meal. Our results showed that this alginate-based formulation is able to reduce the number of acid refluxes and the esophageal exposure time below pH 4.0. Moreover, it is able to decrease significantly, in both the right lateral and supine decubitus position, the number of acid reflux events and their proximal migration. Finally, all patients also reported a lower number of symptoms after treatment, including both heartburn and regurgitation, although the effect on the latter was less evident.

The patients evaluated in the present study were truly representative of the GERD population. Indeed, they had typical reflux symptoms (i.e., heartburn and regurgitation), abnormal acid exposure time, and/or evidence of mucosal breaks at upper GI endoscopy. We opted to include patients with these characteristics in order to be sure of excluding those with functional heartburn. Moreover, we preferred not to enroll patients with normal acid exposure and positive symptom association (i.e., hypersensitive esophagus) for reducing possible confounding factors such as visceral hyperalgesia, overlap with functional disease, autonomic dysfunction and concomitant psychiatric illness that have been more associated with the above condition^[40-43]. Finally, a recent report has suggested caution about overinterpretation

of symptom indexes in reflux monitoring, thus supporting our decision to exclude patients with hypersensitive esophagus in order to avoid confusion^[44].

Previously, Chatfield has reported a comparison of alginate preparation with placebo for the symptomatic relief of reflux esophagitis^[39]. In this multicenter randomized double-blind study, alginate was superior to placebo in reducing symptom severity and increasing symptom-free days. Interestingly, the placebo group recorded a larger number of dropouts due to side effects. This means that alginate is safe and provides better relief of symptoms. An older study that simultaneously used pH-telemetry and X-rays demonstrated that pH within the raft is approximately neutral, while the pH of the gastric contents beneath the raft remains acidic (pH 1-2)^[45]. These data are important because they explain why the alginate formulation is effective in controlling heartburn in the supine decubitus position, as observed in this study, and probably also during the night-time. More recently, using impedance-pH monitoring, we showed a reduction of acid reflux episodes and proximal migration of the refluxate and thereby a relevant decrease of GERD-related symptoms compared with baseline after sodium alginate administration^[7]. However, the results of the latter study were less marked than those of the current investigation, probably because patients were enrolled only on the basis of symptoms without objective documentation of GERD, either by endoscopy or pH monitoring. Moreover, in the previous study, tolerability was not evaluated.

The good control of acid reflux confirms the results obtained in previous studies performed with pH-metry^[7,14,46,47] or scintigraphic methods^[48,49], and represents the main mechanism of the quick and effective relief of heartburn in reflux patients. In a recent study, the positive effect of sodium alginate in reducing acid refluxes has been confirmed using simultaneously stepwise pH pull-throughs, high-resolution manometry and fluoroscopy^[15]. In fact, Kwiatek *et al*^[15] have shown that alginate can also eliminate or displace the “acid-pocket”, which is a phenomenon seen in the proximity of the esophago-gastric junction and is the likely origin of postprandial acid reflux in GERD patients.

Another interesting characteristic of sodium alginate has been emphasized by Manabe *et al*^[50] in NERD patients, who are known to have a lower response rate to PPIs than patients with ERD when gauged by relief of heartburn. In this study, patients who received omeprazole combined with sodium alginate recorded longer symptom relief compared with those receiving omeprazole alone. They concluded that sodium alginate is useful in combination with PPI therapy and has to be considered for treating NERD patients who do not respond completely to PPIs. Also, in our investigation, we evaluated NERD patients and found similar results on symptom relief in this particular group of GERD patients, although we did not study Faringel as an add-on therapy.

It is likely that the positive effect of sodium algi-

nate in controlling GERD-related symptoms is due to a whole equilibrium between raft-forming alginate and antacid substances. Faringel is constituted from sodium alginate and sodium carbonate. A previously published study has shown that, if two different antacid substances are present (e.g., Algicon Liquid), an effective reflux suppressing raft cannot form because a large amount of antacid prevents the raft formation by neutralizing the gastric acid required to react with alginate. Faringel and Gaviscon formulation consist of sodium alginate and sodium carbonate, and they have a lower acid-neutralizing capacity and a complete raft-forming gel reaction^[13,47,49]. These studies have shown that a large amount of antacids is not required for strong raft formation and effective reflux suppression.

On the contrary, various findings suggest that alginate is less effective in reducing nonacid than acid reflux. In our study alginate increased significantly the number of nonacid reflux events compared with baseline. The number of total reflux episodes decreased slightly but significantly in the right lateral decubitus position, whereas no difference was found in the supine decubitus position. Similarly, Zentilin *et al*^[7] have shown no action of alginate on nonacid reflux events. Surprisingly, in our study, the number of nonacid reflux episodes almost doubled after drug intake in 50% of patients. Probably the antacid effect of sodium alginate reduces acid reflux, but seems to increase nonacidic reflux.

Finally, our study shows that the percentage of proximal migration of reflux events decreased significantly both in the right lateral and supine decubitus positions compared with baseline, thus stopping one of the main determinants by which reflux causes symptoms^[51-53]. These results confirm our previous findings with a different sodium alginate formulation, although the study was performed in a smaller sample of patients and without a clear documentation of GERD^[7]. These investigations performed with impedance-pH monitoring technique permitted us to assess the ability of sodium alginate to reduce the proximal extension of refluxed material. The raft obtained with alginate represents a cork in the zone of the LES that prevents any gastric material migration into the esophagus independently of the patient decubitus. This beneficial effect could help in controlling not only typical, but especially extraesophageal symptoms. In particular, the Faringel formulation adds to alginate able to control GERD related typical symptoms a large number of vegetal extracts, which have the potential to promote healing of pharyngo-esophageal mucosal lesions. The anti-flogistic properties of Faringel are due to herbs such as Propolis, *A. vera*, and *Calendula*. Eamlamnam *et al*^[54] have observed that *A. vera* treatment induces a complete reduction in leukocyte adherence and tumor necrosis factor- α levels combined with elevated interleukin-10 levels, which are able to promote healing of gastric ulcers in male Sprague-Dawley rats. Propolis and *A. vera* have also demonstrated pain-killing effects^[55]. Moreover, experimental studies have shown that *C. officinalis* has anti-inflammatory and antibacterial activities as well as angiogenic and fibroblastic

properties acting in a positive way on the inflammatory and proliferative phase of the healing process^[20]. Thus, we can speculate that all these data on the anti-inflammatory properties of the herbal components of Faringel may be relevant for extraesophageal reflux-related symptoms in which a flogistic component seems to be more evident^[56].

In conclusion, our findings demonstrated that Faringel formulation is well tolerated and highly effective in controlling, or at least reducing, heartburn in GERD patients by modifying the number of acid reflux episodes and lowering the proximal migration of reflux events. It was less effective in controlling nonacid reflux and regurgitation. Its action in reducing the proximal extension of reflux events and the combined presence of natural substances (*Calendula*, *Aloe*, honey) that favor mucosal healing could be useful to improve GERD-related extraesophageal symptoms.

COMMENTS

Background

The use of proton pump inhibitors (PPIs) has been considered the best therapeutic option for gastroesophageal reflux disease (GERD), given their high efficacy in inducing symptom relief and esophageal mucosal healing. On the other hand, there is increasing evidence that not all patients (30%-35%) respond satisfactorily to this treatment. Thus, traditional antacids as alginate-based raft-forming formulations are used worldwide as add-on therapy to neutralize gastric acidity and help control heartburn. In recent years, multichannel intraluminal impedance combined with pH-metry (MII-pH) has been applied to assess the effectiveness of drugs or endoscopic devices proposed for the therapy of GERD, particularly if people want to know whether they can affect both acid and nonacid reflux or reduce the proximal migration of the refluxate.

Research frontiers

There are few data available regarding the mechanisms by which antacids as alginate-based raft-forming formulations are able to modify the determinants of reflux symptom perception, therefore, this study tried to evaluate the antireflux properties of an alginate antacid formulation (Faringel) on both acid and nonacid reflux episodes, and the height of proximal extent of reflux events by means of MII-pH monitoring in patients with documented mild to moderate GERD. As a secondary aim, authors assessed the therapeutic efficacy of this novel compound as well as its tolerability using validated questionnaires.

Innovations and breakthroughs

In this prospective, open-label study, 40 patients reporting heartburn and regurgitation with proven reflux disease (i.e., positive impedance-pH test/evidence of erosive esophagitis at upper endoscopy) underwent 2-h impedance-pH test after eating a refluxogenic meal. They were studied for 1 h under basal conditions and 1 h after taking 10 mL Faringel. Patients also completed validated questionnaires in order to evaluate the efficacy of Faringel for symptom relief. Tolerability of the treatment was also assessed.

Applications

The results suggest that Faringel is able to reduce the esophageal acid exposure time, the number of acid reflux events and their proximal migration, thus stopping two of the main determinants by which reflux causes symptoms (i.e., abnormal esophageal acid exposure time and proximal extension of the refluxate). Moreover, Faringel was very well-tolerated and effective in reducing heartburn and regurgitation, although the efficacy on the latter symptom was less evident.

Terminology

GERD is a condition that develops when the reflux of stomach contents causes troublesome symptoms and/or complications; Faringel is an antacid formulation, containing sodium bicarbonate and alginate with the addition of herbal components (i.e., honey, *Chamomille* or *Matricaria recutita* L., *Calendula officinalis*, *Aloe vera*, Propolis gel) that have been recently associated with mild anti-inflammatory and analgesic effects; MII-pH is a novel technique for pH-

independent detection of GER.

Peer review

The study is interesting, well conducted, with a clear statistical analysis and a comprehensive discussion.

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Prevalence of functional dyspepsia and its subgroups in patients with eating disorders

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Abstract

AIM: To study the prevalence of functional dyspepsia (FD) (Rome III criteria) across eating disorders (ED), obese patients, constitutional thinner and healthy volunteers.

METHODS: Twenty patients affected by anorexia nervosa, 6 affected by bulimia nervosa, 10 affected by ED not otherwise specified according to diagnostic and statistical manual of mental disorders, 4th edition, nine constitutional thinner subjects and, thirty-two obese patients were recruited from an outpatients clinic devoted to eating behavior disorders. Twenty-two healthy volunteers matched for age and gender were enrolled as healthy controls. All participants underwent a careful clinical examination. *Demographic* and *anthropometric characteristics* were obtained from a structured *questionnaires*. The presence of FD and, its subgroups, epigastric pain syndrome and

postprandial distress syndrome (PDS) were diagnosed according to Rome III criteria. The intensity-frequency score of broader dyspeptic symptoms such as early satiety, epigastric fullness, epigastric pain, epigastric burning, epigastric pressure, belching, nausea and vomiting were studied by a standardized questionnaire (0-6). Analysis of variance and *post-hoc* Sheffè tests were used for comparisons.

RESULTS: 90% of patients affected by anorexia nervosa, 83.3% of patients affected by bulimia nervosa, 90% of patients affected by ED not otherwise specified, 55.6% of constitutionally thin subjects and 18.2% healthy volunteers met the Postprandial Distress Syndrome Criteria (χ^2 , $P < 0.001$). Only one bulimic patient met the epigastric pain syndrome diagnosis. Postprandial fullness intensity-frequency score was significantly higher in anorexia nervosa, bulimia nervosa and ED not otherwise specified groups compared to the score calculated in the constitutional thinner group (4.15 ± 2.08 vs 1.44 ± 2.35 , $P = 0.003$; 5.00 ± 2.45 vs 1.44 ± 2.35 , $P = 0.003$; 4.10 ± 2.23 vs 1.44 ± 2.35 , $P = 0.002$, respectively), the obese group (4.15 ± 2.08 vs 0.00 ± 0.00 , $P < 0.001$; 5.00 ± 2.45 vs 0.00 ± 0.00 , $P < 0.001$; 4.10 ± 2.23 vs 0.00 ± 0.00 , $P < 0.001$, respectively) and healthy volunteers (4.15 ± 2.08 vs 0.36 ± 0.79 , $P < 0.001$; 5.00 ± 2.45 vs 0.36 ± 0.79 , $P < 0.001$; 4.10 ± 2.23 vs 0.36 ± 0.79 , $P < 0.001$, respectively). Early satiety intensity-frequency score was prominent in anorectic patients compared to bulimic patients (3.85 ± 2.23 vs 1.17 ± 1.83 , $P = 0.015$), obese patients (3.85 ± 2.23 vs 0.00 ± 0.00 , $P < 0.001$) and healthy volunteers (3.85 ± 2.23 vs 0.05 ± 0.21 , $P < 0.001$). Nausea and epigastric pressure were increased in bulimic and ED not otherwise specified patients. Specifically, nausea intensity-frequency score was significantly higher in bulimia nervosa and ED not otherwise specified patients compared to anorectic patients (3.17 ± 2.56 vs 0.89 ± 1.66 , $P = 0.04$; 2.70 ± 2.91 vs 0.89 ± 1.66 , $P = 0.05$, respectively), constitutional thinner subjects (3.17 ± 2.56 vs $0.00 \pm$

0.00, $P = 0.004$; 2.70 ± 2.91 vs 0.00 ± 0.00 , $P = 0.005$, respectively), obese patients (3.17 ± 2.56 vs 0.00 ± 0.00 , $P < 0.001$; 3.17 ± 2.56 vs 0.00 ± 0.00 , $P < 0.001$ respectively) and, healthy volunteers (3.17 ± 2.56 vs 0.17 ± 0.71 , $P = 0.002$; 3.17 ± 2.56 vs 0.17 ± 0.71 , $P = 0.001$, respectively). Epigastric pressure intensity-frequency score was significantly higher in bulimic and ED not otherwise specified patients compared to constitutional thin subjects (4.67 ± 2.42 vs 1.22 ± 1.72 , $P = 0.03$; 4.20 ± 2.21 vs 1.22 ± 1.72 , $P = 0.03$, respectively), obese patients (4.67 ± 2.42 vs 0.75 ± 1.32 , $P = 0.001$; 4.20 ± 2.21 vs 0.75 ± 1.32 , $P < 0.001$, respectively) and, healthy volunteers (4.67 ± 2.42 vs 0.67 ± 1.46 , $P = 0.001$; 4.20 ± 2.21 vs 0.67 ± 1.46 , $P = 0.001$, respectively). Vomiting was referred in 100% of bulimia nervosa patients, in 20% of ED not otherwise specified patients, in 15% of anorexia nervosa patients, in 22% of constitutional thinner subjects, and, in 5.6% healthy volunteers (χ^2 , $P < 0.001$).

CONCLUSION: PDS is common in eating disorders. Is it mandatory in outpatient gastroenterological clinics to investigate eating disorders in patients with PDS?

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Key words: Eating disorders; Functional dyspepsia; Post prandial distress syndrome; Epigastric pain syndrome; Rome III criteria; Upper abdominal symptoms; Anorexia nervosa; Bulimia nervosa; Eating disorders not otherwise specified; Constitutional thinness

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INTRODUCTION

Eating disorders (ED) are highly prevalent health problems in Western countries, especially in young women^[1]. Although no consensus has been yet achieved in the definition of eating disorders^[2], three main ED categories have been identified according to the diagnostic and statistical manual of mental disorders, 4th edition (DSM-IV)^[3]: anorexia nervosa (AN), bulimia nervosa (BN), and eating disorders not otherwise specified (EDNOS). In ED patients there is a significant impairment of both physical health and psychosocial functioning^[4]. Gastrointestinal (GI) symptoms are a common complaint in these patients. Boyd *et al*^[5] interviewed 101 ED patients

(44% AN, 22% BN, 34% EDNOS), using a standardized questionnaire to assess the presence of functional gastrointestinal disorders (FGIDs) such as irritable bowel syndrome (IBS), functional heartburn, functional abdominal bloating, functional constipation, functional dysphagia and functional anorectal pain disorder, showing that 98% of ED patients fulfilled the criteria for at least one FGID. A recent study demonstrated that 68.8% of ED patients met the Manning criteria for IBS^[6]. However, it was suggested that the wide range of FGIDs found in ED were the result of the behavior-associated ED. In fact, these GI symptoms may persist even after the recovery from ED, especially in psychologically distressed patients^[7]. However, the underlying mechanisms that link ED and GI symptoms remain to be elucidated^[8].

It is a common occurrence that patients, before presenting to healthcare services with an ED, seek treatment for GI symptoms^[9]. FGIDs induce high healthcare utilization and negative impact on quality of life^[10]. Dyspeptic symptoms are very common in the general population, with prevalence estimates ranging between 10% and 45%^[11,12]. The results of prevalence studies are strongly influenced by the criteria used to define dyspepsia. Well-performed epidemiological studies have reported a prevalence of approximately 20%-25% in western countries^[13,14], slightly higher in women, with a variable influence of age across studies.

Currently, an internationally accepted clinical standard (Rome III criteria) is extensively used to diagnose FGIDs^[15]. The Rome III Criteria were developed by a Committee that recommended the following pragmatic description of functional dyspepsia (FD) defined as the presence of symptoms thought to originate in the gastroduodenal region, in the absence of any organic, systemic, or metabolic disease that is likely to explain the symptoms. The specific symptoms needed to diagnose FD are: epigastric pain, epigastric burning, post-prandial fullness and early satiation. In addition, the Rome III consensus offers an umbrella definition for FD, and, furthermore, helps to distinguish whether patients report symptom aggravation after ingestion of a meal, meal-related dyspeptic symptoms, the so called postprandial distress syndrome (PDS) characterized by postprandial fullness and early satiation or meal-unrelated dyspeptic symptoms, the so called epigastric pain syndrome (EPS), characterized by epigastric pain and epigastric burning^[16]. A distinction between meal-related and meal-unrelated symptoms might be pathophysiologically and clinically relevant to disclose differences across ED, and other groups of patients with different patterns of abnormal eating behavior such as obese patients (OB) and constitutional thinness subjects (CT) in comparison to healthy volunteers (HV).

Our primary aim was to study the prevalence of FD and its subgroups according to the Rome III criteria across ED in comparison to OB patients, CT subjects and HV. Secondary aims were the evaluation of the frequency-intensity score of broader dyspeptic symptoms

such as early satiety, epigastric fullness, epigastric pain, epigastric burning, epigastric pressure, belching, nausea and vomiting in ED patients compared to the other groups of patients with different patterns of abnormal eating behavior.

MATERIALS AND METHODS

Participants

Five groups of patients matched for age and gender were recruited from an outpatients clinic devoted to eating behavior disorders. The first group consisted of 20 patients (AN-group), the second group of 6 BN patients (BN-group), the third group of 10 EDNOS patients (EDNOS-group), the fourth group of 9 CT subjects (CT-group) and the last group of 32 OB patients (OB-group). Twenty-two HV were recruited among administrative and/or paramedical staff members and patients' friends as the control healthy group (HV-group).

All patients and HV were interviewed to detect lifetime eating disorders in accordance with the criteria of the DSM-IV^[3]. The DSM-IV criteria define anorexia nervosa as self-induced weight loss or refusal to maintain or gain weight normally, with resulting weight more than 15% below normal; intense fear of fatness or gaining weight, even though underweight; deep disturbance in body image; and reproductive hormone abnormality (for example, at least 3 mo of amenorrhea).

Bulimia nervosa is defined as recurrent episodes of binge eating (a large amount of food eaten quickly and privately with lack of control over eating) and recurrent inappropriate compensatory behaviour to prevent weight gain (self-induced vomiting; misuse of laxatives, diuretics, enemas, or other medications; fasting; excessive exercise) at least twice a week for at least 3 mo, and self-evaluation unduly influenced by body shape and weight.

EDNOS represents the third category of ED and involves milder versions of anorexia and bulimia nervosa that do not satisfy all the criteria (for example, a binge episode once a week or for less than 3 mo for bulimia nervosa; weight loss less than 15% for anorexia nervosa).

CT subjects were recruited among the patients evaluated for leanness, using the following inclusion criteria: severely underweight, but stable throughout the post-pubertal period, presence of physiological menstruations without estroprogestative treatment, and the desire for weight gain as the main reason for medical consultation, together with the exclusion of celiac disease, infectious diseases, cancer, or other consumptive diseases^[17].

Obesity is defined if the body mass index (BMI) was ≥ 30 kg/m² according to the National Institute of Health guidelines^[18].

For each patient, demographic (age, smoking habits, alcohol intake) and anthropometric characteristics (weight, height and BMI) were collected.

All patients gave their written consent to participate into the study. The study, fully complied with the Declaration of Helsinki, and was approved by the Ethics Com-

mittee of the Ruggi d'Aragona Hospital AOU University of Salerno.

Questionnaire

All participants underwent a standardized questionnaire testing the presence of FD according to Rome III criteria. The Rome III symptom questionnaire consisted of 18 questions and allowed the diagnosis of FD and its subgroups (PDS and EPS). The characteristic symptoms of PDS were bothersome postprandial fullness or early satiation and those of EPS were unexplained epigastric pain or burning^[16]. The frequency for early satiety, epigastric fullness, epigastric pain and burning (the 4 cardinal symptoms pragmatically described by the Rome III Committee)^[16] and other dyspeptic symptoms such as epigastric pressure, belching, nausea and vomiting was scored from 0 to 3 (0 = absent, 1 = 2 d/wk; 2 = 3-5 d/wk; and 3 = 6 d or 7 d/wk); the intensity for the same symptoms was scored from 0 to 3 (0 = absent; 1 = not very bothersome, not interfering with daily activities; 2 = bothersome, but not interfering with daily activities; and 3 = interfering with daily activities). A frequency-intensity score from 0 up to a maximum of 6 was obtained for each symptom^[19].

Statistical analysis

Data are expressed as mean \pm SE, unless otherwise specified. χ^2 test and, analysis of variance (ANOVA) followed by one way ANOVA for multiple comparisons (Scheffé) were used to compare categorical and continuous data, respectively. The significance level was set at 0.05. The statistical program used was SPSS version 12.0 for Windows.

RESULTS

Anthropometric characteristics of the studied population were shown in Table 1. Eighteen/20 (90%) AN, 5/6 (83.3%) BN, 9/10 (90%) EDNOS, 5/9 (55.6%) CT, and 4/22 (18.2%) HV met Rome III criteria for PDS (χ^2 , $P < 0.001$). Figure 1 shows the distribution of PDS diagnosis in ED, CT and HV. Only one BN patient met the EPS Criteria. None of the patients with ED, CT, OB or HV had both PDS and EPS.

Table 1 shows the intensity-frequency score calculated for each symptom in the studied population. Postprandial fullness intensity-frequency score was significantly higher in AN, BN and EDNOS groups compared to the score calculated in the CT group (4.15 ± 2.08 vs 1.44 ± 2.35 , $P = 0.003$; 5.00 ± 2.45 vs 1.44 ± 2.35 , $P = 0.003$; 4.10 ± 2.23 vs 1.44 ± 2.35 , $P = 0.002$, respectively), OB group (4.15 ± 2.08 vs 0.00 ± 0.00 , $P < 0.001$; 5.00 ± 2.45 vs 0.00 ± 0.00 , $P < 0.001$; 4.10 ± 2.23 vs 0.00 ± 0.00 , $P < 0.001$, respectively) and, HV (4.15 ± 2.08 vs 0.36 ± 0.79 , $P < 0.001$; 5.00 ± 2.45 vs 0.36 ± 0.79 , $P < 0.001$; 4.10 ± 2.23 vs 0.36 ± 0.79 , $P < 0.001$, respectively). Early satiety intensity-frequency score was prominent in anorectic patients compared to bulimic patients (3.85 ± 2.23 vs 1.17 ± 1.83 ,

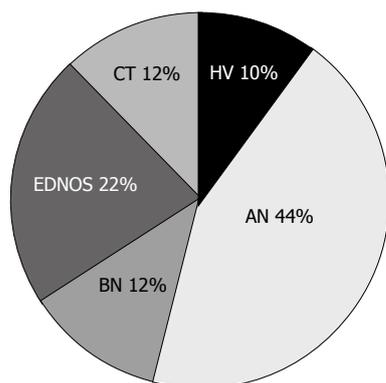


Figure 1 Distribution of the postprandial distress syndrome diagnosis in anorexia nervosa, bulimia nervosa, eating disorders not otherwise specified, constitutional thinners and healthy volunteers ($n = 41$). AN: Anorexia nervosa; BN: Bulimia nervosa; EDNOS: Eating disorders not otherwise specified; CT: Constitutional thinners; HV: Healthy volunteers.

$P = 0.015$), obese patients (3.85 ± 2.23 vs 0.00 ± 0.00 , $P < 0.001$) and, HV (3.85 ± 2.23 vs 0.05 ± 0.21 , $P < 0.001$). Nausea and epigastric pressure were increased in bulimic and EDNOS patients. Specifically, nausea intensity-frequency score was significantly higher in BN and EDNOS patients compared to the score calculated in anorectic patients (3.17 ± 2.56 vs 0.89 ± 1.66 , $P = 0.04$; 2.70 ± 2.91 vs 0.89 ± 1.66 , $P = 0.05$, respectively), Constitutional Thinner subjects (3.17 ± 2.56 vs 0.00 ± 0.00 , $P = 0.004$; 2.70 ± 2.91 vs 0.00 ± 0.00 , $P = 0.005$, respectively), obese patients (3.17 ± 2.56 vs 0.00 ± 0.00 , $P < 0.001$; 3.17 ± 2.56 vs 0.00 ± 0.00 , $P < 0.001$, respectively) and, HV (3.17 ± 2.56 vs 0.17 ± 0.71 , $P = 0.002$; 3.17 ± 2.56 vs 0.17 ± 0.71 , $P = 0.001$, respectively). Epigastric pressure intensity-frequency score was significantly higher in bulimic and EDNOS patients compared to the score calculated in CT subjects (4.67 ± 2.42 vs 1.22 ± 1.72 , $P = 0.03$; 4.20 ± 2.21 vs 1.22 ± 1.72 , $P = 0.03$, respectively), obese patients (4.67 ± 2.42 vs 0.75 ± 1.32 , $P = 0.001$; 4.20 ± 2.21 vs 0.75 ± 1.32 , $P < 0.001$, respectively) and, HV (4.67 ± 2.42 vs 0.67 ± 1.46 , $P = 0.001$; 4.20 ± 2.21 vs 0.67 ± 1.46 , $P = 0.001$, respectively). Vomiting was referred in 100% of BN patients, in 20% of EDNOS patients, in 15% of AN patients, in 22% of CT subjects and, in 5.6% of HV (χ^2 , $P < 0.001$). Epigastric pain intensity-frequency score just failed to reach significance in EDNOS compared to HV ($P = 0.05$), whereas it was significantly higher in EDNOS compared to OB patients ($P = 0.02$). Figure 2 shows the pattern of dyspeptic symptoms that reached the statistical significance in all groups.

DISCUSSION

The novel result of our study was that the diagnosis of PDS according to Rome III Criteria was very common in AN, BN and EDNOS, the three main categories of ED, whilst EPS is incredibly rare. Moreover, BN and EDNOS showed high postprandial fullness, epigastric pressure and nausea intensity-frequency scores, whereas AN patients shared with BN an increase in postprandial fullness

score, but conversely demonstrated a prominent early satiety. OB patients were almost asymptomatic regarding FD symptoms.

The hallmarks of ED are clinical disturbances in body image and eating behavior resulting in physical and psychological impairment. These clinical entities are diagnosed according to DSM-IV criteria. Among them disorders such as AN, BN and EDNOS are more common in women and can result in long-term health consequences even in increased mortality. The core presentation of Anorexia nervosa is characterized by the inability or refusal to maintain a minimally normal weight, a profoundly distorted perception of body weight and shape, and amenorrhea. Under the definition of BN are included individuals who engage in recurrent binge-eating episodes and recurrent inappropriate compensatory behaviours that are intended to rid calories that they voraciously ingested. EDNOS involves milder versions of anorexia and bulimia nervosa that do not satisfy all the criteria. Previous studies have suggested that anorectic patients frequently complain of gastrointestinal symptoms hinting at a disordered gastric motility, especially when they are in a refeeding phase^[20]. Dyspeptic symptoms such as epigastric fullness and distension were found to be significantly more prevalent and intense than in healthy subjects^[21-23] and may serve as an argument for food refusal^[24]. However, they are often overlooked or misinterpreted. In this study the more prevalent and intense dyspeptic symptoms scored by a standardized questionnaire were epigastric fullness and early satiety. In bulimic patients the large quantities eaten during a binge not only lead to a feeling of loss of control but also to a sensation of epigastric distension. The latter as well as the often associated epigastric pain are terminated by self-induced vomiting, which allows either continuation or termination of the binge^[20]. Our findings demonstrated that BN and EDNOS referred postprandial fullness, epigastric pressure and nausea as their most prevalent and intense dyspeptic symptoms. The mechanisms underlying these dyspeptic symptoms in ED are still unclear, although malnutrition and the resultant metabolic myopathy, along with electrolyte depletion seem to play the crucial role in determining the demonstrated abnormalities in gastric emptying^[22], gastric capacity^[25] and, blunted endocrine control^[26]. Conversely, irrespective of the pathophysiology and mechanisms involved, it is intriguing that the association of higher body mass index alone with dyspeptic symptoms was relatively modest also contrary to the study expectation. It is noteworthy that in our OB group no binge behavior has been diagnosed, suggesting that eating patterns are more closely linked to symptom generation in the GI tract^[27]. In addition, to our knowledge this is the first study that demonstrated in ED a high prevalence of PDS using the Rome III criteria, an international accepted instrument. Another novel finding of this study was that 55% of CT subjects met the Rome III criteria for PDS and referred a higher intensity-frequency score for early satiety than healthy volunteers. Individuals with CT belong to a non

Table 1 Anthropometric characteristics and frequency-intensity score (from 0 to 6) calculated for each symptom in the studied population

	AN (n = 20)	BN (n = 6)	EDNOS (n = 10)	CT (n = 9)	OB (n = 32)	HV (n = 22)	P value
Characteristics							
Age (yr)	22.45 ± 0.94	24.83 ± 2.76	24.50 ± 1.82	24.89 ± 2.21	23.84 ± 0.74	23.67 ± 0.71	0.74
Weight (kg)	42.79 ± 1.18	60.80 ± 6.13	54.65 ± 2.51	48.13 ± 1.89	115.40 ± 3.27	60.26 ± 1.87	< 0.001
Symptom							
Postprandial fullness	4.15 ± 0.46	5.00 ± 1.00	4.10 ± 0.71	1.44 ± 0.78	0.00 ± 0.00	0.36 ± 0.17	< 0.001
Early satiety	3.85 ± 0.50	1.17 ± 0.75	3.50 ± 0.72	2.11 ± 0.81	0.00 ± 0.00	0.05 ± 0.05	< 0.001
Nausea	0.89 ± 0.38	3.17 ± 1.05	2.70 ± 0.92	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.17	< 0.001
Epigastric pressure	2.21 ± 0.55	4.67 ± 0.99	4.20 ± 0.70	1.22 ± 0.57	0.75 ± 0.23	0.67 ± 0.34	< 0.001
Epigastric burning	1.05 ± 0.40	1.83 ± 1.17	1.10 ± 0.64	0.00 ± 0.00	0.44 ± 0.23	0.00 ± 0.00	0.02
Epigastric pain	1.32 ± 0.50	1.67 ± 0.80	1.80 ± 0.74	0.22 ± 0.22	0.00 ± 0.00	0.00 ± 0.00	< 0.001
Belching	0.37 ± 0.23	1.33 ± 0.99	0.80 ± 0.53	0.78 ± 0.46	0.31 ± 0.20	0.22 ± 0.22	0.40

Data are expressed as mean ± SE. AN: Anorexia nervosa; BN: Bulimia nervosa; EDNOS: Eating disorders not otherwise specified; CT: Constitutional thinners; HV: Healthy volunteers; OB: Obese patients.

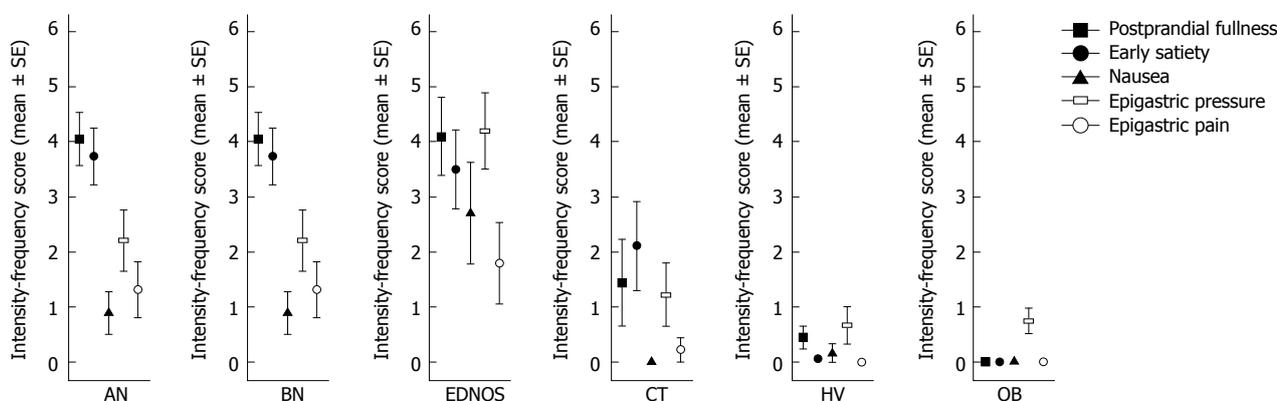


Figure 2 Intensity-frequency scores of post-prandial fullness, early satiety, nausea, epigastric pressure and epigastric pain in anorexia nervosa, bulimia nervosa, eating disorders not otherwise specified, constitutional thinners, healthy volunteers and, obese patients, expressed as mean ± SE. AN: Anorexia nervosa; BN: Bulimia nervosa; EDNOS: Eating disorders not otherwise specified; CT: Constitutional thinners; HV: Healthy volunteers; OB: Obese patients.

pathological state, poorly described^[28]. They are often young women, severely thin that continue to have a close to normal fat mass percentage, normal physiological menstrual cycles, no detectable abnormalities of cortisol, insulin-like growth factor 1, or free T₃ secretory patterns and normal energy metabolism^[17,28]. The mechanism behind low-weight steadiness in CT was not yet elucidated. Multifactorial etiology involves a combination of genetics in addition to as yet unrecognized pathophysiological factors^[29]. CT subjects display an equilibrated energy metabolism similar to that of control subjects. CT subjects attempt to gain weight, often overeating. To assess whether this eating pattern is related to GI symptom generation, further dynamic studies are needed.

Our findings leave room for speculation on the mechanisms underlying FD in patients with an ED. It has been suggested that FD results from a closed interaction of biological, psychosocial and social factors^[30]. The altered eating behavior seen in EDs is strongly associated with disturbed gastrointestinal sensitivity and motor physiology^[8]. ED and FD patients shared a high prevalence of psychiatric comorbidities^[31]. These latter together with the motor and sensitivity disturbances can lay the foundation of an FGID. Once established the psychological and

physiological disturbances can perpetuate and strengthen each other resulting in an FGID that can persist independently of the ED that originally caused the motor and sensitivity disturbances^[7]

It is also conceivable that a large number of individuals presenting for medical treatment for GI symptoms in gastroenterologic outpatient clinics could be better managed by firstly the identification and, secondly by receiving adequate treatment for concurrent ED. This is an important issue given that the ultimate goal of therapy in suspected ED patients is the normalization of gastric motor function with the resumption of normal eating behavior enabling the patient's social reintegration and restoration to an appearance acceptable to the social environment.

We acknowledge the limitations of this study. Firstly, the overall sample size was small. Furthermore, the study was limited by the failure to screen for organic GI disorder which, although quite rare in patients with EDs^[32], could falsely inflate estimates of FD incidence.

In conclusion, the high prevalence of meal-related symptoms in ED patients should encourage in gastroenterology outpatient clinics the routine screening for ED. In addition to perhaps helping design more efficacious

interventions for FD if patterns of food ingestion contribute to the development of unexplained GI symptoms, further studies are necessary to demonstrate whether patterns of food ingestion contribute to the development of unexplained GI symptoms. This attention to eating patterns might provide a simple, safe and potentially effective method to better manage FD patients too.

COMMENTS

Background

Eating disorders (ED) are highly prevalent health problems in Western countries, especially in young women. Three main ED categories have been identified on the basis of the diagnostic and statistical manual of mental disorders, 4th edition: anorexia nervosa (AN), bulimia nervosa (BN), and eating disorders not otherwise specified (EDNOS). Gastrointestinal (GI) symptoms are a common complaint in ED patients. It is a common occurrence that patients, before presenting to healthcare services with an ED, seek treatment for GI symptoms.

Research frontiers

A previous study demonstrated that 98% of ED patients fulfilled the criteria for at least one functional gastrointestinal disorder (FGIDs) such as irritable bowel syndrome, functional heartburn, functional abdominal bloating, functional constipation, functional dysphagia and functional anorectal pain disorder. Recently, a high prevalence of irritable bowel symptoms was confirmed in patients already affected by ED. However, it was suggested that FGIDs were the result of the behaviour-associated ED and that, these GI symptoms may persist even after the recovery from ED, especially in psychologically distressed patients. Currently, the underlying mechanisms that link ED and GI symptoms remain to be elucidated.

Innovations and breakthroughs

The novel result of the study was that AN, BN and EDNOS, the three main categories of ED, had a high prevalence of dyspeptic symptoms fulfilling the Rome III criteria to positively diagnose postprandial distress syndrome (PDS), not epigastric pain syndrome (EPS). Moreover, BN and EDNOS showed high postprandial fullness, epigastric pressure and nausea intensity-frequency scores, whereas AN patients shared with BN an increase in postprandial fullness score, but conversely demonstrated a prominent early satiety. Irrespective of the pathophysiology and mechanisms involved, it is intriguing that the association of higher body mass index alone with dyspeptic symptoms was relatively modest, also contrary to the study expectations. In addition, to the knowledge, this is the first study that demonstrated in ED a high prevalence of PDS using the Rome III criteria, an internationally accepted instrument. Another interesting finding of the study was that 55% of constitutional thinness subjects (CT) met the Rome III criteria for PDS and, referred a higher intensity-frequency score for early satiety than healthy volunteers.

Applications

It is conceivable that a large number of individuals presenting for medical treatment for GI symptoms in gastroenterologic outpatient clinics could be better managed by the identification of a concurrent ED. Their findings leave room for speculation on the mechanisms underlying functional dyspepsia (FD) in patients with an ED. The altered eating behavior seen in EDs is strongly associated with impairment in gastrointestinal sensitivity and motor physiology. ED and FD patients shared a high prevalence of psychiatric comorbidities. These latter together with the motor and sensitivity disturbances can lay the foundation of an FGID. Once established the psychological and physiological disturbances can perpetuate and strengthen each other resulting in an FGID that can persist independently of the ED that originally caused the motor and sensitivity disturbances. Further studies are needed in the future to demonstrate these hypotheses.

Terminology

ED are clinical disturbances in body image and eating behavior resulting in physical and psychological impairment. These clinical entities are diagnosed according to diagnostic and statistical manual of mental disorders, 4th edition criteria; AN is characterized by the inability or refusal to maintain a minimally normal weight, a profoundly distorted perception of body weight and shape, and amenorrhea; BN is a clinical entity that includes individuals who engage in recurrent binge-eating episodes and recurrent inappropriate compensatory behaviours that are intended to rid calories that they voraciously ingested; EDNOS involves milder versions of anorexia and bulimia nervosa that do not satisfy all the criteria; CT is a non

pathological state, poorly described. Subjects constitutionally thin are often young women, severely thin that continue to have a close to normal fat mass percentage and, normal physiological menstrual cycles; Rome III criteria to diagnose FD are defined as the presence of symptoms thought to originate in the gastroduodenal region, in the absence of any organic, systemic, or metabolic disease that is likely to explain the symptoms. The specific symptoms needed to diagnose FD are: epigastric pain, epigastric burning, post-prandial fullness and early satiety; PDS is characterized by bothersome postprandial fullness or early satiety; EPS is characterized by bothersome unexplained epigastric pain or burning.

Peer review

This is a good descriptive study. The results are interesting and suggest that due to the high prevalence of dyspepsia symptoms in patients already diagnosed for ED, it could be recommended to gastroenterologists to evaluate patients seeking treatment for the post-prandial distress syndrome to rule out a possible coexistence of any ED.

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Quadruple therapy with moxifloxacin and bismuth for first-line treatment of *Helicobacter pylori*

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Abstract

AIM: To compare triple therapy vs quadruple therapy for 10 d as first-line treatment of *Helicobacter pylori* (*H. pylori*) infection.

METHODS: Consecutive *H. pylori* positive patients never treated in the past for this infection were randomly treated with triple therapy of pantoprazole (PAN) 20 mg bid, amoxicillin (AMO) 1 g bid and moxifloxacin (MOX) 400 mg bid for 10 d (PAM) or with quadruple therapy of PAN 20 mg bid, AMO 1 g bid, MOX 400 mg bid and bismuth subcitrate 240 mg bid for 10 d (PAMB). All patients were found positive at 13 C-Urea breath test (UBT) performed within ten days prior to the start of the study. A successful outcome was confirmed with an UBT performed 8 wk after the end of treatment. χ^2 analysis was used for statistical comparison. Per protocol (PP) and intention-to-treat (ITT) values were also calculated.

RESULTS: Fifty-seven patients were enrolled in the PAM group and 50 in the PAMB group. One patient in each group did not return for further assessment. Eradication was higher in the PAMB group (negative: 46 and positive: 3) vs the PAM group (negative: 44 and positive: 12). The *H. pylori* eradication rate was statistically significantly higher in the PAMB group vs the PAM group, both with the PP and ITT analyses (PP: PAMB 93.8%, PAM 78.5%, $P < 0.02$; ITT: PAMB 92%, PAM 77.1 %, $P < 0.03$).

CONCLUSION: The addition of bismuth subcitrate can be considered a valuable adjuvant to triple therapy in those areas where *H. pylori* shows a high resistance to fluoroquinolones.

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Key words: *Helicobacter pylori* infection; First-line therapy; Quadruple therapy; Amoxicillin; Moxifloxacin; Bismuth subcitrate

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INTRODUCTION

Helicobacter pylori (*H. pylori*) has an important role in the

development of chronic gastritis and peptic ulcer disease and has been linked to the pathogenesis of gastric lymphoma and gastric cancer^[1-3], hence it is recommended that this infection be cured whenever it is diagnosed. *H. pylori* is susceptible to several antibiotics including clarithromycin, amoxicillin (AMO), metronidazole, tinidazole, tetracycline, rifabutin and fluoroquinolones [levofloxacin and moxifloxacin (MOX)]^[4,5], and is inherently resistant to many other antibiotics such as bacitracin, vancomycin, trimethoprim, polymyxins and nalidixic acid^[6,7]. This bacterial infection, however, has proven challenging to cure. There are several reasons for the loss of eradication efficacy, and antibiotic resistance is the key factor for treatment failure^[8]. Resistance rates vary in different geographic areas and therefore the selection of therapeutic regimes needs adjustments according to local resistance pattern^[9,10]. The prevalence of antibiotic resistance in various regions is correlated with general use of antibiotics in the region^[11,12].

Classical triple therapies with proton pump inhibitors (PPI) clarithromycin and AMO or metronidazole are the mainstay of current treatment, but resistance to clarithromycin has been reducing its effectiveness in recent years, with an eradication rate below 80% of treated cases^[13,14]. In geographic areas with high clarithromycin resistance, bismuth-containing quadruple therapy is superior to standard triple therapy. The original quadruple therapy based on omeprazole, bismuth subcitrate, metronidazole and tetracycline achieves higher eradication rates compared with the standard triple therapy^[15,16]. In a recent randomized, open-label, phase 3 trial, a quadruple regimen with a capsule containing bismuth citrate potassium, metronidazole, and tetracycline given with omeprazole was found more efficacious than a clarithromycin-based triple therapy in patients never treated in the past for the infection^[17].

MOX-based triple therapy has been suggested as an alternative first-line therapy for *H. pylori* infection in geographical areas with clarithromycin resistance exceeding 30% of strains. Eradication rates of up to 92% in the MOX-based triple regimens compared to 79% in the clarithromycin-based regimens were demonstrated^[18]. Primary resistance of *H. pylori* strains collected from patients who have never been treated in the past for *H. pylori* infection to fluoroquinolones has been reported to be lower than that of clarithromycin in several geographic areas^[19]. Resistance to fluoroquinolones, however, rapidly develops in areas where these antibiotics are widely used. In our region in fact, the resistance rate of *H. pylori* to MOX has increased in the last two years from 12% in 2009 to 25% in 2011, while resistance to clarithromycin is stable, ranging from 40% to 59% (unpublished results). Similar results have been registered in other areas of the world. A recent study from Korea reports a steady increase of MOX resistance from 5.6% in 2004 up to 28.2% in 2008 with the need to optimize dosage and duration of treatment^[20]. Treatment with MOX-based triple therapy for 10 d should be preferred over a 7-d course, and there is

evidence that a dose of 800 mg/d is superior to the 400 mg standard dose^[21,22].

The aim of the study was to compare MOX containing triple therapy *vs* MOX and bismuth-containing quadruple therapy for first-line treatment of *H. pylori* infection.

MATERIALS AND METHODS

Eligibility criteria

Patients were enrolled among those with a positive ¹³C urea breath test (UBT) performed with citric acid and 75 mg of ¹³C urea performed within ten days prior to the start of the study. Exclusion criteria included: age <18 years or > 80 years; any previous treatment for *H. pylori* infection, treatment with PPI (omeprazole, lansoprazole, pantoprazole (PAN), rabeprazole, esomeprazole), H₂-blockers (ranitidine, nizatidine, cimetidine, famotidine, roxatidine), and/or antibiotics during the 4 wk before the study; gastrointestinal malignancy, severe concomitant diseases, previous gastric surgery. Further exclusion criteria were a prolongation of the QT interval, defined as the interval between the beginning of the QRS complex and the end of the T wave. QT interval was computed using an electrocardiogram (ECG) performed in all patients within 10 d prior to the beginning of the study.

Study protocol

Consecutive *H. pylori* positive patients never treated in their past for the infection were randomly treated with a triple therapy with PAN, AMO, MOX (PAM) or with a quadruple therapy with PAN, AMO, MOX and bismuth subcitrate (PAMB) for 10 d.

While PAN, AMO and MOX were administered before breakfast and supper, bismuth salts were administered 3 h after the administration of PAN, AMO, MOX, since it may bind in some patients with MOX and prevent its full absorption^[23].

Dosages and time of administration of the studied drugs are summarized in Table 1. Patients were informed that bismuth renders the stools a black dark colour. Successful outcome was confirmed with an UBT performed 8 wk after the end of treatment with a Delta Over Baseline value equal or less than 5.

Determination of sample size

Sample size was predetermined taking the following parameters into consideration: $\alpha = 0.05$; $\beta = 0.20$; lost to follow-up = 5%; expected eradication rate in the PAM group = 72%; expected eradication rate in the PAMB group = 95% (23% increase). With these parameters, the required sample size was equal to 90 patients per group.

Statistical analysis

Statistical evaluation was carried out using the χ^2 analysis. A *P*-value of 0.05 or less was considered statistically significant. Per protocol (PP), in which only data from adherent subjects are analyzed, and intention-to-treat (ITT), in

Table 1 Summary of study data

Triple therapy regimen (PAM)	Quadruple therapy regimen (PAMB)
Pantoprazole 20 mg <i>bid</i> (8.00 am-8.00 pm)	Pantoprazole 20 mg <i>bid</i> (8.00 am-8.00 pm)
Amoxicillin 1 g <i>bid</i> (8.00 am-8.00 pm)	Amoxicillin 1 g <i>bid</i> (8.00 am-8.00 pm)
Moxifloxacin 400 mg <i>bid</i> (8.00 am-8.00 pm)	Moxifloxacin 400 mg <i>bid</i> (8.00 am-8.00 pm)
	Bismuth subcitrate 240 mg <i>bid</i> (11.00 am-11.00 pm)
Number of patients: 57	Number of patients: 50
Female/male: 30/27	Female/male: 28/22
Age: mean 49 yr, range 23-75 yr	Age: mean 50 yr, range 20-72 yr
Follow-up loss: 1	Follow-up loss: 1
UBT negative: 44	UBT negative: 46
UBT positive: 12	UBT positive: 3
Per protocol: 44/56 (78.5%)	Per protocol: 46/49 (93.8%) ^a
Intention to treat: 44/57 (77.1%)	Intention to treat: 46/50 (92%) ^a

^a $P < 0.05$ vs pantoprazole, amoxicillin and moxifloxacin (PAM). PAMB: Pantoprazole, amoxicillin, moxifloxacin and bismuth subcitrate; UBT: Urea breath test.

which all subjects are followed regardless of adherence, evaluations were calculated.

RESULTS

Patient characteristics are summarized in Table 1. Fifty-seven patients were enrolled in the PAM group and 50 in the PAMB group. One patient in each group did not return for further assessment (Table 1). Results of UBT performed 8 wk after the end of treatment are shown in Table 1.

The eradication rate was the following for PP and ITT analysis: PAM group PP: 44/56 (78.5%), PAMB group PP: 46/49 (93.8%); PAM group ITT: 44/57 (77.1%), PAMB group ITT: 46/50 (92%). *H. pylori* eradication rate was statistically significantly higher in the PAMB group vs the PAM group, both with the PP ($P < 0.02$) or ITT analysis ($P < 0.03$) (Table 1).

Adverse effects

Both treatments were well tolerated with no reported side effects.

DISCUSSION

Triple therapy based on a PPI combined with clarithromycin and AMO and/or metronidazole has been the established first-line therapy for *H. pylori* infection over the past years around the world^[24,25]. However, the efficacy of standard triple therapy needs to be reconsidered in areas with a high prevalence of clarithromycin or metronidazole resistant *H. pylori* strains. The geographical prevalence of antibiotic resistance should influence the choice of a first-line regimen for the treatment of infection by *H. pylori*. Alternatively, new regimens with high eradication rates should be identified. Quadruple therapies have been used as second-line therapy, and have also been proven effective as first-line treatment in areas with a high prevalence of clarithromycin-resistant *H. pylori* strains. Quadruple therapy containing bismuth has been used in the first-line therapy of *H. pylori* infection with variable results. Ching *et al.*^[26], from North Wales, United King-

dom, randomized patients to bismuth quadruple therapy or clarithromycin triple therapy. A total of 91% of patients receiving bismuth quadruple therapy and 92% of patients receiving clarithromycin triple therapy achieved successful *H. pylori* eradication. Calvet *et al.*^[27] performed a study in which patients were randomized to receive bismuth quadruple therapy or receive clarithromycin triple therapy. Eradication was achieved in 83% of the bismuth quadruple therapy group and 77% of the clarithromycin triple therapy group. In conclusion, in these two studies, first-line quadruple and triple therapies yielded similar eradication rates in the treatment of *H. pylori* infection.

Other studies have shown that the addition of bismuth to a first-line triple therapy produces high eradication rates despite the presence of high antibiotic-resistant strains. Malfertheiner *et al.*^[17] have recently shown that a quadruple therapy with omeprazole and a single three-in-one capsule containing bismuth citrate potassium, metronidazole and tetracycline for 10 d when compared with a triple therapy for 7 d with omeprazole, AMO, and clarithromycin produces an eradication rate of 80% vs 55% in the standard therapy group. The authors concluded that quadruple therapy needs to be considered as first-line therapy in areas with a high prevalence of clarithromycin-resistant *H. pylori* strains. These data are supported by results from a study from China in which bismuth was added to standard triple-therapy including PPI, clarithromycin and AMO and the *H. pylori* eradication rate was above 90%. This treatment showed a higher efficiency than standard triple-therapy, and the addition of bismuth and prolongation of the treatment from 7 to 14 d helped to overcome clarithromycin resistance in 84% of the patients^[28].

Our study shows that the addition of bismuth subcitrate to a triple therapy that includes PAN, AMO and MOX for first-line treatment of *H. pylori* infection significantly increases the eradication rate of the same therapy without bismuth. With bismuth included, eradication was 93.8% in PP and 92% in ITT analysis, but without bismuth the eradication rate was 78.5% in PP and 71.8% in ITT analysis. Indeed the same triple therapy used three years before the present study, induced a higher eradica-

tion rate^[22]; this change is probably due to an increase in resistance to MOX in our region from 2009-2011 (increase from 12% to 25%). Unfortunately, the absence of a preliminary susceptibility test in our patients does not allow us to understand whether the low rate of eradication in PAM group is effectively due to increased resistance to MOX or if patients infected by *H. pylori* resistant to MOX were equally distributed in the two therapeutic groups. Therefore, a periodic surveillance of antibiotic resistance is necessary since some antibiotics may develop resistance much more easily than others, such as in the case of MOX^[20]. The resistance against fluoroquinolones is mainly generated by mutations in the *gyrA* gene that encodes DNA gyrase^[29,30]. The consequence of such a mutation is the inability of fluoroquinolones to inhibit DNA replication^[31,32].

In our study the addition of bismuth to triple therapy has provided a therapeutic gain of 15% to a standard therapy. Bismuth exerts its antibacterial action by decreasing mucin viscosity, by binding toxins produced by *H. pylori*, and by preventing bacterial colonization and adherence to gastric epithelium^[33,34]. In addition bismuth reduces the bacterial load and has a synergistic effect with antibiotics^[35], particularly with the nitroimidazole family^[36].

The use of bismuth has raised some concerns about its side effects. A meta-analysis of 35 randomized controlled trials was published to assess the safety of bismuth. It showed that no serious adverse events occurred with bismuth therapy, and bismuth for the treatment of *H. pylori* was safe and well-tolerated^[37]. In our study, there have been no side effects reported.

Criticism for our study might include the absence of a preliminary susceptibility test in our patients. In fact, treatment failure in 12 patients in the PAM group was probably due to MOX resistance. However, the addition of bismuth to triple therapy helps to overcome *H. pylori* resistance to MOX: only 3 patients had a positive ¹³C UBT after treatment with quadruple therapy. The addition of bismuth to standard therapy results in an improved eradication rate, therefore, bismuth may be considered as a valuable adjuvant to triple therapy in those areas *H. pylori* shows a high resistance to fluoroquinolones.

The 10-d quadruple therapy consisting of a PPI, bismuth, AMO, and MOX achieved ITT success of 93.8% and can be recommended as the first-line treatment of *H. pylori* infection in regions of high fluoroquinolones resistance.

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COMMENTS

Background

The standard treatment of *Helicobacter pylori* (*H. pylori*) infection is carried out by using a drug that inhibits gastric acid secretion associated with two antibiot-

ics represented by amoxicillin and clarithromycin or metronidazole. The success rate of this triple therapy varies from region to region worldwide, mainly due to a variation in antibiotic resistance. There is, therefore, a continuous search for new antibiotics that show low resistance against *H. pylori* or other substances that, when added to triple therapy, may overcome the obstacle of resistance.

Research frontiers

Fluoroquinolones are a relatively new class of antibiotics that may be highly efficacious against *H. pylori* infection. However, bacterial resistance to fluoroquinolones may rapidly increase over time due to their widespread use, especially for pulmonary and urinary tract infections. Recent studies have shown that the addition of bismuth subcitrate to a standard triple therapy may at least in part overcome the antibiotic resistance and improve the success rate of a standard triple therapy. In this study, the authors showed the adjunction of bismuth subcitrate to a triple therapy that includes the fluoroquinolone derivative, moxifloxacin, improved the success rate of the same therapy given without bismuth in a group of patients with *H. pylori* infection never treated in the past. The *H. pylori* infection had been diagnosed by means of urea breath test (UBT). To verify the effectiveness of the therapy, UBT was repeated in all patients 2 mo after the end of therapy.

Innovations and breakthroughs

To the knowledge, this is the first study that shows that bismuth improves the therapeutic effect of moxifloxacin for the treatment of *H. pylori* infection in patients never treated in the past for this infection.

Applications

The study offers an alternative therapeutic option for all who are involved in the treatment of *H. pylori* infection.

Terminology

UBT is the most accurate non-invasive test for the diagnosis of *H. pylori* infection and does not necessitate endoscopic intervention. The main limit of the test is that it results in false negative results if performed while the patient is taking drugs that may inhibit gastric secretion or antibiotics.

Peer review

The study is an important contribution to the growing body of literature on alternative therapies to treat the increasingly drug-resistant *H. pylori* isolates.

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Adalimumab in prevention of postoperative recurrence of Crohn's disease in high-risk patients

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Abstract

AIM: To evaluate the effectiveness of adalimumab in preventing recurrence after intestinal resection for Crohn's disease in high-risk patients.

METHODS: A multicenter, prospective, observational study was conducted from June 2009 until June 2010. We consecutively included high-risk Crohn's disease patients who had undergone an ileal/ileocolonic resection. High-risk patients were defined as two or more criteria: smokers, penetrating pattern, one or more

previous surgical resections or prior extensive resection. Subcutaneous adalimumab was administered 2 wk (\pm 5 d) after surgery at a dose of 40 mg eow, with an initial induction dose of 160/80 mg at weeks 0 and 2. Demographic data, previous and concomitant treatments (antibiotics, 5-aminosalicylates, corticosteroids, immunomodulators or biologic therapies), smoking status at the time of diagnosis and after the index operation and number of previous resections (type and reason for surgery) were all recorded. Biological status was assessed with C-reactive protein, erythrocyte sedimentation rate and fecal calprotectin. One year (\pm 3 mo) after surgery, an ileocolonoscopy and/or magnetic resonance enterography was performed. Endoscopic recurrence was defined as Rutgeerts score \geq i2. Morphological recurrence was based on magnetic resonance (MR) score \geq MR1.

RESULTS: Twenty-nine patients (55.2% males, 48.3% smokers at diagnosis and 13.8% after the index operation), mean age 42.3 years and mean duration of the disease 13.8 years were included in the study. A mean of 1.76 (range: 1-4) resections previous to adalimumab administration and in 37.9% was considered extensive resection. 51.7% had previously received infliximab. Immunomodulators were given concomitantly to 17.2% of patients. Four of the 29 (13.7%) developed clinical recurrence, 6/29 (20.7%) endoscopic recurrence and 7/19 (36.8%) morphological recurrence after 1-year. All patients with clinical recurrence showed endoscopic and morphological recurrence. A high degree of concordance was found between clinical-endoscopic recurrence ($\kappa = 0.76$, $P < 0.001$) and clinical-morphological recurrence ($\kappa = 0.63$, $P = 0.003$). Correlation between endoscopic and radiological findings was good (comparing the 5-point Rutgeerts score with the 4-point MR score, a score of i4 was classified as MR3, i3 as MR2, and i2-i1 as MR1) ($P < 0.001$, $r_s = 0.825$). During follow-up, five (17.2%) patients needed adalimumab dose intensification (40 mg/wk); Mean time to intensi-

fication after the introduction of adalimumab treatment was 8 mo (range: 5 to 11 mo). In three cases (10.3%), a biological change was needed due to a worsening of the disease after the dose intensification to 40 mg/wk. One patient suffered an adverse event.

CONCLUSION: Adalimumab seems to be effective and safe in preventing postoperative recurrence in a selected group of patients who had undergone an intestinal resection for their CD.

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Key words: Crohn's disease; Postoperative recurrence; Prevention; Tumor necrosis factor alpha agents; Adalimumab

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INTRODUCTION

Crohn's disease (CD) is a complex chronic inflammatory bowel disease with an unpredictable course, in most cases accompanied by periodic recurrence and exacerbations^[1]. Up to 70% of patients with CD have to undergo surgery due to complications of the disease at least once in their lifetime, frequently resulting in ileocolonic anastomosis^[2,3]. Unfortunately, postsurgical recurrence, defined as the appearance of new lesions detected endoscopically, radiologically or pathologically, is common after surgery. One year after intestinal resection, 70%-90% of patients have endoscopic evidence of recurrent disease in the neo-terminal ileum. Despite the high endoscopic recurrence rate, symptomatic recurrence is delayed, with only 20% of patients having symptoms within the first year, increasing to one third after 3 years^[4].

Ileocolonoscopy was considered the gold standard in the evaluation of postoperative recurrence, being a useful tool to predict clinical recurrence, CD-related complications and the need for repeat surgery in the future. Rutgeerts *et al*^[4] devised a 5-point score ranging from i0 to i4 to measure the presence and severity of endoscopic lesions in the neoterminal ileum and anastomosis and reported a correlation between endoscopic recurrence and the likelihood of clinical recurrence, especially in those patients with more severe (i3-i4) endoscopic score.

The main limitations of ileocolonoscopy are its restriction to the level of intestinal mucosa and to the co-

lon and neoterminal ileum area due to stenosis at the ileocolonic anastomosis site. Recently, magnetic resonance (MR) enteroclysis, a safe and non-invasive new imaging technique for small bowel, has proven to be a useful tool for detecting unsuspected penetrating disease, reaching areas restricted to ileocolonoscopy. A 4-point MR-enteroclysis-based score (MR0-MR3) has been validated by Sailer *et al*^[5] and studies comparing ileocolonoscopy and MR enteroclysis suggest that they are of similar value in terms of predicting the risk of clinical recurrence in postoperative CD patients^[6].

Different studies have assessed the efficacy of the available medications in the prevention of postoperative CD recurrence. Mesalamine, nitroimidazole antibiotics, azathioprine and 6-mercaptopurine have been reported to be effective treatments but have shown limited efficacy in reducing postoperative recurrence^[7-12]. Recent results from a randomized placebo-controlled trial and a prospective pilot study with the chimeric anti-tumor necrosis factor alpha (TNF- α) antibody infliximab (IFX) support the utility of biologic therapies for the prevention of postoperative CD recurrence^[13-15].

Adalimumab is a fully human anti-TNF- α monoclonal antibody that has been shown to be highly effective in inducing and maintaining remission in patients with moderate-to-severe CD. Adalimumab is effective not only in naïve patients but also in patients with loss of response or intolerance to IFX^[16-19]. However, its efficacy in preventing postoperative recurrence has yet to be assessed.

The aim of this study is to evaluate the effectiveness and safety of adalimumab in preventing endoscopic and morphological recurrence at 12 mo after intestinal resection for CD in patients at high risk of recurrence, as well as to assess the influence of different risk factors associated with recurrence in CD patients treated with adalimumab. Additionally, we investigated the effectiveness of adalimumab in preventing clinical CD recurrence 1 year after surgery and compared endoscopic and radiological findings for predicting clinical recurrence in CD patients who had undergone intestinal resection.

MATERIALS AND METHODS

Study design

A multicenter, prospective, observational study was conducted in order to assess the effectiveness and safety of adalimumab in the prevention of postoperative recurrence of CD in high-risk patients. Four Spanish referral hospitals participated in the study.

Patients

All patients undergoing intestinal resection (macroscopically normal lines of resection) for ileal or ileocolonic CD from June 2009 to June 2010 were assessed and invited to participate. Inclusion criteria were: (1) patients aged between 15 and 70 years; (2) undergoing intestinal resection for CD; (3) high risk of recurrence, defined as at least two of the following criteria: smoker; penetrating pattern; prior extensive resection (> 100 cm); or one

or more previous surgical resections; (4) monitored for at least 1 year; and (5) if on concomitant treatments, a stable dose 12 wk before surgery and constant throughout the duration of the study. Exclusion criteria included the following: (1) patients with active ileocolonic or anorectal disease at entry; (2) more than 10 years of CD requiring first respective intestinal surgery for short (< 10 cm) fibrostenotic stricture; and (3) prior adverse events related to adalimumab.

Demographic data (gender and age), previous and concomitant treatments (antibiotics, 5-aminosalicylates, corticosteroids, immunomodulators or biologic therapies), smoking status at the time of diagnosis and after the index operation and number of previous resections (type and reason for surgery) were all recorded. Clinical data and inflammatory parameters C-reactive protein (CRP) (level in milligrams per liter), erythrocyte sedimentation rate (level in millimeters per hour) and fecal calprotectin (level in micrograms per gram) were evaluated at each visit (months 0, 3, 6, 9 and 12 after surgery). Ileocolonoscopy and magnetic resonance enterography (MRE), if available at the site, were performed at one year (\pm 3 mo) after treatment initiation, or withdrawal if clinical recurrence was suspected by the clinician, with or without elevated biological parameters. Adalimumab was administered 2 wk (\pm 5 d) after surgery with an induction dose of 160/80 mg at weeks 0 and 2 and at a maintenance dose of 40 mg subcutaneous eow. During follow-up, if at the discretion of the physician, the patient required intensified treatment, the adalimumab was increased to 40 mg every week.

Definitions

Index operation was defined as the last ileal or ileocolonic resection prior to the study. Baseline data, month 0, refers to the month when the index operation was performed. Clinical postoperative recurrence was defined as the onset of symptoms (diarrhea, abdominal pain and decreased well-being) or complications that led to changes in medical or surgical management.

Endoscopic recurrence refers to the existence of new mucosal (endoscopic) lesions in the neoterminal ileum after surgery. Ileocolonoscopy was performed by an experienced endoscopist and classified according to Rutgeerts score^[4] (Table 1). Endoscopic recurrence was defined as i2-i4 classified endoscopic findings.

Morphological recurrence refers to the occurrence of new lesions assessed by imaging techniques. MRE was performed with administration of oral contrast (1500 mL of 5% mannitol solution) as described Leyendecker *et al*^[20]. The presence of irregularities in mucosal surface, enhancement of mucosal contrast, thickening of the bowel wall, stenosis and extramural abnormalities (abscesses and fistulas) were evaluated and MRE findings were classified into the four grade MR score (Table 1)^[5]. Morphological recurrence was defined as MR1-MR3 classified MRE findings. Radiologist and endoscopist were blinded to the results of each other and to the in-

flammatory marker testing to assure correct and non-biased results.

Statistical analysis

Continuous variables were summarized as mean \pm SD or range when appropriate. Test of normality was developed using the D'Agostino test. Categorical variables were summarized as frequencies and percentages. Concordance between clinical-endoscopic recurrence and clinical-morphological recurrence was calculated with the weighted kappa statistic (κ). Kappa values were interpreted as follows: 0.99-0.81 "almost perfect agreement"; 0.80-0.61 "substantial agreement"; 0.60-0.41 "moderate agreement"; 0.40-0.21 "fair agreement" and less than 0.2 "slight agreement"^[21]. Correlation between endoscopic and morphological recurrence was calculated using Spearman rank correlation. Univariate logistic regression was performed to assess the influence of variables collected on the development of recurrence. Logistic regression multivariate analysis could not be performed due to the low number of observations. Differences were considered significant if *P* value < 0.05.

RESULTS

Demographics and clinical history

Twenty-nine patients were included in the study, 16 (55.2%) of whom were male. Mean age at diagnosis of CD was 28 years (range: 13-60 years). Demographic and clinical characteristics are shown in Table 2. The mean time from diagnosis to the last resection was 166 mo (range: 7 to 365 mo). Mean age at the last resection was 42.3 \pm 11.18 years. The indication for resection was therapeutic failure in 10/29 (34.5%), stenosis in 17/29 (58.6%) and penetrating pattern in 2 (6.9%) cases. Almost all patients (28 of 29) had been treated with a course of systemic corticosteroids at some point for the disease (mean No. courses: 5.7; range: 1-10) and 12 (42.9%) had received corticosteroids prior to the index operation. In addition, 41.4% of patients were taking antibiotics at the time of the index operation. IFX had been taken previously by 15 (51.7%) patients and aminosalicylates by 13 (44.8%) patients. Concomitant treatment with thiopurines was given to five (17.2%) patients and enteral nutrition therapy (elemental and/or semi-elemental formulas) in six (20.7%) patients. Patients' smoking status, at diagnosis and after the index operation was evaluated. At diagnosis, almost half (48.3%) were smokers while after the index operation, only 4 (13.8%) continued smoking.

Adalimumab intervention

All patients were treated with an induction dose of 160/80 mg subcutaneous adalimumab at weeks 0 and 2 and at a maintenance dose of 40 mg eow after intestinal resection. During follow-up, colonoscopy and MRE were necessary to continue in five (17.2%) patients because of suspected clinical recurrence and/or elevated

Table 1 Rutgeerts and magnetic resonance score for classification of postoperative recurrence in Crohn's disease

Rutgeerts score	Description	MR score	Description
i0	No lesions	MR0	No findings
i1	Less than 5 aphthous lesions	MR1	Minor mucosal irregularities: Slight wall thickening Slight mural contrast enhancement No stenosis
i2	More than 5 aphthous lesions with normal mucosa between the lesions or skip areas or larger lesions or lesions confined to ileo-colonic anastomosis		
i3	Diffuse aphthous ileitis with diffusely-inflamed mucosa	MR2	Major mucosal abnormalities: Distinct bowel wall thickening Distinct mural contrast enhancement Low grade stenosis without prestenotic dilatation
i4	Diffuse inflammation with already large ulcers, nodules and/or narrowing	MR3	Same finding as MR 2 plus: Transmural edema with T2w signal increase and contrast enhancement of the perienteric fat High grade stenosis without prestenotic dilatation Extramural complications (fistula, abscess, conglomeration of bowel loops)

MR: Magnetic resonance; MR0-MR3: A 4-point MR-enteroclysis-based score; i0-i4: A 5-point score to measure the presence and severity of endoscopic lesions in the neoterminal ileum and anastomosis.

Table 2 Patient characteristics at baseline (n = 29) (%)

Patient characteristics	Value
Gender	
Female	13 (44.8)
Age (yr), mean (range)	42.3 (19.8-61.1)
Duration of the disease (mo), median (range)	166 (7-365)
Montreal classification:	
A1 (< 17 yr)	6 (20.7)
A2 (17-40 yr)	17 (58.6)
A3 (> 40 yr)	6 (20.7)
L1 (ileal)	15 (51.7)
L3 (ileocolonic)	9 (31.0)
L1 + L4 (ileal + upper gastrointestinal)	5 (17.2)
B1 (non-stricturing/penetrating)	9 (31.0)
B2 (stricturing)	14 (48.3)
B3 (penetrating)	6 (20.6)
Perianal disease	10 (34.4)
Extensive resection	11 (37.9)
Immunomodulators (AZA, 6-MP) concomitant to adalimumab	5 (17.2)
Concomitant enteral nutrition	6 (20.7)
Previous infliximab	15 (51.7)
Previous resections (including index operation)	
1	15 (51.7)
2	7 (24.1)
3	6 (20.7)
4	1 (3.4)
Smoking status at diagnosis	
Smokers	14 (48.3)
Ex-smokers	2 (6.9)
Non-smokers	13 (44.8)
Smoking status after the index operation	
Smokers	4 (13.8)
Ex-smokers	12 (41.4)
Non-smokers	13 (44.8)

AZA: Azathioprine; 6-MP: 6-mercaptopurine.

biological parameters. All patients had endoscopic and morphological recurrence and needed adalimumab

dose intensification (40 mg every week). Mean time to intensification after the introduction of adalimumab treatment was 8 mo (range: 5 to 11 mo). The 5 patients had received thiopurine drugs and 4 of them, biological treatment with IFX, before index surgery. Concomitant treatment with immunomodulators was given to one of the 5 patients.

In three cases (10.3%), a biological change was needed due to the persistence of symptoms and progressive elevation of acute-phase reactants after the dose had been increased to 40 mg every week. Two of the three patients had previously received IFX and changed to certolizumab, and the third had switched to IFX.

Primary endpoint: Endoscopic and morphological recurrence at 12 mo

Six of the 29 (20.7%) showed endoscopic recurrence (i2-i4). Two patients had an endoscopic grade score of i2, two patients had a score of i3 and the other two, i4. Additionally, 19 of the 29 (65.5%) patients had MRE 1 year after resection. In 7 of the 19 (36.8%), morphological recurrence (MR1-MR3) was observed. Three had an MR score of MR1, three of MR2 and the other patient, MR3 (Table 3).

Influence of clinical parameters in endoscopic recurrence:

In the univariate analysis (Table 4), two variables were found to be associated with endoscopic recurrence. Patients with extensive resection (over 100 cm) [odds ratio (OR): 14.17, 95% CI: 1.12-708.0, P = 0.026] and with more than two previous resections (OR: 13.3, 95% CI: 1.7-107.4, P = 0.015) had increased risk of endoscopic recurrence. Other variables studied, such as the disease pattern, presence of perianal disease, number of previous surgical resections, indication of surgery and IFX treatment failure had no significant influence on the endoscopic recurrence rate.

Table 3 Correlation between endoscopic and radiological findings *n* (%)

Rutgeerts score	MR score			
	MR0	MR1	MR2	MR3
i0	9 (100)	0	0	0
i1	3 (75)	1 (25)	0	0
i2	0	1 (50)	1 (50)	0
i3	0	1 (50)	1 (50)	0
i4	0	0	1 (50)	1 (50)

MR: Magnetic resonance; MR0-MR3: A 4-point MR-enteroclysis-based score; i0-i4: A 5-point score to measure the presence and severity of endoscopic lesions in the neoterminal ileum and anastomosis.

Table 4 Univariate analysis for endoscopic recurrence *n* (%)

Endoscopic recurrence		Yes	No	<i>P</i> value
Gender	Female	3 (23.1)	10 (76.9)	0.775
	Male	3 (18.8)	13 (81.3)	
Duration of the disease	≤ 10 yr	3 (23.1)	10 (76.9)	0.775
	> 10 yr	3 (18.8)	13 (81.3)	
Pattern	Non-stricturing, non-penetrating	2 (22.2)	7 (77.8)	0.318
	Stricturing	4 (28.6)	10 (71.4)	
	Penetrating	0 (0)	6 (100)	
Extensive resection	Yes	5 (45.5)	6 (54.5)	0.026
	No	1 (5.6)	17 (94.4)	
Immunomodulators concomitant to ADA	Yes	1 (20)	4 (80)	0.967
	No	5 (20.8)	19 (79.2)	
Prior infliximab	Yes	5 (33.3)	10 (66.7)	0.111
	No	1 (7.1)	13 (92.9)	
Previous resections	≤ 2	2 (9.1)	20 (90.9)	0.015
	> 2	4 (57.1)	3 (42.9)	
Smoking status at diagnosis	Smokers	3 (21.4)	11 (78.6)	0.516
	Non-smokers	3 (23.1)	10 (76.9)	
	Ex-smokers	0 (0)	2 (100)	
Smoking status after the index operation	Smokers	2 (50)	2 (50)	0.119
	Non-smokers	4 (16)	21 (84)	
Adverse events	Yes	0 (0)	1 (100)	0.454
	No	6 (22.2)	21 (77.8)	

ADA: Adalimumab.

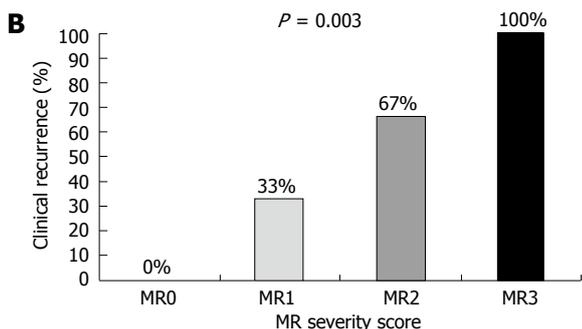
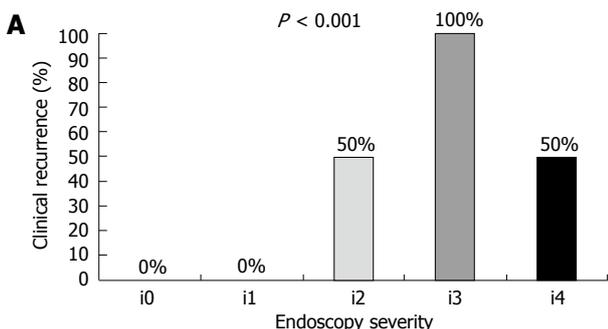


Figure 1 Concordance after ileocolonic resection for Crohn's disease. A: Between clinical-endoscopic recurrence; B: Between clinical-morphological recurrence. MR: Magnetic resonance; MR0-MR3: A 4-point MR-enteroclysis-based score; i0-i4: A 5-point score to measure the presence and severity of endoscopic lesions in the neoterminal ileum and anastomosis.

Secondary endpoints

Clinical recurrence at 12 mo: Four of the 29 (13.7%) patients developed clinical recurrence 1 year after intestinal surgery. In the univariate analysis, active smoker status after the index operation was significantly correlated with higher clinical recurrence rate (smokers *vs* non-smokers OR: 11.50; 95% CI: 1.01-131.29; *P* = 0.049). Patients with extensive resection (OR: 14.17; 95% CI: 1.12-708.0; *P* = 0.014) were significantly correlated with the development of clinical recurrence. No other significant correlations were found with the other variables.

Correlation between clinical, endoscopic and morphological recurrence: After 12 mo of follow-up, all patients with clinical recurrence (four patients) showed

endoscopic and morphological recurrence. Patients with clinical recurrence were classified into i2-i4 groups (1 in i2, 2 in i3 and 1 in i4) using the Rutgeerts score for Post-operative Endoscopic Classification of CD^[4]. There was significant concordance (substantial agreement) between the endoscopy severity and the clinical recurrence rate (κ = 0.76, *P* < 0.001) (Figure 1A).

Similarly, with the MR score, the four patients with clinical recurrence showed radiological recurrence (1 in MR1, 2 in MR2 and 1 in MR3). Also, substantial agreement between MR-score severity and the clinical recurrence rate was observed (κ = 0.63, *P* = 0.003) (Figure 1B).

Endoscopic and MRE evaluation was possible in 19 patients. Correlation between endoscopic and radiological findings was good. The Spearman rank correlation coefficient between the two techniques was r_s = 0.889 (*P* < 0.001) when dichotomous outcome “recurrence, yes or no” was correlated. To compare the 5-point Rutgeerts score with the 4-point MR score, a score of i4 was classified as an MR score of MR3, i3 was defined as equivalent to MR2, and i2-i1 were classified as MR1, consistent with the fact that MR imaging does not provide sufficient spatial resolution to differ between i1 and i2^[6]. In this case, the Spearman rank correlation coefficient was r_s = 0.825, *P* < 0.001.

Using endoscopy as the gold standard, the sensitivity of MRE for detecting moderate-to-severe endoscopic findings (Rutgeerts score i3 and i4) was 75%; specificity 93.3%, positive predictive value 75% and negative predictive value 93.3%.

Safety: One (3.4%) patient reported an adverse event during the 12 mo of follow-up: episodes of peripheral

vertigo. Although the etiology for this adverse event was not identified, adalimumab treatment was discontinued at month 6. This patient continued without treatment and remained in remission at month 12.

Ethical considerations

The study was carried out in accordance with the Declaration of Helsinki principles of good clinical practice. The study protocol was reviewed and approved by the local independent ethics committee.

DISCUSSION

In this study, we found adalimumab to be effective and safe in preventing recurrence after surgery for CD in a special subgroup of patients at high risk of recurrence. Intestinal resection is not curative and the majority of patients develop endoscopic recurrence even before clinical symptoms become apparent. Early detection and treatment of endoscopic lesions is essential to improve the long-term outcome for these patients and thus, to improve their quality of life. One year after intestinal resection, 70%-90% of patients have endoscopic recurrence and clinical recurrence occurs in nearly 20%^[4]. A number of studies have evaluated the efficacy of active treatment (mesalamine, nitroimidazole antibiotics and immunomodulators) for prevention of postoperative CD recurrence and most of them reported endoscopic recurrence to be between 40% and 60% at 12 mo after surgery^[22]. Recently, the anti-TNF- α antibody, IFX, has been associated with a significant reduction in postoperative recurrence in CD^[14,15,23,24]. However, to date, there have been no published results with adalimumab, only a case series of six patients who underwent resection for an ileocecal stricture in which adalimumab was successfully used to prevent postsurgical recurrence of CD^[25]. For this reason, we decided to evaluate the effectiveness and safety of adalimumab in preventing postoperative endoscopic and morphological recurrence as the primary study endpoint. This cohort of patients developed clinical recurrence in 13.7% of cases, endoscopic recurrence in 20.7% and MRE recurrence in 36.8%, although MRE was only evaluated in 19 patients and could be overestimated. In the clinical trial conducted by Regueiro *et al*^[14], 9.1% of patients in the IFX-treated group had endoscopic recurrence and 84.6% in the placebo group. In our study, the rate of endoscopic recurrence was higher, at 24.1%. It is important to take into account that our results refer to a special subgroup of patients with high risk factors for recurrence (two or more of: smoker, penetrating pattern, prior extensive resection and one or more previous surgical resections), so higher percentages are expected. In addition, there were several differences between the two studies in terms of design. In our cohort, only 5 of the 29 patients (17.2%) were on concomitant immunomodulator treatment whereas this applied to 4 of the 11 (36.4%) patients in the IFX-treated group in the Regueiro study. Also, in the clinical trial with IFX,

only one (9.1%) patient had previously undergone more than two intestinal resections compared to 7 (24.1%) patients in this study.

Different risk factors have been associated with postoperative CD recurrence. However, cigarette smoking is the only modifiable risk factor to be identified to date, and the most significant, with twice the risk of presenting a clinical recurrence compared with non-smokers^[26,27]. We found an increased clinical recurrence risk in patients who reported smoking at the time of the index operation compared with non-smokers. When we evaluated patients who were smokers at the time of diagnosis, no differences in risk were found. This was consistent with other studies that found no increased risk in ex-smokers^[26,27] or reported a reduction in the recurrence rate after stopping^[28,29] and suggests that the effect of smoking disappears some time after giving up the habit. These results support the fact that physicians should advise, encourage and assist CD patients to stop smoking. Another factor to consider is the length of the previous intestinal resection. In our study, the clinical and endoscopic recurrence rate was higher in patients with prior extensive resection (> 100 cm) and with more than two previous resections, consistent results with those obtained from the study about the impact of azathioprine on the prevention of postoperative CD recurrence^[9]. Other risk factors for postoperative CD recurrence, such as penetrating pattern, duration of CD or complex perianal disease, did not influence the proportion of patients with endoscopic and/or morphological recurrence.

Clinical postoperative recurrence was defined as the presence of symptoms, such as diarrhea, abdominal pain or decreased well-being, or complications which led to changes in medical or surgical management. All patients with clinical recurrence showed endoscopic and morphological recurrence 1 year after intestinal resection.

We avoided using the clinical activity index because it is difficult to perform in this setting. Some studies in which clinical postoperative recurrence was measured with the Crohn's Disease Activity Index did not correlate with endoscopic recurrence one year after ileocolonic resection, while inflammatory parameters, such as CRP values, showed good correlation with endoscopic recurrence at one year^[30,31]. We decided to use serum and fecal biological markers because they represent objective and quantifiable estimates of inflammatory activity. The main advantages are its simplicity and reproducibility; they can be repeated frequently to monitor changes in inflammatory activity, whether spontaneous or induced by treatment. We found that two or more elevated inflammatory parameters showed good correlation with endoscopic and morphological recurrence, especially in those scores indicating more severe lesions (i3-i4 or MR2-MR3). These techniques have been compared previously and our results are consistent with available data^[5,6].

The incidence of side effects (3.4%) was no different from other published series. The patient that developed

an adverse event also had to discontinue treatment with IFX previously. This is consistent with a recent study that concluded that elective switching from IFX to adalimumab may be associated with loss of tolerance within one year, and the recommendation is therefore to adhere to the first anti-TNF agent^[32].

We are aware of the potential limitations of our study. The main one is that there was no control group, so we cannot accurately compare our results with those obtained in other studies such as Regueiro *et al*^[14]. Secondly, the number of patients included in this cohort study was small, which may compromise statistical validity and not allow differences to be established in some of the variables measured. Lastly, five patients were treated concomitantly with immunomodulators, although there was no change in the dose regimen for these drugs before or during the study. It is difficult to assess the added benefit of co-treatment in preventing postoperative recurrence. In fact, in the Regueiro clinical trial, with a higher percentage of patients on immunomodulators, the concomitant use of immunomodulators did not appear to influence postoperative recurrence.

In summary, a significant percentage of CD patients require intestinal resection surgery over the course of their disease, and almost all show endoscopic recurrence one year after resection. This is the first published study on the effectiveness of adalimumab in preventing endoscopic and morphological recurrence after intestinal resection for CD. Our results strongly suggest that adalimumab is effective in patients with several risk factors associated with postoperative recurrence, such as smokers, penetrating pattern, or previous and extensive intestinal resections. Obviously, further randomized, controlled trials with better design and a larger number of patients are needed to confirm our conclusions and determine the duration of adalimumab maintenance treatment after surgery.

COMMENTS

Background

Crohn's disease (CD) is a complex chronic inflammatory bowel disease with an unpredictable course, in most cases accompanied by periodic recurrence and exacerbations. Up to 70% of patients with CD have to undergo surgery due to complications of the disease at least once in their lifetime, frequently resulting in ileocolonic anastomosis.

Research frontiers

Ileocolonoscopy was considered the gold standard in the evaluation of postoperative recurrence, being a useful tool to predict clinical recurrence, CD-related complications and the need for repeat surgery in the future.

Innovations and breakthroughs

A multicenter, prospective, observational study was conducted from June 2009 until June 2010. Authors consecutively included high-risk CD patients who had undergone an ileal/ileocolonic resection.

Applications

In this study, authors found adalimumab to be effective and safe in preventing recurrence after surgery for CD in a special subgroup of patients at high risk of recurrence. Intestinal resection is not curative and the majority of patients develop endoscopic recurrence even before clinical symptoms become apparent. Early detection and treatment of endoscopic lesions is essential to improve the long-term outcome for these patients and thus, to improve their quality of life.

Peer review

This manuscript is a prospective observational study that evaluated the effectiveness of adalimumab (ADA) for the prevention of postoperative recurrence of CD. Although this study lacks control group, this is the first prospective study on the efficacy of ADA for postoperative prevention of CD, so the presented data are important for the clinical practice and for the future controlled trial.

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Tissue transglutaminase levels above 100 U/mL and celiac disease: A prospective study

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Abstract

AIM: To investigate whether a tissue-transglutaminase antibody (tTGA) level ≥ 100 U/mL is sufficient for the diagnosis of celiac disease (CD).

METHODS: Children suspected of having CD were prospectively included in our study between March 2009 and September 2011. All patients with immune globulin A deficiency and all patients on a gluten-free diet were excluded from the study. Anti-endomysium antibodies (EMA) were detected by means of immunofluorescence using sections of distal monkey esophagus (EUROIMMUN, Luebeck, Germany). Serum anti-tTGA were measured by means of enzyme-linked immunosorbent assay using human recombinant tissue transglutaminase (ELIA Celikey IgA kit Phadia AB, Uppsala, Sweden). The

histological slides were graded by a single experienced pathologist using the Marsh classification as modified by Oberhuber. Marsh II and III lesions were considered to be diagnostic for the disease. The positive predictive values (PPVs), negative predictive values (NPVs), sensitivity and specificity of EMA and tTGA along with their 95% CI (for the cut off values > 10 and ≥ 100 U/mL) were calculated using histology as the gold standard for CD.

RESULTS: A total of 183 children were included in the study. A total of 70 (38.3%) were male, while 113 (61.7%) were female. The age range was between 1.0 and 17.6 years, and the mean age was 6.2 years. One hundred twenty (65.6%) patients had a small intestinal biopsy diagnostic for the disease; 3 patients had a Marsh II lesion, and 117 patients had a Marsh III lesion. Of the patients without CD, only 4 patients had a Marsh I lesion. Of the 183 patients, 136 patients were positive for EMA, of whom 20 did not have CD, yielding a PPV for EMA of 85% (95% CI: 78%-90%) and a corresponding specificity of 68% (95% CI: 55%-79%). The NPV and specificity for EMA were 91% (95% CI: 79%-97%) and 97% (95% CI: 91%-99%), respectively. Increased levels of tTGA were found in 130 patients, although only 116 patients truly had histological evidence of the disease. The PPV for tTGA was 89% (95% CI: 82%-94%), and the corresponding specificity was 78% (95% CI: 65%-87%). The NPV and sensitivity were 92% (95% CI: 81%-98%) and 97% (95% CI: 91%-99%), respectively. A tTGA level ≥ 100 U/mL was found in 87 (47.5%) patients, all of whom were also positive for EMA. In all these 87 patients, epithelial lesions confirming CD were found, giving a PPV of 100% (95% CI: 95%-100%). The corresponding specificity for this cut-off value was also 100% (95% CI: 93%-100%). Within this group, a total of 83 patients had symptoms, at least gastrointestinal and/or growth retardation. Three patients were asymptomatic but were screened because they belonged to a group at risk for CD (diabetes mellitus type 1 or positive family history). The fourth patient who

lacked CD-symptoms was detected by coincidence during an endoscopy performed for gastro-intestinal bleeding.

CONCLUSION: This study confirms based on prospective data that a small intestinal biopsy is not necessary for the diagnosis of CD in symptomatic patients with tTGA \geq 100 U/mL.

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Key words: Celiac disease; Diagnosis, Serology; Anti-tissue-transglutaminase antibodies; Anti-endomysium antibodies

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INTRODUCTION

Celiac disease (CD) is an immune-mediated enteropathy affecting approximately 1% of the worldwide population^[1-3]. The immune reaction occurs when genetically susceptible individuals ingest gluten, which is a storage protein in wheat and the related grain species barley and rye, and this reaction is completely reversible upon gluten withdrawal, which is currently the only available treatment for CD^[3-5]. The gold standard for the diagnosis of CD has been considered to be a small intestinal biopsy since the histological lesions of CD were discovered in 1954^[6,7]. However, a small intestinal biopsy is not only expensive, time-consuming and stressful for children and their parents but may also provide inconclusive or even false results, due to patchy disease or to inadequate quality or orientation of the biopsy specimen^[8-10]. Therefore, there has long been research focused on finding non-invasive markers to diagnose CD. For this purpose, the disease-associated auto-antibodies, especially anti-endomysium antibodies (EMA) and anti-tissue-transglutaminase antibodies (tTGA), have proven to be highly sensitive and specific^[6,11-14]. In fact, according to the new ESPGHAN guidelines for the diagnosis of CD, a confirmatory small intestinal biopsy is no longer necessary in genetically predisposed individuals who are symptomatic and who have a tTGA of at least 10 times the upper limit of normal, a positive EMA and a good clinical response to the gluten free diet^[15]. However, these new guidelines for children are mainly based on retrospective data^[16-18]. Because such study designs are subject to selection bias, and because

the diagnosis of CD implies a lifelong gluten free diet, the diagnosis of CD should be based on serology only when the chance of a false positive result is close to zero. The aim of the present study was therefore to evaluate prospectively whether the new diagnostic approach in patients with high tTGA levels is justified.

MATERIALS AND METHODS

Study population

All patients who were referred to the Wilhelmina Children's Hospital in Utrecht, the Netherlands with the suspicion of having CD were prospectively included in the study between March 2009 and September 2011. Patients were referred to us because of symptoms associated with CD (e.g., abdominal symptoms, growth retardation) or because they belonged to a group at risk for CD, e.g., patients with Down syndrome or Diabetes Mellitus and patients with a positive family history for CD. In this patient group, serology (both EMA and tTGA) was performed, and any patient with abnormal serology was biopsied, as were patients with negative serology but a high clinical suspicion of CD. Patients with immunoglobulin A (IgA) deficiency ($n = 8$) and patients on gluten restriction during the diagnostic work-up were excluded from the study. The study was performed according to the guidelines of the local medical ethics board.

Serological assessment

IgA EMA values were detected by indirect immunofluorescence using sections of distal monkey esophagus mounted on glass slides (EUROIMMUN, Luebeck, Germany). Serum IgA tTGA values were measured using the ELiA Celikey IgA kit (Phadia AB, Uppsala, Sweden). As recommended by the manufacturer, serum samples containing an antibody titer greater than 10 U/mL were considered positive. Total IgA was measured in all patients, and a serum IgA concentration below 0.07 g/L was regarded as IgA deficiency.

Histological evaluation

Duodenal biopsies were obtained by upper gastrointestinal endoscopy. An average of 3.1 biopsies (range: 1-8 biopsies) per patient were taken from the distal duodenum. Starting at the end of 2009, duodenal bulb biopsies were also routinely obtained during endoscopy, as recent studies suggested that this region could be the only affected site in CD^[19]. On average, 1.9 biopsies per patient were taken from this location with a range of 0 to 5.

Histological diagnosis for all patients was performed by a single experienced pathologist using the Marsh classification as modified by Oberhuber^[20,21]. The pathologist had no knowledge of the serological results or of the clinical presentation of the patients. An increased number of intraepithelial lymphocytes (Marsh I) were considered not to be diagnostic for CD. By contrast, Marsh I combined with crypt hyperplasia (i.e., Marsh II) or findings with villous atrophy (Marsh III) were consid-

Table 1 Results of small-intestinal biopsy and serology *n* (%)

		Biopsy data		
		Patients <i>n</i> = 183 (100.0)	Patients with CD <i>n</i> = 120 (65.6)	Patients with normal histology <i>n</i> = 63 (34.4)
IgA EMA	Negative	47 (25.7)	4 (3.3)	43 (68.3)
	Positive	136 (74.3)	116 (96.7)	20 (31.7)
IgA tTGA > 10	Negative	53 (29.0)	4 (3.3)	49 (77.8)
	Positive	130 (71.0)	116 (96.7)	14 (22.2)
IgA tTGA ≥ 100	Negative	96 (52.5)	33 (27.5)	63 (100)
	Positive	87 (47.5)	87 (72.5)	0 (0.0)

CD: Celiac disease; IgA: Immunoglobulin A; EMA: Anti-endomysium antibodies; tTGA: Anti-tissue-transglutaminase antibodies.

ered to be diagnostic for CD.

Statistical analysis

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the screening tests, which exhibited 95% confidence intervals (CI), were calculated using the histological evaluation as the gold standard. It was subsequently determined whether a tTGA level ≥ 100 U/mL is associated with a nearly perfect PPV.

RESULTS

A total of 183 patients met the inclusion criteria of the study. Of those patients, 70 (38.3%) were male, and 113 (61.7%) female with an age range of between 1.0 and 17.6 years and a mean age of 6.2 years. A total of 120 (65.6%) patients had a biopsy diagnostic for CD, of whom only 3 patients had a Marsh II lesion. In the remaining 63 (34.4%) patients, the diagnosis of CD could be excluded. Of the patients without CD, only 4 patients had Marsh I histology.

Of the total study population, 138 patients had positive EMA and/or tTGA antibodies, while 45 patients were negative for both antibodies. The patients who were negative for both antibodies underwent a small intestinal biopsy because of a strong clinical suspicion of CD (CD-like symptoms). Three of these patients had a Marsh III lesion, and one patient had a Marsh II lesion, while the diagnosis of CD could be excluded in the remaining 41 patients.

A positive EMA was found in 136 (74.3%) patients; 20 (31.7%) of them did not meet the histological criteria for CD (Table 1), giving a specificity of only 68% (Table 2). The corresponding PPV was 85%. The specificity of tTGA was slightly better (78%), with 116 of 130 positive patients being correctly diagnosed (Table 1). The corresponding PPV was also better at 89% (Table 2).

EMA was undetectable in 47 (25.7%) patients, of whom 43 indeed showed normal histology (Table 1). Consequently, the sensitivity and NPV of EMA were high with values of 97% and 91%, respectively (Table 2). These values were equally high for tTGA, i.e., 97% and

Table 2 Sensitivity, specificity, positive predictive value and negative predictive value of anti-endomysium antibodies and anti-tissue-transglutaminase antibodies (%)

	Sensitivity	Specificity	PPV	NPV
IgA EMA	97 (91-99)	68 (55-79)	85 (78-90)	91 (79-97)
IgA tTGA >10	97 (91-99)	78 (65-87)	89 (82-94)	92 (81-98)
IgA tTGA ≥ 100	73 (63-80)	100 (93-100)	100 (95-100)	66 (55-75)

The 95% CI are given in parentheses. IgA: Immunoglobulin A; EMA: Anti-endomysium antibodies; tTGA: Anti-tissue-transglutaminase antibodies; PPV: Positive predictive value; NPV: Negative predictive value.

92%, respectively. Illustratively, 49 of the 53 patients with negative tTGA did not have CD (Table 1).

A total of 42 patients (23.0%) had tTGA levels between 10 and 100 U/mL. Of those patients, only 28 (66.7%) had CD, while the diagnosis could be histologically excluded in 14 (33.3%) patients. Of the latter group, 3 patients had a Marsh I lesion. By contrast, the 87 patients with a tTGA level ≥ 100 U/mL all met the histological criteria for CD (Table 1), yielding a PPV of 100% (Table 2). All were also positive for EMA. Among these 87 patients, only 4 patients were asymptomatic. Three patients were screened because they belonged to a group at risk for CD (diabetes mellitus type 1 or a positive family history for CD). The fourth patient who lacked CD-symptoms was detected by coincidence during an endoscopy performed for gastro-intestinal bleeding. All other patients (*n* = 83) had typical symptoms (at least gastro-intestinal symptoms and/or growth retardation). After the diagnosis of CD was made, all patients adhered to the gluten-free diet, and the vast majority showed clinical improvement.

DISCUSSION

In patients with high tTGA levels, there is increasing evidence that a small intestinal biopsy is not needed to confirm the diagnosis of CD, as these increased levels are highly suggestive of the disease. This conclusion was also stated in the new ESPGHAN guidelines for the diagnosis of CD in the pediatric population^[15]. Briefly, these guidelines suggest that in symptomatic individuals who have tTGA levels of at least 10 times the upper limit of normal and who respond well to the gluten free diet, histological confirmation is unnecessary. However, prospective studies are needed to confirm the applicability of these guidelines in clinical practice.

The sole reliance on serology for the diagnosis of CD is appropriate only if the PPV is close to 100%. In this study, it was prospectively shown that 87/87 patients with a tTGA of at least 100 U/mL did indeed suffer from CD, giving a PPV of 100%. However, in this cohort, most of the patients had typical CD symptoms and responded well to the diet, while only 4 patients lacked any CD associated symptoms. Therefore, due to the underrepresentation of asymptomatic patients in this cohort, it can be questioned whether this perfect PPV will also be

observed in asymptomatic patients.

Comparable results were found in previous retrospective studies, showing that high tTGA levels are associated with histological lesions compatible with CD^[16-18]. Barker *et al*^[22] showed that 48 of 49 mostly symptomatic children with a tTGA level ≥ 100 U/mL had at least Marsh II enteropathy. Comparably, Donaldson *et al*^[23] showed that 38 of the 38 pediatric patients with tTGA ≥ 100 U/mL had Marsh III histopathology. A subsequent retrospective study, also in a pediatric population, showed that all symptomatic patients with tTGA of at least 100 U/mL who responded well to the diet had CD ($n = 111$), thereby reaching a PPV of 100%^[24].

Similarly, in a study conducted in a mixed adult/pediatric population, it was shown that a tTGA ≥ 100 U/mL occurs almost exclusively in the setting of Marsh III (73 of 76 patients) and that the 3 patients without villous atrophy had either a Marsh II ($n = 2$) or a Marsh I ($n = 1$) lesion^[25]. Likewise, a study performed in adults showed that 91 patients with a tTGA level of at least 10 times the upper limit all had at least Marsh II enteropathy^[16]. By contrast, Freeman reported that 3 of 14 adult patients with tTGA ≥ 100 U/mL did not have CD^[26]. Notably, in the latter 3 studies, an exact description of the clinical presentation of the patients was lacking^[16,25,26].

To the best of our knowledge, only one other prospective study has been performed in a mixed pediatric and adult population. This study showed that 1 of the 72 patients with a tTGA of at least 11.4 times the upper limit of normal had a normal small intestinal biopsy, yielding a PPV of 98.6%, which the authors considered to be insufficient for omitting a biopsy^[27]. However, in this study, the presence of symptoms was not taken into consideration, which may influence the PPV. In fact, the patient with this high level of tTGA and a normal biopsy did have an excellent clinical and serological response to the diet, suggesting that CD may have been missed histologically.

In conclusion, the current study shows that 87/87 patients with tTGA ≥ 100 U/mL had CD, which confirms the new ESPGHAN guidelines and other retrospective studies. However, because almost all studied patients in this study were symptomatic, omitting a biopsy should only be considered in this group. By contrast, in asymptomatic individuals, a small intestinal biopsy should still be performed, at least until more studies become available studying this specific group.

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COMMENTS

Background

In celiac disease (CD), the ingestion of gluten leads to a typical enteropathy characterized by an increase in the number of intra-epithelial lymphocytes, hyperplasia of the crypts and, in most cases, atrophy of the villi. The detec-

tion of these lesions in small intestinal biopsy specimens obtained by upper endoscopy is used to identify CD and is considered to be the gold standard investigation for the diagnosis of the disease. Serologically, the immune reaction to gluten can be detected by measuring disease-specific antibodies, the anti-endomysium antibodies (EMA) and the anti-tissue-transglutaminase antibodies (tTGA). In clinical practice, the measurement of these antibodies is used to screen for CD, as they are highly sensitive and specific. The aim of this study is to determine to what extent serology can replace a small intestinal biopsy for the diagnosis of CD.

Research frontiers

Retrospective studies have shown that a notably high titer of tTGA is strongly associated with the degree of enteropathy. Thus, high levels of tTGA seem to be sufficient for the diagnosis of CD. Therefore, the ESPGHAN guidelines for the diagnosis of CD in children and adolescents were revised this year, recommending that the diagnosis of CD can be made without histological confirmation in genetically predisposed children who are symptomatic, have a tTGA level of at least 10 times the upper limit of normal, as well as a positive EMA, and who respond well to the diet.

Innovations and breakthroughs

Because the new guidelines are based only on retrospective data, which allow a chance of selection bias, there was need for a prospective study to evaluate the guidelines in clinical practice. The authors therefore prospectively investigated the positive predictive value of tTGA ≥ 100 U/mL (= 10 times the upper limit) and demonstrated that it indeed reached 100%; i.e., all patients with these high tTGA values showed the small intestinal lesions that are diagnostic for CD. Therefore, in these patients, histological confirmation seems to be unnecessary. Because the majority of the studied patients had typical CD-symptoms and all of them were positive for EMA, omitting a biopsy should only be considered for symptomatic patients who also have increased levels of EMA.

Applications

The results of this study show that the new ESPGHAN guidelines in patients with very high tTGA levels can be used safely without being at risk for over-diagnosing the disease. Because a significant number of patients fulfill these criteria, i.e., almost half of the patients in this study, applying this guideline will have great clinical implications. Omitting a biopsy will not only reduce the stress and inconvenience associated with the diagnosis, but will also save time and significantly reduce costs.

Terminology

tTGA: These anti-bodies are directed against the enzyme tissue-transglutaminase, which is the auto-antigen in CD. This enzyme plays an essential role in eliciting the immune response against gluten; EMA: The endomysium is the intercellular matrix, which lies between the smooth muscle cells of the muscularis mucosae throughout the gastro-intestinal tract. It is rich in the enzyme tissue-transglutaminase. Antibodies directed against the endomysium are actually directed against tissue-transglutaminase.

Peer review

The authors present data from a prospective study on application of new cut-off values for diagnosing celiac disease in a population of children with high pre-test likelihood of disease. In the presented population, the specificity reaches 100 % if cut-off is raised to 10 times the regular cut-off. The draw-back is loss of specificity, but the subgroup with high titre can be diagnosed without duodenal biopsies. Upper endoscopy is demanding for children and mostly is performed in general anaesthesia, with the implications that may have for complications *etc.* The manuscript is well written, with a clear aim and conclusion.

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Comparison of bacterial quantities in left and right colon biopsies and faeces

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Abstract

AIM: To compare quantities of predominant and pathogenic bacteria in mucosal and faecal samples.

METHODS: Twenty patients undergoing diagnostic colonoscopy with endoscopically and histologically normal mucosa were recruited to the study, 14 subjects of which also supplied faecal (F) samples between 15 d to

105 d post colonoscopy. Mucosal biopsies were taken from each subject from the midportion of the ascending colon (right side samples, RM) and the sigmoid (left side samples, LM). Predominant intestinal and mucosal bacteria including clostridial 16S rRNA gene clusters IV and XIVab, *Bacteroidetes*, *Enterobacteriaceae*, *Bifidobacterium spp.*, *Akkermansia muciniphila* (*A. muciniphila*), *Veillonella spp.*, *Collinsella spp.*, *Faecalibacterium prausnitzii* (*F. prausnitzii*) and putative pathogens such as *Escherichia coli* (*E. coli*), *Clostridium difficile* (*C. difficile*), *Helicobacter pylori* (*H. pylori*) and *Staphylococcus aureus* (*S. aureus*) were analysed by quantitative polymerase chain reaction (qPCR). Host DNA was quantified from the mucosal samples with human glyceraldehyde 3-phosphate dehydrogenase gene targeting qPCR. Paired *t* tests and the Pearson correlation were applied for statistical analysis.

RESULTS: The most prominent bacterial groups were clostridial groups IV and XIVa+b and *Bacteroidetes* and bacterial species *F. prausnitzii* in both sample types. *H. pylori* and *S. aureus* were not detected and *C. difficile* was detected in only one mucosal sample and three faecal samples. *E. coli* was detected in less than half of the mucosal samples at both sites, but was present in all faecal samples. All detected bacteria, except *Enterobacteriaceae*, were present at higher levels in the faeces than in the mucosa, but the different locations in the colon presented comparable quantities (RM, LM and F followed by P_1 for RM vs F, P_2 for LM vs F and P_3 for RM vs LM: $4.17 \pm 0.60 \log_{10}/g$, $4.16 \pm 0.56 \log_{10}/g$, $5.88 \pm 1.92 \log_{10}/g$, $P_1 = 0.011$, $P_2 = 0.0069$, $P_3 = 0.9778$ for *A. muciniphila*; $6.25 \pm 1.3 \log_{10}/g$, $6.09 \pm 0.81 \log_{10}/g$, $8.84 \pm 1.38 \log_{10}/g$, $P_1 < 0.0001$, $P_2 = 0.0002$, $P_3 = 0.6893$ for *Bacteroidetes*; $5.27 \pm 1.68 \log_{10}/g$, $5.38 \pm 2.06 \log_{10}/g$, $8.20 \pm 1.14 \log_{10}/g$, $P_1 < 0.0001$, $P_2 \leq 0.0001$, $P_3 = 0.7535$ for *Bifidobacterium spp.*; $6.44 \pm 1.15 \log_{10}/g$, $6.07 \pm 1.45 \log_{10}/g$, $9.74 \pm 1.13 \log_{10}/g$, $P_1 < 0.0001$, $P_2 \leq 0.0001$, $P_3 = 0.637$ for *Clostridium* cluster IV; $6.65 \pm 1.23 \log_{10}/g$, $6.57 \pm 1.52 \log_{10}/g$, $9.13 \pm 0.96 \log_{10}/g$, $P_1 <$

0.0001, $P_2 \leq 0.0001$, $P_3 = 0.9317$ for *Clostridium* cluster XIVa; $4.57 \pm 1.44 \log_{10}/g$, $4.63 \pm 1.34 \log_{10}/g$, $7.05 \pm 2.48 \log_{10}/g$, $P_1 = 0.012$, $P_2 = 0.0357$, $P_3 = 0.7973$ for *Collinsella* spp.; $7.66 \pm 1.50 \log_{10}/g$, $7.60 \pm 1.05 \log_{10}/g$, $10.02 \pm 2.02 \log_{10}/g$, $P_1 \leq 0.0001$, $P_2 = 0.0013$, $P_3 = 0.9919$ for *F. prausnitzii*; $6.17 \pm 1.3 \log_{10}/g$, $5.85 \pm 0.93 \log_{10}/g$, $7.25 \pm 1.01 \log_{10}/g$, $P_1 = 0.0243$, $P_2 = 0.0319$, $P_3 = 0.6982$ for *Veillonella* spp.; $4.68 \pm 1.21 \log_{10}/g$, $4.71 \pm 0.83 \log_{10}/g$, $5.70 \pm 2.00 \log_{10}/g$, $P_1 = 0.1927$, $P_2 = 0.0605$, $P_3 = 0.6476$ for *Enterobacteriaceae*). The *Bifidobacterium* spp. counts correlated significantly between mucosal sites and mucosal and faecal samples (Pearson correlation coefficients 0.62, $P = 0.040$ and 0.81, $P = 0.005$ between the right mucosal sample and faeces and the left mucosal sample and faeces, respectively).

CONCLUSION: Non-invasive faecal samples do not reflect bacterial counts on the mucosa at the individual level, except for bifidobacteria often analysed in probiotic intervention studies.

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Key words: Gastrointestinal microbiota; Mucosa; Faeces; Real-time quantitative polymerase chain reaction; Sampling

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INTRODUCTION

Within the gastrointestinal tract, the bacterial community living dispersed in the luminal content differs from those living on the mucosal surface^[1] and reflects the health status of the gastrointestinal tract^[2]. The mucosal microbiota, intimately located on the host epithelium, has an active role in the host's immunity and forms an essential part of the protective mucosal barrier against invading pathogens^[3,4]. In general, the same main bacterial groups, *Firmicutes*, *Bacteroidetes* and *Proteobacteria*, dominate on the mucosa and in faeces, with the bacterial families of *Ruminococcaceae*, *Actinobacteria*, *Prevotellaceae*, *Porphyromonadaceae*, *Lachnospiraceae* and *Bacteroidaceae* being characteristic for the mucosal microbiota^[5,6].

Durban and colleagues assessed the microbial community from four randomly located, pooled mucosal biopsy samples and faecal samples retrieved from 9 volunteers between 2 wk to 8 wk post colonoscopy^[6]. They found that on family level taxonomy the mucosal microbiota was higher in richness and diversity and was presented by

a comparatively steep rarefaction curve, whereas on species level taxonomy no clear distinction between the two sample types was seen. This could imply that the mucosal environment allows for a variety of microbes to thrive with less exhaustive competition and that, in faeces, the niches are less compartmentalized and thus the most efficiently growing bacterial families dominate. Although both types of microbiota were predominant in *Firmicutes* and *Bacteroidetes*, the microbial composition was clearly more dependent on the sample type (biopsy or faeces) than the individual being sampled and the mucosal microbiota was found to be underrepresented in the faecal samples^[6].

Hong *et al.*^[5] recently published a study in which they applied an elaborate sampling schema which enabled the comparison of closely (1 cm apart) and distantly (left and right colon) located mucosal biopsies from 5 (five) subjects. Unexpectedly, the microbiota on the mucosal surface appeared to be unique, even when comparing closely situated sampling sites (1 cm distance)^[5], even though the intestinal microbiota had previously been shown to be subject-specific in several studies^[7-9]. Thus the study by Hong *et al.*^[5] raises further concerns regarding the representativeness of mucosal samples from a certain anatomical location and of faecal samples in relation to the overall mucosal microbiota. Possibly a single mucosal biopsy gives a less reliable picture of the status of the overall gastrointestinal tract than a faecal sample, as faeces represents an end-point view of the ecosystem.

Clearly, for a thorough evaluation of the species composition of the mucosa, faecal material is not a representative sample. However, in many cases the alterations in the quantities of selected bacterial groups or species in the gastrointestinal tract are of interest and, in such a setting, the alterations in bacterial quantities at different mucosal locations and in faeces may be more uniformly expressed, depending on the target species. Thus, the present study focused on the quantification of selected gastrointestinal bacterial groups or species being either dominant, potentially pathogenic, or often encountered on the mucosal surface.

MATERIALS AND METHODS

Subjects

Twenty patients (8 men and 12 women, aged 61 ± 15 years, range: 33-85 years), who underwent colonoscopy between June 2010 and Feb 2011 at the Sahlgrenska University Hospital Östra, Gothenburg, were included in the study. Colonoscopy was performed due to various abdominal complaints, such as diarrhoea, constipation and/or abdominal pain as well as lower gastrointestinal bleeding and/or iron-deficiency anaemia (Table 1). The prerequisite for inclusion into the study was normal-appearing mucosa in the entire colon, and thus patients with any significant pathology, such as colonic polyps, inflammatory bowel disease, malignancy, ischemic colitis *etc.*, were excluded. The possibility of microscopic colitis

Table 1 Demographic and clinical characteristics of study subjects

Patient No.	Age	Gender	Days passed ¹	Reason for referral to colonoscopy	Diverticulosis
1	53	F	105	Iron deficiency anaemia	Yes
2	41	M	NA	Constipations	No
3	43	M	NA	Functional diarrhoea	No
4	64	M	98	IBS	No
5	85	M	NA	Rectal bleeding	Yes
6	75	M	15	Iron deficiency anaemia	Yes
7	63	M	NA	IBS	No
8	62	F	29	IBS, constipation	No
9	81	M	NA	Iron deficiency anaemia	Yes
10	72	F	23	IBS, diarrhoea	Yes
11	41	F	21	Rectal bleeding	Yes
12	74	F	26	Iron deficiency anaemia	Yes
13	75	F	26	Follow-up after diverticulitis	Yes
14	68	F	19	IBS, diarrhoea	No
15	47	F	19	Follow-up after diverticulitis	Yes
16	80	F	32	Iron deficiency anaemia	Yes
17	54	M	NA	Rectal bleeding	No
18	57	F	21	Rectal bleeding	Yes
19	33	F	24	Diffuse abdominal pain	No
20	51	F	28	Rectal bleeding	No

¹From colonoscopy to faeces sampling. F: Female; M: Male; NA: Not analysed; IBS: Irritable bowel syndrome.

was ruled out through light microscopic examination of biopsy specimens obtained from the mid-portion of the colon ascendens, as well as from the sigmoid. On the other hand, the presence of colonic diverticula was accepted provided there were no signs of acute diverticulitis and/or diverticulosis-associated colitis. Eight tissue specimens for analysis were obtained from the midportion of the ascending colon, as well from the sigmoid colon, using regular biopsy forceps. One of these specimens from each site was used for analysis of the microbiota. There were no complications related to the colonoscopy or biopsy procedures. In addition, faecal samples were

collected post-colonoscopy (15 d to 105 d and unknown for 6 subjects) from 14 subjects. The ethics committee of the University of Gothenburg approved the study and written informed consent was obtained from each of the patients.

Isolation of DNA and microbial quantification

Bacterial DNA was extracted from the mucosal and faecal samples with the Promega Wizard[®] Genomic DNA Purification Kit, A1125, (Promega Corporation, Madison, WI, United States) with some minor modifications applied. The mucosal samples were cut in half with scalpel knives and DNA was extracted from both pieces. Homogenisation of the samples was done by bead beating for 3 × 30 s at 6800 g in a 1.4 mL Bertin VK01 glass bead tube, before continuing according to the protocol. Extraction of bacterial DNA from faecal samples was performed as described previously^[10]. The DNA concentrations were measured with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States) and samples were stored at -20 °C until quantitative polymerase chain reaction (qPCR) analysis.

The qPCR reactions were performed using Applied Biosystems Real-Time PCR system equipment (7500 Fast, Applied Biosystems, Foster City, CA, United States) and software applying in-house optimized assay conditions for the primer sequences presented in Table 2. Reactions were run in a 25 µL volume, except for the *Helicobacter pylori* (*H. pylori*) and *Clostridium difficile* (*C. difficile*)-targeting qPCR analysis, which were run in a 15 µL volume. Mucosal or faecal microbial DNA was applied as template in quantities of 25 ng or 5 ng respectively. All reactions were run in triplicate. For the human glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene assay, 5 ng of mucosal microbial DNA was used as template. In order to obtain standard curves, ten-fold serial dilutions ranging from 1 pg to 10 ng of the genomic DNA of selected bacterial species or human DNA (Table 2) were used. Results were expressed as log₁₀ genomes per gram of sample (wet weight), taking into account the size and the 16S rDNA copy number of the standard species genome.

Statistical analysis

For mucosal samples, the proportion of host DNA was estimated according to the *GAPDH* qPCR result and subtracted prior to calculations. Outlier values and target bacteria that were not normally distributed due to too low prevalence were removed from the data set. Normality of the data was checked with the D'Agostino and Pearson omnibus *K*² test and comparisons within bacterial groups between sampling sites were done using paired *t* tests. Correlations between different sample types for each qPCR assay were analysed using Pearson's correlation coefficient. Statistical analysis were performed with Prism 5 Version 5.01 (GraphPad Software, Inc., San Diego, United States).

Table 2 Real-time polymerase chain reaction primers, probes and assay conditions

qPCR assay	Primers	Chemistry ¹	Annealing temperature (°C)	Standard species	Primer reference	Reaction condition reference
<i>Akkermansia muciniphila</i>	CAGCACGTGAAGGTGGGGAC	FAST SYBR Green Mastermix; 300 nmol/L each primer	58	<i>Akkermansia muciniphila</i> ATCC BAA-835	Png <i>et al.</i> ^[20]	This study
	CCTTGGCGGTGGCTTCAGAT					
<i>Bacteroidetes</i>	GGCGACCGGCGCACGGG	Power SYBR Green Mastermix; 300 nmol/L each primer	65	<i>Bacteroides fragilis</i> ATCC 25285	Nakanishi <i>et al.</i> ^[21]	This study
	GRCCTTCTCTCAGAACCC					
<i>Bifidobacterium spp.</i>	CCTGGTAGTCCACGCCGTAA	FAST TaqMan Mastermix; 300 nmol/L each primer, 200 nmol/L probe	60	<i>Bifidobacterium adolescentis</i> JCM 1275	Mäkiyuokko <i>et al.</i> ^[22]	Mäkiyuokko <i>et al.</i> ^[22]
	CAGGCGGGATGCTTAACG ATCCAGCATCCACCG					
<i>Clostridium cluster IV</i>	GCACAAGCAGTGGAGT	SYBR Green Core Reagents; 1.5 nmol/L MgCl ₂ , 250 nmol/L each primer	62	<i>Clostridium leptum</i> DSM 753	Matsuki <i>et al.</i> ^[23]	This study
	CTTCTCCGTTTTGTCAA					
<i>Clostridium cluster XIVab</i>	GAWGAAGTATYTCGGTATGT	Power SYBR Green Mastermix; 300 nmol/L each primer	52	<i>Clostridium boltae</i> DSM 15670	Song <i>et al.</i> ^[24]	Lahtinen <i>et al.</i> ^[25]
	CTACGCWCCCTTTACAC					
<i>Clostridium difficile</i>	TTGAGCGAATTACTTCGGTAAAGA	FAST SYBR Green Mastermix; 300 nmol/L each primer	60	<i>Clostridium difficile</i> ATCC 9689	Lahtinen <i>et al.</i> ^[25]	Lahtinen <i>et al.</i> ^[25]
	CCATCCTGTACTGGCTCACCT					
<i>Collinsella aerofaciens</i>	CCCGACGGGAGGGGAT	Power SYBR Green Mastermix; 300 nmol/L each primer	60	<i>Collinsella aerofaciens</i> ATCC25986	Kassinen <i>et al.</i> ^[26]	This study
	CTTCTGCAGGTACAGICTTGA					
<i>Domain bacteria</i>	CATRHHYTCGTCAGCTCGT	FAST SYBR Green Mastermix; 200 nmol/L each primer	60	<i>Enterococcus faecium</i> DGCC 2063	This study	This study
	GCGGTGTGTRCAAGRCCC					
<i>Enterobacteriaceae</i>	TGCCGTAACITCGGGAGAAGGCA	SYBR Green Core Reagents; 2 nmol/L MgCl ₂ , 200 nmol/L each primer	58	<i>Enterococcus faecium</i> DGCC2063	Matsuda <i>et al.</i> ^[27]	This study
	TCAAGGACCAGTGTTCAGTGTC					
<i>Escherichia coli</i>	ACTGGAATACTTCGGATTACAGATACGT	FAST TaqMan Mastermix; 100 nmol/L each primer, 30 nmol/L probe	60	<i>Escherichia coli</i> ATCC 11775	Kačlíková <i>et al.</i> ^[28]	This study
	ATCCCTACAGATTCATTCACGAAA					
	CAGCAGCTGGGTGGCATCAGTTATTCG-tamra					
<i>Faecalibacterium prausnitzii</i>	CCCTTCAGTGCCGCAGT	SYBR Green Core Reagents; 4 nmol/L MgCl ₂ , 250 nmol/L each primer	62	<i>Faecalibacterium prausnitzii</i> ATCC 27768	Rinttilä <i>et al.</i> ^[16]	This study
	GTCGCAGGATGTCAAGAC					
Human GAPDH	GGTAAGGAGATGCTGCATTCCG	Power SYBR Green Mastermix; 300 nmol/L each primer	60	Human DNA	Png <i>et al.</i> ^[20]	This study
	CGCCCAATACGACCAAAATCTAA					
<i>Helicobacter pylori</i>	GAAGATAATGACGGTATCTAACGAATAA	FAST SYBR Green Mastermix; 400 nmol/L each primer	58	<i>Helicobacter pylori</i>	Modified from Rinttilä <i>et al.</i> ^[16]	This study
	CATAGGATTCACACCTGACTGACTAT					
<i>Staphylococcus aureus</i>	GCGATTGATGGTGATACGGTT	Power SYBR Green Mastermix; 300 nmol/L each primer	60	<i>Staphylococcus aureus</i> ATCC 29213	Brakstad <i>et al.</i> ^[29]	This study
	AGCCAAGCCTTGACGAATAAAGC					
<i>Veillonella</i>	AYCAACCTGCCCTTCAGA	Power SYBR Green Mastermix; 200 nmol/L each primer	60	<i>Veillonella parvula</i> DSM 2008	Rinttilä <i>et al.</i> ^[16]	This study
	CGTCCCGATTAACAGAGCTT					

¹Manufactured by (Applied Biosystems, Foster City, CA). GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; qPCR: Quantitative polymerase chain reaction.

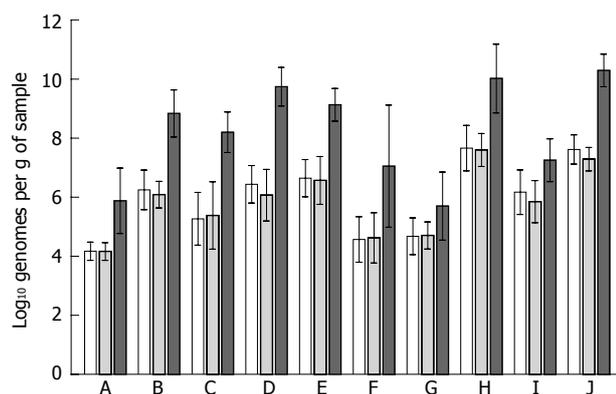


Figure 1 Quantities of bacterial groups detected on the mucosa and in faeces. The bacterial targets A: *Akkermansia muciniphila*; B: *Bacteroidetes*; C: *Bifidobacterium* spp.; D: *Clostridium* cluster IV; E: *Clostridium* cluster XIVab; F: *Collinsella aerofaciens*; G: *Enterobacteriaceae*; H: *Faecalibacterium prausnitzii*; I: *Veillonella* spp.; J: Eubacteria are represented with the three sample types side-by-side (biopsies from the right colon as white bars with pattern; biopsies from the left colon as grey bars; faecal samples with dark grey bars). The bacterial quantities between the two mucosal samples did not differ according to paired *t*-tests, whereas the faecal quantities of all analysed bacteria were significantly higher than those detected for either mucosal site ($P < 0.05$), except for *Enterobacteriaceae*. The error bars denote the 95% CI.

RESULTS

Preliminary qPCR analysis from six mucosal and three faecal samples, showed an average percentage of human DNA of $60.74\% \pm 12.26\%$ and $0.02\% \pm 0.02\%$ respectively. Thus, the proportion of bacterial DNA was not further analysed for faecal samples as they were assumed to demonstrate 100% bacterial DNA. Among the bacterial groups and species analysed in this study, no alterations were detected between the colonic samples originating from the right and left sides of the colon (Figure 1). The clostridial clusters XIVab and IV, *Bacteroidetes* and *Faecalibacterium prausnitzii* (*F. prausnitzii*) were the most abundant bacteria in all sample types.

H. pylori and *Staphylococcus aureus* were not detected in any of the samples. *C. difficile* was detected in four samples, all originating from different subjects: one mucosal sample originating from the left side of the colon and three faecal samples. The *C. difficile* positive subjects were all female, aged 47, 74, 57 and 33 and subject to colonoscopy due to diverticulitis follow-up, iron deficiency anemia, rectal bleeding and diffuse abdominal pain. All, however, had endoscopically and histologically normal appearing mucosa. *Escherichia coli* (*E. coli*) was detected in less than half of the mucosal samples at both sites, while present in all faecal samples at $\log_{10} 5.92 \pm 1.04$ genomes per gram of faeces. *H. pylori*, *Staphylococcus aureus* (*S. aureus*), *C. difficile* and *E. coli* were not included in the statistical analysis due to low prevalence.

For the whole subject group, the abundances of different bacteria appeared to follow the same trend in the mucosa and faeces (Figure 1), whereas at the individual level, only *Bifidobacterium* spp. quantities correlated significantly between the two mucosal sampling sites and faeces (Table 3). The two mucosal sites also correlated signifi-

cantly for the quantities of *Bacteroidetes*, *Clostridium* cluster XIVab and *F. prausnitzii* (Table 3).

DISCUSSION

The right and left segments of the colon show differences in physiology and motility, creating different environments for bacteria in the murine^[11] and human^[1,5] mucosa. Our aim was to analyse the quantities of predominant gastrointestinal bacteria and putative pathogenic species in relation to the site of mucosal sampling. We studied 20 patients undergoing diagnostic colonoscopy that displayed, both endoscopically and histologically, normal appearing mucosa. In addition, faecal samples were obtained from 14 subjects between 15 d to 105 d post colonoscopy to assess how well a faecal sample can represent the mucosal microbiota with a 16S rRNA gene-based qPCR. Since in whole community analysis (i.e., 16S rDNA pyrosequencing and metagenomics) the abundance data represents relative proportions of the whole with all groups affecting the result, a targeted analysis, such as qPCR, which quantifies the target independently, could allow for a less biased comparison of quantities. This possibly also results in more uniform representation between different mucosal sampling sites.

The selected bacterial quantities analysed in the present study were comparable between the two mucosal sampling sites for each individual, although previous analysis covering the overall mucosal microbiota with higher taxonomic precision have shown definite heterogeneity between different sampling sites in both humans^[5] and rodents^[11,12]. However, cleansing of the colon prior to colonoscopy may have distorted the mucosal microbiota at the genus level^[13] and possible faecal contamination of the mucosal biopsies may diminish the degree of heterogeneity between mucosal biopsy samples. In addition, the 20 subjects that were analysed, had a considerably heterogeneous background in relation to gastrointestinal health and age, possibly resulting in a wide range of detected microbial quantities reducing the sensitivity of comparative analysis. Of the analysed bacterial groups for both mucosal and faecal quantities, only *Bifidobacterium* spp. correlated significantly between the different sample types (i.e., a high abundance in faeces predicted a high abundance in mucosal samples at both sites and *vice versa*, although the faecal quantities were on average higher than the mucosal quantities). As *Bifidobacterium* spp. have previously been associated with both compromised functional gastrointestinal health^[7] and, in some studies, with aging^[14], the subjects of the present study may present a substantially wide range of abundance for gastrointestinal bifidobacteria, enabling more evident correlation: 6 of the 20 subjects had irritable bowel syndrome or abdominal pain, and the subjects' ages varied broadly. The two mucosal sites, the midportion of the ascending colon and the sigmoid, were also comparable in terms of *Bacteroidetes*, *Clostridium* cluster XIVab and *F. prausnitzii* for each subject. The wide time range between colonoscopy and faecal sampling post

Table 3 Pearson correlations between sample types

Bacterial group/study period	Right colon vs left colon		Right colon vs faecal sample		Left colon vs faecal sample	
	Correlation coefficient	P value	Correlation coefficient	P value	Correlation coefficient	P value
<i>Akkermansia muciniphila</i>	0.14	0.63	-0.01	0.97	0.36	0.26
<i>Bacteroidetes</i>	0.61	0.02	0.45	0.12	0.17	0.61
<i>Bifidobacterium</i>	0.71	0.01	0.62	0.04	0.81	0.00
Clostridium Cluster IV	0.26	0.39	0.26	0.44	0.17	0.64
Clostridium Cluster XIVab	0.71	0.00	0.54	0.06	0.50	0.09
<i>Collinsella aerofaciens</i>	0.38	0.25	0.63	0.13	-0.87	0.13
<i>Eubacteria</i>	0.19	0.52	0.01	0.97	-0.31	0.33
<i>Enterobacteriaceae</i>	0.38	0.20	0.59	0.03	0.31	0.35
<i>Faecalibacterium prausnitzii</i>	0.55	0.04	0.76	0.00	0.28	0.38
<i>Veillonella</i>	0.33	0.46	0.64	0.09	0.46	0.54

Significant correlations ($P < 0.05$) are designated with bold font.

colonoscopy may bias the correlation analysis. Nevertheless, no statistically significant correlations were found for age, reason for referral to colonoscopy, or for the time that elapsed between colonoscopy and faecal sampling for any of the bacteria analysed (data not shown). Due to the invasive and burdensome nature of colonoscopy, no timely follow-up was possible.

In general, average levels of bacteria were higher in the faeces than in the mucosa and comparable with previously published 16S rRNA gene-targeting qPCR data^[15-17]. The clostridial clusters XIVab and IV and *Bacteroidetes* were the most abundant bacterial groups in both sample types, in accordance with the present view of human mucosal and faecal microbiota^[5-7,18]. For *Enterobacteriaceae* the higher abundance in faeces was not statistically significant, but a similar trend was evident between the left side mucosal and faecal samples. When analysed in relation to the eubacterial counts (i.e., as proportional values), the majority of the analysed bacteria were as prominent on the mucosa as in the faeces (data not shown), as has previously been shown with RNA-targeting fluorescent in situ hybridization for a selected set of bacterial groups^[19]. However, only non-parametric analysis of the target bacteria were possible using proportional values as the data was no longer normally distributed. Nevertheless, even though *Bifidobacterium spp.* was the only bacterial group that correlated within individuals, for the subject group as a whole the average faecal and mucosal bacterial quantities appeared to be associated, as abundant mucosal bacteria were also abundant in faeces (Figure 1). As for the prevalence of the different bacteria, only *Collinsella aerofaciens* was significantly more prevalent on the mucosa (right side of the colon) than in faeces according to Fisher's test (data not shown). *F. prausnitzii* was detected in all sample types with quantities above the eubacterial count (\log_{10} 7.6 ± 1.5 , 7.6 ± 1.1 and 10.0 ± 2.0 bacteria per gram of sample for right colon, left colon and faecal sample, respectively; Figure 1), implying technical issues related to the analysis, as it has previously been detected at the level of \log_{10} 8 to 9^[16]. The potentially pathogenic bacteria (*H. pylori*, *S. aureus*, *C. difficile*) were rarely detected even though 11 of the 20 study subjects were over 60 years of age and all had compromised gastrointestinal health prior

to colonoscopy. *E. coli*, which is a common commensal gastrointestinal species, in addition to being a potential pathogen, was more prominent.

Taken together, faecal samples did not reflect quantities of bacteria in the intestinal mucosa at the individual level, except for *Bifidobacterium spp.* which has often been analysed in pro- and prebiotic intervention studies. Although the mucosal microbiota is site-specific in terms of use of community profiling methods, selected bacterial quantities did not differ, even between distant locations in the colon and thus less exhaustive biopsy sampling may be sufficient to evaluate bacterial quantities on the mucosa. At the group level, faecal sampling may be adequate.

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COMMENTS

Background

The intestinal microbiota has been recognized as an important factor in the maintenance of good health and in the prevention of disease and has thus received a steadily increasing amount of attention in research. It has been widely acknowledged that the mucosal and faecal microbiotas are not alike and that even closely situated mucosal samples differ from each other. Thus sampling schemas highly affect the outcome when analyzing intestinal bacteria and an important research focus has been to gain a better insight into the selection of the most appropriate methodologies in each setting and to understand how the techniques compare and complement one another.

Research frontiers

The aim of the present study was to test whether quantities of distinct bacterial groups, genera or species, as opposed to a whole community analysis, could be quantified in a representative manner from mucosal samples originating from different sites in the colon and from faecal samples. Comparable bacterial quantities at different mucosal sites would allow less exhaustive biopsy sampling during colonoscopy while a correlation between mucosal and faecal quantities would allow predictions to be made on the mucosal microbiota from non-invasive faecal samples.

Innovations and breakthroughs

Real-time quantitative polymerase chain reaction (qPCR) allows independent

comparison of each target bacterial group, genera and species between the different samples, whereas whole community approaches are restricted to proportional quantities. In the present study, selected gastrointestinal bacterial groups or species being either dominant, potentially pathogenic, or often encountered on the mucosal surface were quantified from three kinds of samples of up to twenty subjects. Distantly situated mucosal sites were found to have comparable bacterial quantities in an individual, whereas the faecal quantities did not reflect mucosal quantities at the individual level for most bacteria.

Applications

With quantitative analysis of selected bacteria, mucosal biopsies taken from different parts of the colon are comparable, allowing less exhaustive biopsy sampling. Faecal samples, however, poorly reflect mucosal quantities.

Terminology

Quantitative real-time PCR is based on detecting the amount of amplified product during each PCR cycle and comparing the detection threshold cycle to a standard dilution series. Primer and probe design allows a vast array of target selection and taxonomic depth to be applied.

Peer review

This study reports the analysis of several bacterial species, including resident and pathogenic bacteria present in the right and left segments of the human colon, compared with species present in faeces. The study is well conducted and the results are interesting, improving knowledge of the microbiome present in the human colon.

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Significant decrease in prevalence of *Helicobacter pylori* in the Czech Republic

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Abstract

AIM: To study possible decrease in prevalence of *Helicobacter pylori* (*H. pylori*) infection in the Czech Republic within a 10-year period.

METHODS: A total of 22 centres entered the study. The catchment areas of these centres covered cities and towns with more than 20 000 inhabitants, smaller towns ($\leq 20\ 000$ inhabitants) with surrounding villages and rural areas, and were spread over the

whole country, corresponding well to the geographical distribution of the Czech population. A total of 1 837 subjects (aged 5-98 years) took part in the study, randomly selected out of 38 147 people from the general population. *H. pylori* infection was investigated by means of a ^{13}C -urea breath test. Breath samples in duplicates were analysed using isotope ratio mass spectrometry. The cut-off point was 3.5. Social and demographic characteristics were based on data from self-completed questionnaires.

RESULTS: The overall prevalence of *H. pylori* infection was 23.5% (430/1826), and 4.8% (20/420) in children aged 15 or less. There was no statistically significant difference in prevalence between males (24.3%; 208/857) and females (22.9%, 222/969, $P = 0.494$). *H. pylori* infection was strongly associated with higher age, among subjects aged 55+ years, prevalence of *H. pylori* infection was 39.8% (252/633, $P < 0.001$). The highest prevalence of *H. pylori* infection was found among persons aged 55-64 years (43.9%, 97/221) and 75+ years (37.9%, 58/153). Among study subjects aged 15+ years, prevalence of *H. pylori* infection was significantly increased in those with lowest education (odds risk 3.19, 95% CI 1.87-5.47). Compared to never married (14.1%), the prevalence of *H. pylori* infection was statistically significantly higher among married (35.4%, 246/694, $P < 0.001$), divorced (36.8%, 49/133, $P < 0.001$) and widowed study subjects (40.2%, 45/112, $P < 0.001$), both in minimally and fully adjusted analysis. There was no significant difference in the prevalence of *H. pylori* infection between married and widowed subjects (35.4%, 246/694 vs 40.2%, 45/112, $P = 0.389$). There was little variation in smoking prevalence across categories of smoking and there was no evidence of an increased risk of *H. pylori* infection among current or past smokers in our data (odds risk 1.04 with 95% CI 0.78-1.40 for current smokers; odds ratio 0.83 with 95% CI 0.60-1.16 for former smokers). The current prevalence of *H. pylori*

in 2011 was significantly lower compared to the prevalence reported from identical geographical areas in 2001 (23.5% vs 41.7%, $P < 0.001$).

CONCLUSION: The overall prevalence of *H. pylori* infection in the general population has fallen substantially in the Czech Republic over the past 10 years.

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Key words: Epidemiology; *Helicobacter pylori*; Czech Republic; ^{13}C -urea breath test; Decline of prevalence

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is still the most common human infection worldwide. It is estimated that 50%-80% of the population is infected^[1-3]. The prevalence in developed countries of Europe is 10%-30% (with an increase of 1%-3% for each age decade). Up to 95% of the adult population is infected in developing countries^[4-8].

Our group accomplished a large multi-centre epidemiologic study on the prevalence of *H. pylori* and dyspepsia in the Czech Republic, a Central European country, in 2001^[9-11]. The Project was executed on a total of 2509 persons aged 5-100 years, randomly selected out of 30 012 subjects from the general population. *H. pylori* infection was investigated by means of ^{13}C -urea breath test. The overall prevalence of *H. pylori* was 41.7% in 2001^[9]. Meanwhile, several concerns have been raised about the possible decrease of *H. pylori* prevalence in developed countries. The aim of our current multi-centre prospective study was to evaluate the prevalence of *H. pylori* infection in the Czech Republic using the same methods in a representative sample of general unselected population from the same geographical areas 10 years later.

MATERIALS AND METHODS

Study population

A total of 22 centres entered the study. They included 15 centres of general practitioners for adults and 7 for children and adolescents. These centres covered cities and towns with more than 20 000 inhabitants (10 centres),

smaller towns ($\leq 20\ 000$ inhabitants) with surrounding villages (9 centres) and rural areas (3 centres), and were spread over the whole country, corresponding well to the geographical distribution of the Czech population. A total of 1837 subjects (aged 5-98 years) took part in the study, randomly selected out of 38 147 registered males and females in this age range.

Urea breath test

Urea breath tests were performed in the morning after overnight fasting by means of ^{13}C -urea breath test^[12]. Citric acid solution (3 g dissolved in 150 mL of still water) was given initially as a test drink. Five min later two baseline exhaled breath samples were collected into 20-mL vacutainers using a straw. Thereafter all persons ingested 75 mg ^{13}C -urea (*Helicobacter Test* INFAI, INFAI GmbH, Köln, Germany) dissolved in 50 mL of still water with 1 g citric acid (at time 0). Breath samples were collected in duplicates using a straw into 20-mL vacutainers after 30 min. Tubes with breath samples were sent to a single analytical centre by post and measured within a one-week period. Breath samples in duplicates were analysed using isotope ratio mass spectrometry (AP 2003, Analytical Precision Products, Cambridge, United Kingdom). The cut-off point was 3.5. Results of ^{13}C -urea breath test for *H. pylori* status were obtained for 99.4% (1826/1837) of subjects.

Questionnaires

Data were collected by self-completed questionnaires distributed to adults and parents of children aged 5-15 years. The questionnaire included information on place of residence in childhood, mother's and father's education, access to running warm water in childhood, crowding in childhood and number of siblings. Information on the study subjects' current place of residence, education, marital status, self-reported socio-economic group and smoking habits was also collected in the questionnaire and was used in the analysis of determinants of *H. pylori* positivity in subjects aged above 15 years.

Ethical approval

The study was approved by the University Ethics Committee. All participants received detailed written information about the Project in advance and signed written consent (parents on behalf of their children). For all data obtained, all personal identification information was deleted in compliance with the laws for the protection of confidentiality of the Czech Republic.

Statistical analysis

The data was analysed using STATA statistical software (StataCorp. 2011. Stata Statistical Software: Release 12, College Station, TX). Education was analysed in six categories (university, secondary, vocational higher and lower, elementary and students) and marital status in five categories (single, first time married, re-married, divorced and widowed). In the analysis, we used three categories

Table 1 Prevalence of *Helicobacter pylori* by age and gender in a representative sample of Czech population *n* (%)

	Males	Females	Total
Age (yr)			
5-14	188 (4.8)	197 (5.1)	385 (4.9)
15-24	124 (10.5)	151 (7.9)	275 (9.1)
25-34	97 (16.5)	107 (13.1)	204 (14.7)
35-44	84 (30.9)	79 (19.0)	163 (25.1)
45-54	73 (42.5)	93 (34.4)	166 (37.9)
55-64	94 (42.5)	127 (44.9)	221 (43.9)
65-74	122 (38.5)	137 (36.5)	259 (37.4)
≥ 75	75 (34.7)	78 (41.0)	153 (37.9)

Czech population examined in 2011 (*n* = 1826).

of smoking (never smoker, past smoker and current smoker). Proportions were compared using Chi-square test and the relative risk of *H. pylori* infection was studied in logistic regression with adjustment for age, gender and other social variables. Multivariable analyses were restricted to subjects with non-missing data on the variables studied.

RESULTS

The overall prevalence of *H. pylori* infection was 23.5% (430/1826), and 4.8% (20/420) in children aged 15 or less. There was no statistically significant difference in prevalence between males (24.3%, 208/857) and females (22.9%, 222/969, *P* = 0.494). *H. pylori* infection was strongly associated with higher age (Table 1).

Among study subjects aged above 15 years, prevalence of *H. pylori* infection was significantly increased in those with lowest education (Table 2). Compared to never married, the prevalence of *H. pylori* infection was statistically significantly higher among married and divorced study subjects, both in minimally and fully adjusted analyses (Table 2). There was little variation in smoking prevalence across categories of smoking and there was no evidence of an increased risk of *H. pylori* infection among current or past smokers in our data (Table 2).

The current prevalence of *H. pylori* in 2011 was significantly lower compared to the prevalence reported from identical geographical areas in 2001 (23.5% *vs* 41.7%, *P* < 0.001).

DISCUSSION

Our multi-centre prospective study proved that the current prevalence of *H. pylori* in 2011 is significantly lower compared to the prevalence of identical geographical areas in 2001. Both our studies were based on a representative sample of the general population, covering large and small urban population as well as rural areas and using ¹³C-urea breath test as a gold standard for *H. pylori* diagnostics. Thus our results are reliable and trustworthy. The decline of *H. pylori* was found not only in overall prevalence but also in particular age decades. Explanation of this phenomenon is not easy and unequivocal. Although

Table 2 Prevalence of *Helicobacter pylori* by social characteristics and smoking in a representative sample of Czech population

	<i>n</i>	<i>H. pylori</i> (%)	OR ¹ (95% CI)	OR ² (95% CI)
Education				
University	351	25.6	1.0	1.0
Secondary	435	26.9	0.99 (0.71,1.38)	1.02 (0.73,1.43)
Vocational (higher)	66	31.8	1.31 (0.73,2.33)	1.33 (0.74,2.39)
Vocational (lower)	307	40.1	1.63 (1.16,2.29)	1.68 (1.18,2.37)
Elementary	80	53.7	2.84 (1.69,4.75)	3.19 (1.87,5.47)
Studying	188	8.0	0.51 (0.27,0.96)	0.60 (0.31,1.16)
Marital status				
Never married	488	14.1	1.0	1.0
First time married	566	34.8	1.75 (1.18,2.59)	1.55 (1.03,2.33)
Re-married	128	38.3	1.91 (1.15,3.20)	1.77 (1.05,2.99)
Divorced	133	36.8	1.88 (1.13,3.13)	1.74 (1.03,2.92)
Widowed	112	40.2	1.60 (0.88,2.92)	1.20 (0.65,2.20)
Smoking				
Never smoker	871	28.5	1.0	1.0
Former smoker	232	30.6	0.83 (0.60,1.16)	0.78 (0.55,1.09)
Current smoker	324	27.8	1.04 (0.78,1.40)	0.84 (0.61,1.15)

Czech population aged above 15 years and examined in 2011 (*n* = 1427).

¹Adjusted for age and gender; ²Adjusted for age, gender and mutually adjusted for other variables. OR: Odds ratio; *H. pylori*: *Helicobacter pylori*.

there are no strictly unimpeachable epidemiologic data available, the decrease of *H. pylori* prevalence probably already started in the Czech Republic in the late 1990s^[9]. It could be explained mostly by the relatively favourable and improving socio-economic conditions and standards of living together with falling fertility rates. In the same period, the age-standardised death rate from stomach cancer in the Czech Republic fell from 17.3/100 000 (in 1992) to 11.9/100 000 (2001) followed by further current decline to 8.7/100 000 (in 2010)^[13]. Two large Czech seroprevalence studies found decreased *H. pylori* prevalence in the mid 2000s among subjects screened for thyroid disease^[14] and in patients with peptic ulcer disease, functional dyspepsia and gastro-oesophageal reflux disease^[15].

Decrease in the prevalence of *H. pylori* infection was also reported in other former communist countries such as Estonia (in children from 42% in 1991 to 28% in 2002)^[16], Slovakia (in adults from 52% in 1992 and 41% in 2002 to 35% in 2007)^[17,18] and Russia (in children from 44% in 1995 to 13% in 2005)^[19]. This decline in *H. pylori* prevalence was explained by the profound socio-economic changes after the fall of communist regimes.

However, significant decrease of the prevalence of *H. pylori* infection was also found in stable developed European countries like the Netherlands^[20-22], Finland^[23,24], the United Kingdom^[25,26], Germany^[27], Norway^[28] and Denmark^[29]. This decrease could not be explained only by the birth-cohort phenomenon either. In developed countries, spontaneous subsequent clearance of *H. pylori* infection seems to be common^[30]. In Finland, decrease of prevalence of *H. pylori* infection is believed to be due to the "screen and treat" project^[31,32]. The main explanation for the decrease in *H. pylori* prevalence cannot merely be wide use of antibiotics for various indications.

The prevalence of *H. pylori* infection is significantly lower in patients with inflammatory bowel disease but not in those with chronic obstructive pulmonary disease (despite of extensive antibiotic use)^[33]. There was no difference in *H. pylori* prevalence in Crohn's disease with or without previous treatment with sulfasalazine or antibiotics^[34].

In our previous study, we did not find a birth-cohort phenomenon of *H. pylori* infection after the Second World War^[35], in contrast to developed countries^[8,36-41]. This could be explained by the great migration of population in former Czechoslovakia after the Second World War, forced unnatural collectivisation (in fact expropriation) of agriculture, equalisation of society and mostly low socio-economic status under the communist government^[35]. den Hoed *et al*^[42] found a new birth-cohort phenomenon of *H. pylori* infection in the Netherlands. They observed a decline in *H. pylori* prevalence in 6- to 8-year-old Dutch children from 19% in 1978 to 9% in 1993. The further prevalence of *H. pylori* in childhood has remained stable in the Netherlands from 1993 to 2005, suggesting a stabilisation of the previously decreasing trend in subsequent birth cohorts. This finding may reflect stabilisation in determinants such as family size, housing, and hygienic conditions (or offset by day care)^[42].

Several interesting data came from other studies outside Europe. Decreased prevalence of *H. pylori* was also found for instance in the United States^[43,44], China^[45,46], Japan^[47], South Korea^[48] and Singapore^[49]. After fifteen years, the prevalence of *H. pylori* infection among both children and adults in China remained significantly higher in areas with a high incidence of gastric cancer compared with that in areas with a low incidence of gastric cancer. *H. pylori* infection rates have decreased in the general Chinese population during recent years^[50].

Poor standard of living, low socio-economic status, overcrowded families, low education of parents and smoking (in adults) are still major risk factors of *H. pylori* infection. Acquisition, chronic infection and possible spontaneous clearance of *H. pylori* are influenced by several other factors like ethnicity and genetic factors. Extensive broad use of antibiotics for different indications might be less important^[9].

It is necessary to revive the so-called hygiene hypothesis^[51]. It was suggested that with the higher hygiene standards in developed countries, people (especially raising children) are less exposed to helminths and this fact modulates development of the immune system (Th1/Th2 shift of CD4+ T-lymphocytes) and thereby increases the risk of several diseases like inflammatory bowel disease, bronchial asthma, food allergy and many others. The high prevalence of *H. pylori* and low rate of gastric cancer in Africa and some parts of Asia were called the "African or Asian (Indian) Enigma" and explained merely by the high prevalence of helminth infections^[52]. Subsequent prospective studies found that no such dissociation existed^[53]. Nowadays the "African Enigma" is considered to be a myth^[54]. Nevertheless, hygiene theories should be considered in explanation of the gradual decline of *H. pylori* infection. Blaser and his group pointed out an

inverse association between *H. pylori* infection and bronchial asthma^[55-57], even though not confirmed by some others^[58]. Sonnenberg *et al*^[59] found low prevalence of *H. pylori* infection in inflammatory bowel disease.

Sýkora *et al*^[60] published an excellent study on *H. pylori* among children in the Western Bohemian region (based on *H. pylori* stool antigen testing). The overall prevalence was 7.1% among 1545 children (aged 0-15 years; in the period 2003-2005). Breast-feeding was an important protective factor, *H. pylori* was found in 12.4% children that were not breast-fed. The prevalence of *H. pylori* was 80.8% among subjects living in children's homes in this study^[60].

Smoking is another independent risk factor for *H. pylori* infection in adults^[61,62]. The lower number of current smokers in Europe might also influence the decline of *H. pylori* infection. In our previous study in 2001, smoking habits were strongly related to risk of *H. pylori* positivity in adults, men and women with lowest education and heavy smokers being at the highest risk of *H. pylori* infection^[9]. Surprisingly, there was no evidence of an increased risk of *H. pylori* infection among current or past smokers in our present study. It is necessary to mention another interesting phenomenon: the decreased prevalence of *H. pylori* represents a prominent decline of CagA positive *H. pylori* strains^[42]. Explanation for this finding still remains unclear.

However, several studies recently showed that the prevalence of *H. pylori* is also declining among children in developing countries like Uganda, Brazil and the Middle East region despite persisting poor hygiene, standard of living and low socio-economic conditions^[5,6,63-65].

On top of all those aspects mentioned above, it is necessary to admit that the reasons for decline of *H. pylori* infection have not been fully clarified yet^[66,67]. It is necessary to also consider the fundamental determinants of "modern times" that could cause gradual disappearance of *H. pylori* from the human microbiome^[68-72].

In summary, the overall prevalence of *H. pylori* infection in the general population has fallen substantially in the Czech Republic over the past 10 years. This decrease can be explained mostly by the relatively favourable and improving socio-economic status and high standard of living conditions. However, it is necessary to also consider that both environmental factors and the human host create an unfavourable milieu responsible for the decline of *H. pylori* infection.

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COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection is still the most common human infection worldwide. It is estimated that 50%-80% of the population is infected. The prevalence in developed countries of Europe is 10%-30%. Up to 95% of the adult population is infected in developing countries.

Research frontiers

The authors accomplished a large multi-centre epidemiologic study on the prevalence of *H. pylori* and dyspepsia in the Czech Republic, a Central European country, in 2001. *H. pylori* infection was investigated by means of ¹³C-urea breath test. The overall prevalence of *H. pylori* was 41.7% in 2001. The aim of the current multi-centre prospective study was to evaluate the prevalence of *H. pylori* infection in the Czech Republic using the same methods in a representative sample of general unselected population from the same geographical areas 10 years later.

Innovations and breakthroughs

The current prevalence of *H. pylori* in 2011 was significantly lower compared to the prevalence of identical geographical areas in 2001 (23.5% vs 41.7%, *P* < 0.001).

Applications

This decrease can be explained mostly by the relatively favourable and improving socio-economic status and high standard of living conditions. However, it is necessary to also consider that both environmental factors and the human host create an unfavourable milieu responsible for the decline of *H. pylori* infection.

Peer review

The paper aims at assessing the prevalence of *H. pylori* infection based on ¹³C urea-breath test in a large random sample of Czech population. The study results show that the prevalence is declining in comparison with a similar previous study from the same group. Potential reasons for this fall are considered in the discussion. The paper is interesting, scientifically sound and well written.

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Development of a quantum-dot-labelled magnetic immunoassay method for circulating colorectal cancer cell detection

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Abstract

AIM: To detect of colorectal cancer (CRC) circulating tumour cells (CTCs) surface antigens, we present an assay incorporating cadmium selenide quantum dots (QDs) in these paper.

METHODS: The principle of the assay is the immunomagnetic separation of CTCs from body fluids in conjunction with QDs, using specific antibody biomarkers: epithelial cell adhesion molecule antibody, and monoclonal cytokeratin 19 antibody. The detection signal was acquired from the fluorescence signal of QDs. For the

evaluation of the performance, the method under study was used to isolate the human colon adenocarcinoma cell line (DLD-1) and CTCs from CRC patients' peripheral blood.

RESULTS: The minimum detection limit of the assay was defined to 10 DLD-1 CRC cells/mL as fluorescence was measured with a spectrofluorometer. Fluorescence-activated cell sorting analysis and Real Time RT-PCR, they both have also been used to evaluate the performance of the described method. In conclusion, we developed a simple, sensitive, efficient and of lower cost (than the existing ones) method for the detection of CRC CTCs in human samples. We have accomplished these results by using magnetic bead isolation and subsequent QD fluorescence detection.

CONCLUSION: The method described here can be easily adjusted for any other protein target of either the CTC or the host.

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Key words: Circulating tumor cells; Cancer; Quantum dots; Nanoprobes; Micrometastasis

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INTRODUCTION

Colorectal cancer (CRC) is ranked as the second most common cause of cancer-related death worldwide^[1]. Cancer-related death is most commonly caused by metastases derived from epithelial tumors like CRC^[2]. The process of metastasis requires the potential and ability of cancer cells to enter into circulation, attach to the endothelium, invade the target organ and subsequently form metastases. The concept and existence of circulating tumour cells (CTCs) and the dissemination and settlement of these cells in secondary organs have been widely accepted^[3]. Approximately 10⁶ tumour cells per gram of primary tumour are daily released into the bloodstream^[4]. However, shear forces in the physiological range can induce lethal damage in a high percentage of CTCs, thus only the 0.1% of those are viable and the 0.01% of the viable cells are responsible for the metastasis. A possible explanation why CTCs are still detectable in the blood months to years after complete removal of the primary tumour may therefore be the circulation and exchange of tumour cells between different metastatic sites and compartments^[5].

The first report with regard to the identification of CTC specific biomarker and genes in CRC was published by Smirnov *et al.*^[6]. Many studies tried to identify biomarkers for CRC-derived CTCs, but further investigations are needed to evaluate the use of these biomarkers in an automated clinical practice^[7,8]. Additionally, the potential prognostic significance of CTCs in CRC has been intensively and extensively reviewed^[9]. Rahbari *et al.*^[10] have supported that the detection of CTCs in the peripheral blood is significantly associated with poor prognosis in CRC. Therefore, the identification of CTCs would be extremely useful to clinical practice, allowing for early cancer detection, as well as, early therapeutic intervention, monitoring and detection of disease recurrence.

Identifying CTCs in peripheral blood, however, has been proven to be more difficult than expected, due to the low concentration of CTCs in blood and lack of technology with sufficiently high sensitivity and specificity^[11]. To date, the reverse transcriptase polymerase-chain reaction (RT-PCR) has been used for CTC detection in a variety of cancers. However, there are difficulties using RT-PCR for CTC detection. High RT-PCR sensitivity is associated with a susceptibility to false positive results in up to 5% of samples^[12]. In addition, RT-PCR based methods are time-consuming, expensive and difficult to standardise^[13]. Flow cytometry (FACS) has been used for CTC detection in several cancers^[14]. CTC quantification is possible with FACS, and this might provide a more accurate measure of the risk of recurrence than current RT-PCR based techniques. The introduction of immunomagnetic detection devices, such as the CellSearch[®] System (Veridex, Warren, NJ), has made possible the detection and at the same time the quantification of CTCs^[15]. How-

ever, the equipment required for FACS and CellSearch[®] System is very bulky, expensive, and difficult to operate at the point-of care. Therefore, the development of alternative sensitive, speedy, specific and low cost methods for CTC detection is important for cancer prognosis.

Quantum dots (QDs) have been developed as a new class of high-sensitivity and high-specificity probes lacking the intrinsic limitations of organic dyes and fluorescent proteins^[16,17]. In comparison with organic fluorophores, the QDs have unique optical and electronic properties, such as size- and composition-tunable fluorescence emission from visible to infrared wavelengths, large absorption coefficients across a wide spectral range and very high levels of brightness and photostability^[16]. QDs have been applied in fluorescence labelling for cancer imaging in living animals, and cellular imaging^[16,17].

The recent introduction of fluorescence detection technology using multifunctional magnetic beads and QDs has been reported^[18-20]. In the present study, we demonstrate a sensitive assay that combines magnetic beads isolation and QDs fluorescence detection for the identification of CRC CTCs surface antigens. The principle of the assay is the separation of CTCs from body fluids using magnetic beads coupled with epithelial cell adhesion molecule (EpCAM) antibody, and monoclonal cytokeratin 19 (CK19) antibody. These complexes are then tagged with streptavidin-conjugated QDs which lead to the detection of a fluorescent signal. For the evaluation of the performance, the method under study was used to isolate human colon adenocarcinoma cell line (DLD-1) human CRC cell line from and CTCs from CRC patient's peripheral blood. FACS analysis and Real Time RT-PCR have also been used to evaluate the performance of the described method. This method provides a simple, low cost and sensitive means of CTCs detection that can be easily adjusted for any other protein target, and can be directly applicable on clinical samples.

MATERIALS AND METHODS

Antibodies

The following antibodies were incorporated in the assay under study: mouse anti-human EpCAM biotin conjugated monoclonal antibody (Acris Antibodies Inc. Acris GmbH, San Diego, CA, United States), mouse anti-human CK19 biotin conjugated monoclonal antibody (Novus Biologicals, Littleton, Colorado, United States), mouse anti-human IgG (Fc specific) biotin conjugated monoclonal antibody (Acris), IgG2b negative control antibody (Santa Cruz, CA, United States), and mouse anti-human CD45 Alexa Fluor 488 conjugated monoclonal antibody (Acris).

Cell culture

The DLD-1 cell line (American Type Culture Collection: CCL-221) was used in our experiments. The DLD-1 colorectal cancer cell line was cultured in RPMI-1640 (Invitrogen), supplemented with 10% foetal bovine

Table 1 Histopathological characteristics of colorectal cancer samples at diagnosis

Characteristics	Colorectal cancer patients (n)
Tumor location	
Rectum	2
Left colon	5
Right colon	2
Tumor size	
≤ 4 cm	5
> 4 cm	4
Differentiation	
Well	1
Moderate	7
Poor	1
TNM stage	
I	0
II	1
III	3
IV	3

TNM: Classification of Malignant Tumors, T describes the size of the tumor and whether it has invaded nearby tissue, N describes regional lymph nodes that are involved, M describes distant metastasis.

serum (Invitrogen). Prior to testing, the cells were trypsinized, washed and then resuspended in Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS). Peripheral blood samples were obtained from 9 CRC patients (Table 1) and 5 healthy donors that gave informed consent to be included in this study. Concerning CRC patients, peripheral venous blood was sampled immediately after patients were anaesthetised and prior surgery's commencement. In all patients, an intravenous cannula was used to collect blood into 7-mL vacutainers containing sodium ethylenediaminetetraacetic acid (EDTA), discarding the first 7-mL aliquot of blood to reduce the risk of contamination of blood by skin epithelial cells. Three 30-mL samples were then collected at one-minute intervals (five 6-mL aliquots per 30-mL sample). Each 30-mL aliquot was thoroughly mixed and then divided into three 10-mL aliquots, one for analysis by RT-PCR, one by the proposed method and one by flow cytometry (FACS analysis). Human white blood cells were isolated from peripheral blood using Ficoll-Hypaque PLUS reagents (Amersham Biosciences, Little Chalfont, NA, United Kingdom). Cells were counted manually using a Burkert-Turk haemocytometer. Trypan blue (0.4%, Sigma, LO, United Kingdom) exclusion test was used to ensure cell viability was above 90% in experiments.

Conjugation of magnetic beads with anti-EpCAM antibody

Streptavidin coated magnetic beads (MBs) (Dynabeads M-280, Invitrogen) were functionalized with the biotinylated antibody EpCAM. For this purpose, 40 mg of antibody were added to 200 mL (10 g/L) streptavidin coated MBs and incubated at room temperature for 30 min. For the removal of unbound antibody, conjugated MBs were washed 5 times with PBS (1X) with the aid of a magnetic device (Dyna MPC-s, Invitrogen) and dis-

solved in 200 mL of PBS containing 0.1% bovine serum albumin (BSA). For negative control IgG2b antibody was used to replace EpCAM on MBs.

Functionalization of QDs with streptavidin

Cadmium selenide (CdSe) QDs (15-20 nm in size) with a maximum emission wavelength of 655 nm, shelled with ZnS and a polymer coating presenting carboxylic groups were purchased by Invitrogen. QDs were coated with streptavidin prior to testing according to the manufacturer's instructions. Briefly, 50 mL of QDs were diluted in 400 mL of borate buffer 10 mmol/L (pH 7.4), 96 mL of streptavidin solution (10 g/L) (Invitrogen) and 11.4 mL of EDC (10 g/L) (Sigma-Aldrich, MO, United States) and incubated at room temperature for 90 min. Streptavidin coated QDs were washed 5 times with 500 mL of borate buffer 50 mmol/L (pH 8.3) on an Amicon Ultra-4 Filter (Millipore, MA, United States) and dissolved into 50 mmol/L borate buffer (pH 8.3) to a final volume of 500 mL.

Limit of detection

For the assessment of the limit of detection (LOD) of the method, our team had to add DLD-1 cells and duplicate solutions of DLD-1 cells in whole blood, both obtained from healthy control individuals, in order to test them. Two series of ten-fold dilutions ranging from 10^4 to 10 cells/mL were prepared. Two negative controls were used in our experiments. The first has consisted of white blood cells from healthy donors, and the second MBs were coupled with IgG2b antibody. The LOD was defined as the lowest concentration level that could be determined to be statistically different from the negative controls (Analytical detection limit guidance 1996).

Cell detection

The principle of the assay is based upon the separation of cells using MBs coupled with anti-EpCAM (epithelial cell adhesion molecule). EpCAM is a carcinoma-associated antigen and is a member of a family that includes at least two type I membrane proteins and functions as a calcium-independent cell adhesion molecule. Like most other tumor-associated antigens, EpCAM is expressed on normal tissue. However, EpCAMs' expression on a wide variety of carcinomas, mostly in gastrointestinal carcinomas, exceeds the expression and accessibility of the antigen compared with normal cells, thereby establishing a useful therapeutic and diagnostic window for a targeted antibody approach. These complexes are then tagged with anti-CK19 biotinylated antibody that specifically recognizes CTCs and finally streptavidin-conjugated QDs leading to the detection of a fluorescent signal (Figure 1). To this purpose, 500 μL of each dilution of the cells comprising positive and negative controls or an equal volume of PBS BSA 0.1% (blank) were coupled at first with 40 mL of EpCAM antibody conjugated MBs, and then with 10 mL (0.2 g/L) of CK19 biotinylated antibody, and finally 40 mL of streptavidin coated QDs. For each hy-

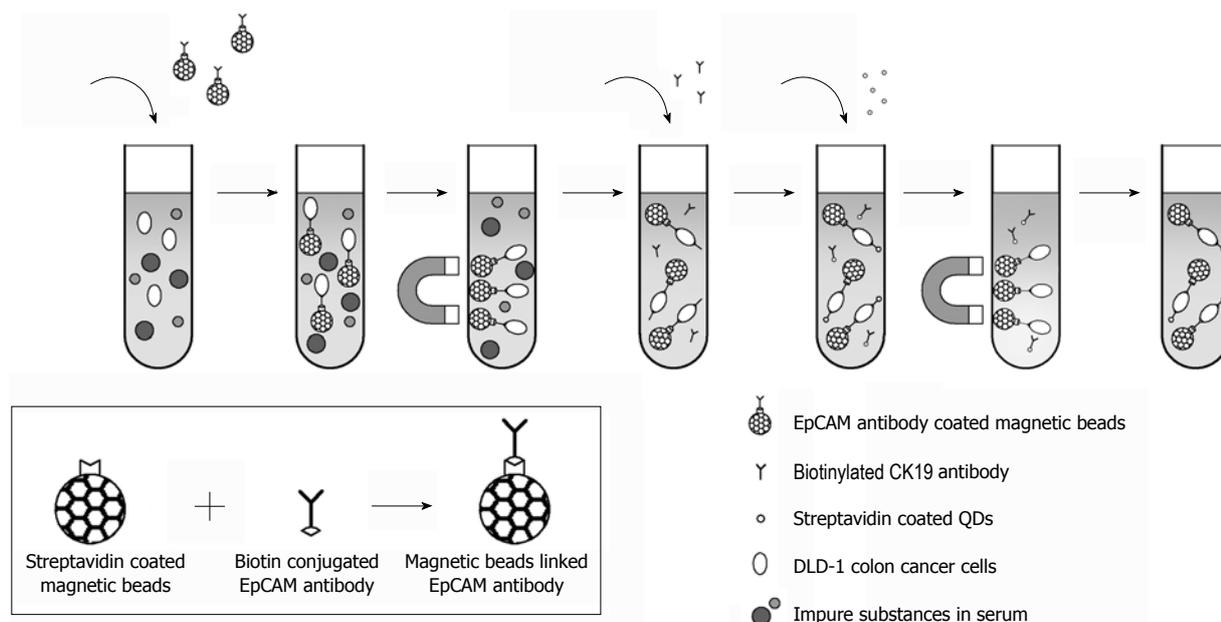


Figure 1 Circulating tumour cells are separated from the leucocytes using magnetic beads coupled with epithelial cell adhesion molecule antibody and a magnetic device. These complexes are then tagged with cytokeratin-19 (CK19) biotinylated antibody and streptavidin-conjugated quantum dots (QDs) which lead to the detection of a fluorescent signal. EpCAM: Epithelial cell adhesion molecule; DLD-1: Human colon adenocarcinoma cell line.

bridization step the dilutions were incubated at 37 °C for 20 min with gentle agitation, separated by the matrix with the aid of a magnetic device (DynaL MPC-s, Invitrogen), washed once with PBS-BSA 0.1% and then re-suspended in 200 mL PBS 0.1% BSA. For the direct visual observation of fluorescence, the samples were transferred on a UV transilluminator with UV emission at 312 nm (Vilber Loumat, France). Fluorescence was also detected with a spectrofluorometer (excitation 480 nm emission 655 nm, PMT 450V), (Infinite M200, Tecan United States).

FACS analysis

All samples were analyzed using a FACS Calibur BD Biosciences (United States) and the software BD FACStation™ System with the program Cell Quest, Mac. The antibodies used are described above and the staining of the isolated peripheral blood mononuclear cells samples was proceeded according to routine protocols. The anti-CK19 biotin conjugated monoclonal antibody with the QDs was used to evaluate the number of CTCs in the sample of interest and the CD45 Alexa Fluor 488 conjugated monoclonal antibody for the detection of non-specific interactions. Concerning the cell counting, for each sample 10 000 events were acquired. The parameter being used to describe individual samples was the % percentage of positive cells. In the first diagram (FSC/SSC) of FACS analysis the monocytes were gated, given that the CTCs are generally found in this population, in order to exclude most of the negative events. From the diagram of FSC/FL3 we have calculated the percentage of the cells that are fluorescent in FL3 (CK19+). In order to avoid non-specific binding, we have calculated at the same time the percentage of the cells that are fluorescent in FL3, but not in FL1 (CK19+/CD45-). In the FSC/FL3 diagram

the cells that are fluorescent in FL3 (CK19+) were gated, and screened them in another diagram FL1/FL3. At last, the percentage of the cells that are fluorescent in FL3, but not in FL1 was calculated and the percentage in absolute cell number was converted. We deemed that if we found > 1 positive cell/mL in a sample, this would be a CTC, considering that the mean average of the healthy samples was 1000 cell/L (Figure 2).

Reverse transcriptase and real time RT-PCR

Total RNA was isolated from DLD-1 cells, as well as RBC samples with the use of Trizol (Invitrogen, TRI Reagent) according to the manufacturer's instructions. The isolated RNA was dissolved in diethylpyrocarbonate-treated water and stored at -80 °C until used. Reverse transcription was performed by incubating 1 µg total RNA for 1 h at 42 °C in the presence of 500 mg/L of OligodT 12-18, 10 mmol/L deoxyribonucleotide triphosphates, 5 × first-strand buffer, 0.1 mol/L dithiothreitol, and 200 U/mL MMLV reverse transcriptase (Invitrogen). Assessment of the CK19 and EpCAM mRNA levels was performed by employing the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels as a reference gene. The following pair of primers were used: (1) GAPDH (Fw) 5'-GAAGGTGAAGGTCGGAGT-3' and (Rv) 5'-GAAGATGGTGTATGGGATTTTC-3' resulting in a 228-bp fragment; (2) CK19 (Fw) 5'-CCC GCGAC-TACAGCCACTA-3' and (Rv) 5'-GCTCATGCGCA-GAGCCTGTT-3' resulting in a 193-bp fragment; and (3) EpCAM (Fw) 5'-GCCAAATAATAACGGGACCTA-3' and (Rv) 5'-CCAGCTGAGAGACCAGGAGAA-3' resulting in a 130-bp fragment. Real-time PCR was performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, United States),

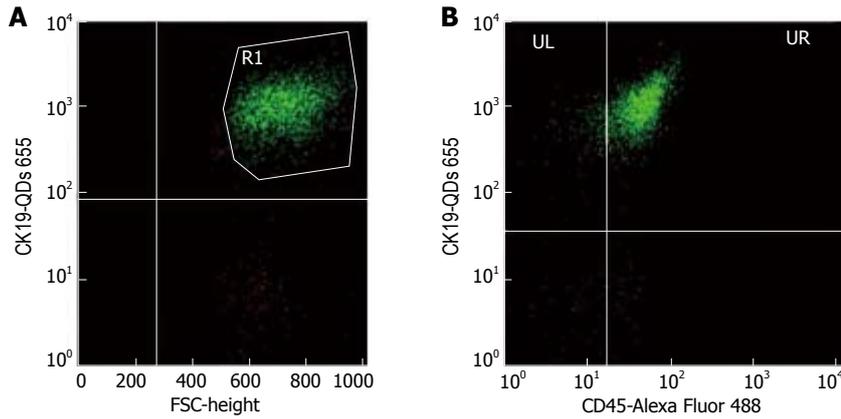


Figure 2 Representative fluorescence-activated cell sorting analysis of peripheral blood samples. A: Events which fell within region R1 are confirmed to be cytokeratin 19 (CK19)+ and fluorescent in FL3 (anti-CK19 conjugated with QDs); B: From the R1 gate, the events confirmed to be CK19+CD45+ are those in the UL region, in UR region the events represent those that are CK19+CD45+ and fluorescent in FL1 (anti-CD45 conjugated with Alexa Fluor). We deemed that the cells in the UL are those meeting the criteria for circulating tumor cells.

as follows: initial denaturation for 2 min at 50 °C and for 10 min at 95 °C, followed by 40 cycles of PCR (95 °C for 15 s; 60 °C for 1 min). Reactions were performed in duplicate, using the SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Data were analyzed using the comparative CT method for the relative quantitation of results^[21]. Post-amplification denaturation curves showed that the primer pairs generated single products.

Statistical analysis

The consistency of the methods and comparison of total positive rate in the methods were analyzed using Cohen's kappa statistic and χ^2 test, respectively. All statistical analyses were carried out using GraphPad version 3.00 (GraphPad Software, San Diego, Calif). $P < 0.05$ was considered significant.

RESULTS

Our method for CTCs isolation and detection using EpCAM antibody coated MBs and CK19 antibody coated QDs is schematically illustrated in Figure 1. The assessment of the immobilization of antibodies on the surface of MBs was performed by using spectrophotometric measurement at 280 nm of the antibody solution before and after conjugation with MBs (data not shown).

For the evaluation of performance, the method under study was used to detect DLD-1 human CRC cells. The DLD-1 cells expressed EpCAM and CK19, as identified by RT-PCR. The direct visual observation of negative control did not reveal any fluorescent signal (Figure 3) as opposed to the DLD-1 cells reported above that reacted positively at or above the LOD concentration. The LOD of the methodology described in the present study was defined to 10 DLD-1 cells/mL of sample when results were assessed by visual observation of the test tubes.

In order to obtain an indication of the method's performance with clinical samples, we applied the optimized

assay to the detection of CTCs from human white blood cells that were isolated from peripheral blood of CRC patients, in comparison with real-time PCR and FACS analysis. Several studies used QDs-probes for FACS analysis^[22-24]. The concordance of the positive results recorded by the proposed assay on CRC samples with those of real-time PCR and FACS was 78.57% (11 of 14) and 85.71% (12 of 14) respectively. The relevant percentage with regard to negative results was 100% in both cases (Table 2). Despite the fact that the number of clinical samples was limited, it was noted that the samples with tumor node metastasis (TNM) stage IV had increased number of CTCs compared to those with TNM stage II and III.

The repeatability of the method was defined as 100% since the results recorded for the samples included in this type of evaluation were identical for all assessments ($n = 3$).

DISCUSSION

The spreading of tumor cells is one of the primary causes of recrudescence at distant sites and of death from cancer. Thus, the detection of circulating metastatic cells is important to predict recurrence and improve survival. In the present study, we describe a newly developed technique for the detection of CTCs, incorporating CdSe QDs for the detection of CTCs specific surface antigens. In the aforementioned method for CTCs detection, we used EpCAM antibody coated MBs and CK19 antibody coated QDs. We used EpCAM since the anti-EpCAM-based immunomagnetic enrichment technology, have showed significant better recovery rates compared to other cytometric technologies in spiking experiments. In addition, EpCAM-based enrichment methods of CTCs in CRC patients have been successfully applied in several cases^[25-27]. Regarding CK19, the later is a widely used biomarker to detect tumor cells which derive from epithelial tissues^[27].

The developed methodology does not require sample processing for DNA isolation, which facilitates its

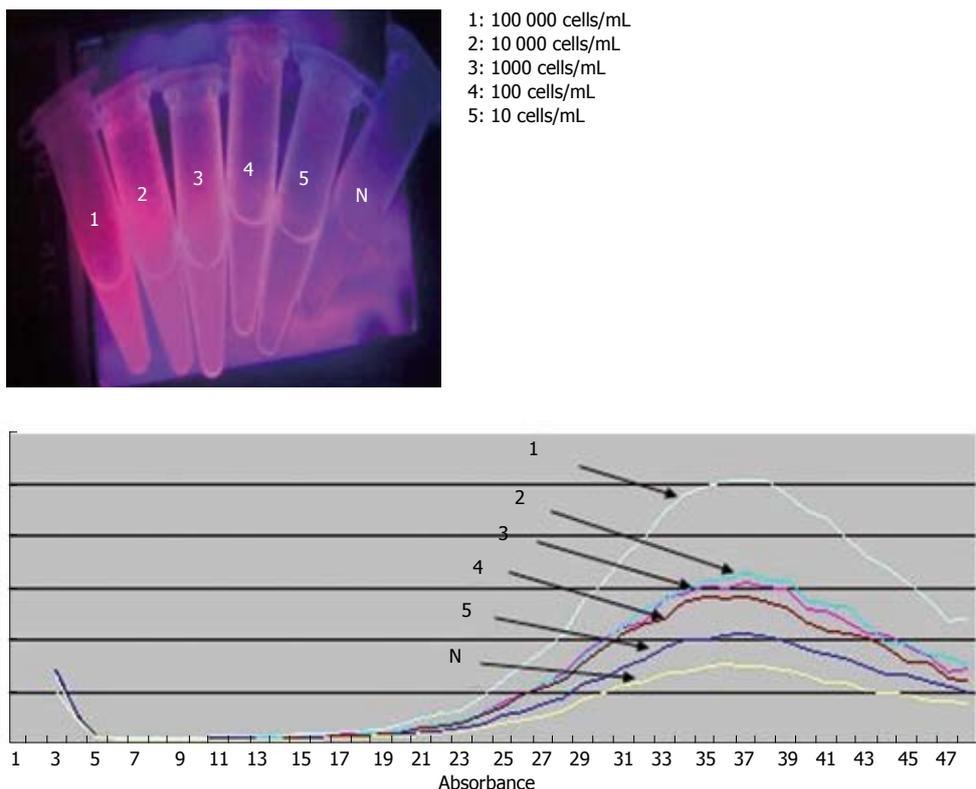


Figure 3 Representative results recorded by the proposed method for the human colon adenocarcinoma cell line serial dilutions ranging from 10^4 to 10 cells/mL (numbers 1-5) and negative controls. Fluorescence is evident in the human colon adenocarcinoma cell line samples with concentration up to 10 cells/mL, as illustrated at the fluorescent emission spectra obtained after magnetic separation.

Table 2 Results recorded by fluorescence-activated cell sorting, real time reverse transcriptase polymerase chain reaction, and proposed method for detection of circulating tumor cells in clinical samples

Sample	FACS (CTCs/ mL blood)	Real-time PCR ¹	Results with MBs- EpCAM/QDs-CK19 ²
CRC 1	34	12.96	+++
CRC 2	20	8.64	++
CRC 3	51	17.28	++++
CRC 4	57	18.52	++++
CRC 5	63	19.75	++++
CRC 6	19	9.87	++
CRC 7	9	0	+/-
CRC 8	3	0	-
CRC 9	2	0	-
Healthy 1	0	0	-
Healthy 2	0	0	-
Healthy 3	0	0	-
Healthy 4	0	0	-
Healthy 5	0	0	-
DLD-1 cells	500	100	+++++
Negative control (1X PBS)	0	0	-

¹% expression CK19/CK19 of DLD-1 normalized to GAPDH); ²The number of "+" is proportional to the fluorescence intensity (from low to high) as evaluated by two independent observers who were blinded to the patients and the fluorescence-activated cell sorting (FACS) and real-time polymerase chain reaction data. CRC: Colorectal cancer; DLD-1: Colorectal adenocarcinoma cell line; EpCAM: Epithelial cell adhesion molecule; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; MBs: Magnetic beads; PCR: Polymerase chain reaction; CTCs: Circulating tumour cells; CK19: Cytokeratin 19; QDs: Quantum dots; PBS: Phosphate buffered saline.

incorporation at point-of-care. The use of QDs in the proposed approach bypasses the disadvantages of fluorescent dyes often incorporated into immuno-detection tests such as rapid photobleaching, narrow excitation spectrum and low signal intensity.

The accuracy of the QD detection system, as evaluated on clinical samples from CRC patients, compared to reverse transcriptase real-time PCR and FACS analysis applied to blood samples has ranged between 78.57% and 85.71%. Admittedly, PCR-based techniques as well as FACS analysis are among the most reliable and useful methodologies for the detection of CTCs in a variety of cancers. However, both techniques require trained personnel, dedicated space, and high-cost equipment.

It should be noted that while our method shows great promise in sensitive detection of CTCs, it is still far from achieving to detect a single CTC in the whole human circulating blood. At the present time, according to our knowledge, the most sensitive system in probably CellSearch™, whose detection limit is claimed to be 1 CTC/7.5 mL^[25]. However, this system is very expensive and partially subjective by distinguishing the CTC shapes from those of normal cells. Recently, the detection of CTC has also been achieved using surface enhanced Raman spectroscopy with a detection limit of 50 CTC/mL in whole blood^[28-30]. The method we presented here, might provide a fast and low-cost alternative way for CTC cell detection. For reasons of applicability the method described here incorporates a combination of

cancer and CTC antibodies that increases specificity and at the same time facilitates its adjustment for any other protein target, either of the tumor or the host. Evidently the method can be easily extended to the detection of any other tumor as the adaptation of the method would require only the incorporation of specific antibodies for the cancer or disease in question. Once fully developed, this method will be directly applicable on clinical samples in the context of the one reported above. This implies the potential use of the proposed methodology as a diagnostic technology platform.

COMMENTS

Background

The detection of circulating tumor cells (CTCs) is of great importance for the clinical management of patients with solid cancers like colorectal cancer (CRC), due to the fact that they have long been considered as a reflection of tumor aggressiveness. However, owing to the rarity of CTCs in peripheral blood, their detection requires methods combined with high sensitivity and specificity, which sets tremendous challenges for the implementation of these assays into clinical routine.

Research frontiers

The concept and existence of CTCs as well as the dissemination and settlement of these cells in secondary organs have been widely accepted. The development of alternative sensitive, speedy, specific and low cost methods for CTC detection is important for cancer prognosis. In the present study, authors demonstrate a sensitive assay that combines magnetic beads isolation and quantum dots (QDs) fluorescence detection for the identification of CRC CTCs surface antigens.

Innovations and breakthroughs

The developed methodology does not require sample processing for DNA isolation, which facilitates its incorporation at point-of-care. The use of QDs in the proposed approach bypasses the disadvantages of fluorescent dyes often incorporated into immuno-detection tests such as rapid photobleaching, narrow excitation spectrum and low signal intensity.

Applications

This method can be easily extended to the detection of any other tumor as the adaptation of the method would require only the incorporation of specific antibodies for the cancer or disease in question.

Peer review

The topic is of significant clinical importance as the detection of circulating cancer cells may be used for early diagnosis of the recurrence of the disease.

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Contrast-enhanced ultrasonography parameters in neural network diagnosis of liver tumors

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Abstract

AIM: To study the role of time-intensity curve (TIC) analysis parameters in a complex system of neural networks designed to classify liver tumors.

METHODS: We prospectively included 112 patients with hepatocellular carcinoma (HCC) ($n = 41$), hypervascular ($n = 20$) and hypovascular ($n = 12$) liver metastases, hepatic hemangiomas ($n = 16$) or focal fatty changes ($n = 23$) who underwent contrast-enhanced ultrasonography in the Research Center of Gastroenterology and Hepatology, Craiova, Romania. We recorded full length movies of all contrast uptake phases and post-processed them offline by selecting two areas

of interest (one for the tumor and one for the healthy surrounding parenchyma) and consecutive TIC analysis. The difference in maximum intensities, the time to reaching them and the aspect of the late/portal phase, as quantified by the neural network and a ratio between median intensities of the central and peripheral areas were analyzed by a feed forward back propagation multi-layer neural network which was trained to classify data into five distinct classes, corresponding to each type of liver lesion.

RESULTS: The neural network had 94.45% training accuracy (95% CI: 89.31%-97.21%) and 87.12% testing accuracy (95% CI: 86.83%-93.17%). The automatic classification process registered 93.2% sensitivity, 89.7% specificity, 94.42% positive predictive value and 87.57% negative predictive value. The artificial neural networks (ANN) incorrectly classified as hemangiomas three HCC cases and two hypervascular metastases, while in turn misclassifying four liver hemangiomas as HCC (one case) and hypervascular metastases (three cases). Comparatively, human interpretation of TICs showed 94.1% sensitivity, 90.7% specificity, 95.11% positive predictive value and 88.89% negative predictive value. The accuracy and specificity of the ANN diagnosis system was similar to that of human interpretation of the TICs ($P = 0.225$ and $P = 0.451$, respectively). Hepatocellular carcinoma cases showed contrast uptake during the arterial phase followed by wash-out in the portal and first seconds of the late phases. For the hypovascular metastases did not show significant contrast uptake during the arterial phase, which resulted in negative differences between the maximum intensities. We registered wash-out in the late phase for most of the hypervascular metastases. Liver hemangiomas had contrast uptake in the arterial phase without agent wash-out in the portal-late phases. The focal fatty changes did not show any differences from surrounding liver parenchyma, resulting in similar TIC patterns and extracted parameters.

CONCLUSION: Neural network analysis of contrast-enhanced ultrasonography - obtained TICs seems a promising field of development for future techniques, providing fast and reliable diagnostic aid for the clinician.

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Key words: Hepatocellular carcinoma; Liver tumors; Contrast enhanced ultrasound; Time-intensity curve; Artificial neural network; Computer-aided diagnosis system

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INTRODUCTION

Rapid classification of liver masses is of utmost importance, as early diagnosis drastically improves survival chances of oncologic patients, determined by the appropriate curative therapy. Hepatocellular carcinoma (HCC) is the sixth cancer as incidence worldwide, being the third cause of cancer-related mortality^[1,2].

Current guidelines include at least one dynamic imagistic method for establishing a positive HCC diagnosis^[3,4], as with the widespread use in recent years of imaging techniques, the differential diagnosis of a newly discovered liver mass became less invasive for the patient.

Contrast-enhanced ultrasonography (CEUS) with second generation blood-pool contrast agents is one of the most cost-effective methods for determining tumor vascularization patterns^[5-9]. Arterial contrast uptake followed by late portal wash-out is considered the radiological hallmark of HCC. Various other benign and malignant liver tumors show different uptake patterns, thus providing differential diagnosis. Since other tumors can sometimes mimic the filling patterns specific to HCC, different studies reported lower sensitivities for CEUS when compared to other dynamic imaging methods such as contrast-enhanced magnetic resonance imaging (CE-MRI) or 4-phase computed tomography (CT)^[9,10]. Time intensity curves (TIC) are the graphical representation of contrast intensity represented in every moment of a CEUS investigation. Comparative TIC analysis between a tumoral region of interest (ROI) and a parenchymal equivalent ROI could enhance the diagnostic accuracy of CEUS, thus establishing its role in HCC diagnosis^[11,12].

Artificial neural networks (ANN) emerged in recent

years as potent diagnostic tools for malignant lesions, mainly because of their adaptability and excellent problem solving-oriented architecture. They have been employed in complex medical image analysis tasks with various degrees of success. Computer-aided diagnosis (CAD) systems have gained a reputation for providing integrative solutions for the diagnosis of several types of malignancies^[13-15], with many applications in gastroenterology and tumor pathology associated with the digestive tract.

Our aim here was to establish the role of TIC analysis parameters in a complex system of neural networks designed to classify liver tumors.

MATERIALS AND METHODS

Patient inclusion and final diagnosis

Between September 2008 and May 2011 we prospectively included 112 patients with hepatocellular carcinoma ($n = 41$), hypervascular ($n = 20$) and hypovascular ($n = 12$) liver metastases, hepatic hemangiomas ($n = 16$) or focal fatty changes ($n = 23$) who underwent CEUS in the Research Center of Gastroenterology and Hepatology, Craiova, Romania. Positive diagnosis was reached through a combination of other imagistic methods (CT and CE-MRI), liver biopsy in uncertain cases or follow-up for a minimum period of six months. The study was performed in accordance with the Declaration of Helsinki and received necessary approval of the Ethic Committee of the University of Medicine and Pharmacy of Craiova. All patients gave informed consents on all procedures and agreed in writing so that their anonymized CEUS investigations were to be used in the ANN model. An overview of the study protocol can be observed in Figure 1.

Data collection and pre-processing

Full length CEUS recordings were retrieved in an uncompressed video for offline post-processing and subsequent TIC analysis. This ensured an optimal preservation of image features, color intensities and hues.

Digital files were transferred to a high-end graphical station where they were analyzed with in-house developed software (programmed by Ionescu M and Streba CT). At first, the team of gastroenterologists with extensive US and CEUS experience (Sandulescu L, Ciurea T, Saftoiu A, Vere CC and Rogoveanu I) supervised Streba CT in the selection of tumor and normal parenchyma ROIs at equal tissue depths, on each CEUS recording. The software recorded median color intensity values inside the ROIs for each frame and presented an accurate TIC for visual interpretation. The locations of the two ROIs were manually adjusted after a breathing motion, thus removing breathing artifacts. The team also visually analyzed the TICs and provided a diagnosis, blinded to any other patient details.

ANN design and methodology

Raw intensity values were extracted as data sequences

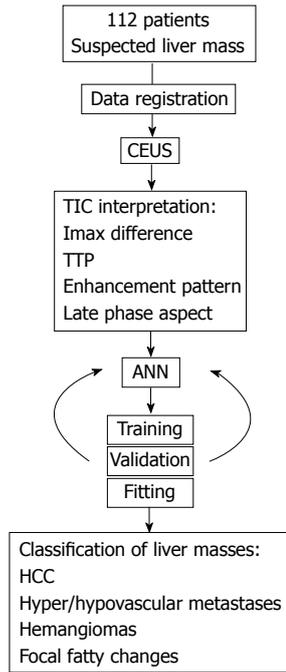


Figure 1 Study protocol. The patients were registered and contrast-enhanced ultrasonography (CEUS) was performed, with subsequent movie registration and offline time-intensity curve (TIC) analysis. Relevant parameters were fed to the artificial neural networks (ANN) which divided the dataset into training, validation and fitting lots. A back propagation and 10-fold cross-validation algorithm assured a high accuracy for the classifications obtained by the ANN system. Imax: Maximum intensities; TTP: Time to reaching peak intensities; HCC: Hepatocellular carcinoma.

and analyzed by a feed forward back propagation multi-layer ANN which was trained to classify data into distinct classes, corresponding to each type of liver lesion. Based on previous studies which concluded that the best suited ANN layout for classification tasks should be as simple as possible, the network architecture of choice contained only one hidden layer, with an input layer and one layer dedicated for the output (Figure 2A)^[14,16].

The first layer of the ANN consisted of several groups of input neurons (Figure 2B) corresponding to each imputed parameter, as follows: difference in maximum intensities, corresponding time to reaching peak intensities and the aspect of the late/portal phase, as quantified by the ANN on individual frame-by-frame differences between median intensities starting from the 45th second of the recording. As various tumors can be diagnosed by evaluating central versus peripheral enhancement, the software also calculated a ratio between median intensities of the central and peripheral areas, thus quantifying relative changes in contrast uptake.

Each neuron in an ANN was connected to every neuron of the consecutive layer hidden layer through connections called “synapses”, which are attributed different “weights”, based on strength of the connection^[14,17,18]. These weights were calculated by the ANN in a hierarchical manner (basically, the system determined the importance of each parameter in the analysis).

A hidden 2nd layer contained neurons with associ-

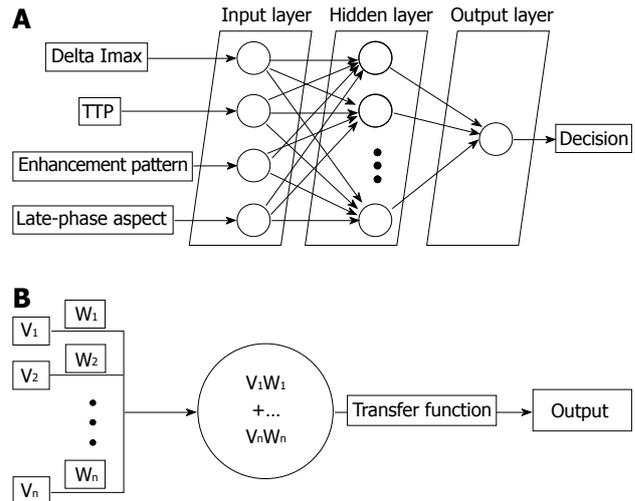


Figure 2 Graphical representation of an artificial neural networks and a neuron from the 2nd hidden layer. A: The four classes of parameters are imputed to corresponding neurons in the first layer of the artificial neural networks, which in turn establish synaptic connections with all neurons of the 2nd hidden layer. These neurons provide a value for the output layer, which in turn presents the user with a classification decision; B: Neurons in the hidden layer receive multiple inputs (V) which are attributed specific weights (W) and all products between these two values are summed. The corresponding result (output) is forwarded through a transfer function of the efferent synapse. Imax: Maximum intensities; TTP: Time to reaching peak intensities.

ated transfer and processing functions which received weighted parameters as well as activity of the previous neurons. The sum of the products between synaptic weights and neuron activity decided an output category (in this case, any real number between 1 and 5, corresponding to the five distinctive tumor classes), similar to information processing within a human brain^[17-20].

A final output layer was organized and provided five distinct values, one for each type of liver mass identified. This layer could have been varied in size depending on the total number of categories, if other types of tumors would have been present. This presented the user with a possible classification suggestion and probability score, calculated as a percentage from the total ideal score for the ideal value (i.e., if the total was 0.8, it would present the user with an 80% probability for the tumor to be HCC, as “1” was the assigned score for this diagnosis).

All 112 cases were randomly divided into training, validation and testing sets. The training phase represented the moment when the system learned from a selected data-set which varied in size and composition, thus determining respective weights for each synapses. The ANN was trained with the back propagation algorithm and 10-fold cross-validation was used to assess its performance, as previously described elsewhere^[18,19]. This method minimized the risk of over-fitting, which is an increasingly rigid structure that could ultra-classify cases. Operators chose the learning rate, determining the epoch (that is, the number of iterations needed for going through the training phase), as previously experience showed^[18,19]. Also, all cases were assessed by the team of experts according to the visual TIC representation, thus

Table 1 Descriptive statistics of the study lot and description of the artificial neural networks classification

	HCC	Hypervascular metastasis	Hypovascular metastasis	Hepatic hemangioma	Fatty focal change
Sex (<i>n</i>)					
Male	30	9	5	4	16
Female	11	11	7	12	7
Age (yr), median (range)					
Male	64 (52-83)	68 (45-89)	66 (42-89)	48 (32-64)	43 (31-61)
Female	66 (59-77)	62 (38-72)	62 (41-80)	50 (41-62)	44 (29-61)
Tumor size (cm), median (range)					
Male	5.08 (2-10.7)	5.09 (2-13.1)	5.11 (2.1-12.7)	3.1 (2-7)	4 (2-6.5)
Female	4.8 (2.3-11)	5.7 (2-11.8)	5.6 (2-13.01)	3.2 (2-8.5)	4.6 (2-6.1)
ANN classification					
HCC	38	0	0	3	0
Hypervascular metastasis	0	18	0	2	0
Hypovascular metastasis	0	0	12	0	0
Hepatic hemangioma	1	3	0	12	0
Fatty focal change	0	0	0	0	23

When accounting for multifocal tumors, only the largest median sizes was taken into the calculation. ANN: Artificial neural networks; HCC: Hepatocellular carcinoma.

enabling a direct comparison with the diagnostic accuracy of TIC analysis as a stand-alone method.

Statistical analysis

The statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software Inc, LaJolla, CA). We calculated the respective sensitivities, specificities, positive (PPV) and negative (NPV) predictive values for both human visual and computerized ANN interpretations. We evaluated the differences between the sensitivity and specificity of the ANN-guided and human TIC evaluation-based diagnosis by using the McNemar's test. A *P* value below 0.05 was considered significant. Moreover, we calculated the training and testing accuracy of the ANN and we performed a fitting analysis in order to better characterize the classification characteristics of the system.

RESULTS

The study lot consisted of 69 men and 43 women, their detailed characteristics being illustrated in Table 1.

HCC cases showed contrast uptake during the arterial phase, followed by wash-out in the portal and first seconds of the late phases (Figure 3A). For the hypovascular metastases we recorded negative peak values which resulted in negative differences being imputed in the ANN (Figure 3B). For hypervascular metastases, wash-out in the late phase was registered in the majority of cases. Hemangiomas showed contrast uptake in the arterial phase, followed by the absence of wash-out during the portal-late phases (Figure 3C). Focal fatty changes registered similar TIC patterns to the surrounding parenchyma (Figure 3D).

The ANN containing TIC parameters showed similar diagnostic capabilities to human interpretation of TICs. The automatic classification process registered 93.2% sensitivity, 89.7% specificity, 94.42% PPV and 87.57% NPV. Comparatively, human visual interpreta-

tion of TICs showed 94.1% sensitivity, 90.7% specificity, 95.11% PPV and 88.89% NPV. The ANN incorrectly classified as hemangiomas three HCC cases and two hypervascular metastases, while in turn misclassifying four liver hemangiomas as HCC (one case) and hypervascular metastases (three cases) (Table 1). Overall, the ANN correctly identified 67/72 (93%) malignant lesions and 36/40 (90%) of all benign focal liver lesions. In turn, human evaluation based solely on TIC parameters misclassified four hypervascular metastases as hemangiomas and four hemangiomas as either HCC (two cases) or hypervascular metastases (two cases). The differences between the automatic ANN classification and human interpretation of TIC data were not statistically significant in terms of specificity (*P* = 0.225) and sensitivity (*P* = 0.451).

The neural network had 94.45% training accuracy (95% CI: 89.31%-97.21%) and 87.12% testing accuracy (95% CI: 86.83%-93.17%). Fitting coefficients demonstrated the overall classification capabilities of the ANN, with *r* = 0.989 (training); 0.991 (validation); 0.984 (testing) and 0.993 (overall).

DISCUSSION

We present here the first report on using an ANN-driven computer aided diagnostic system in conjunction with CEUS TIC analysis for the correct classification of liver masses. Our pilot study on an initial lot of 112 patients compared the diagnostic capability of such a system with visual TIC analysis by a team of gastroenterologists blinded to any other patient data. We obtained similar results, the ANN scoring 93.2% sensitivity and 89.7% specificity, compared to 94.1% sensitivity and 90.7% specificity for human visual interpretation, respectively.

Early correct diagnosis and appropriate staging of liver malignancies is of utmost importance for patient survival, as curative surgical interventions have narrow indications and are extremely specific to certain types

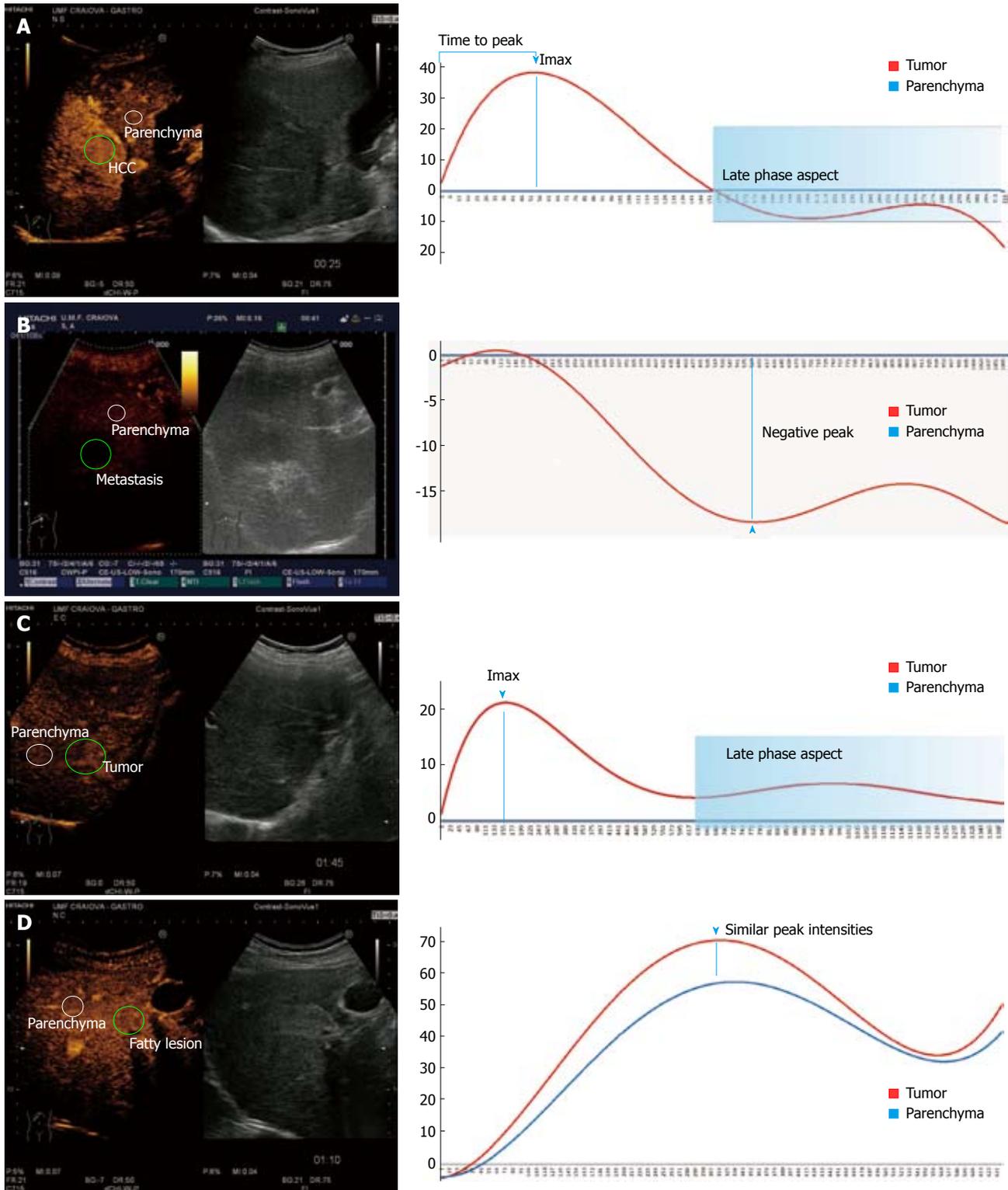


Figure 3 Examples of contrast-enhanced ultrasonography aspects and the selection of the two regions of interest, corresponding to the liver tumor and normal parenchyma, respectively. Graphical representation of the time-intensity curve (TIC) and the most important parameters extracted and later fed to the artificial neural networks. A: Hepatocellular carcinoma (HCC)-positive contrast uptake in the early arterial phase followed by wash-out in the portal/late phase; B: Hypovascular metastasis-hypo-enhancement of the tumor compared to normal parenchyma; C: Hepatic hemangioma-absence of the wash-out and positive peak intensity; D: Focal fatty change-similar TIC parameters for the two selected areas of interest.

of tumors^[1-4]. HCC currently ranks third in terms of mortality worldwide and fifth in incidence with almost 750 000 new cases diagnosed each year, while being second in mortality among digestive cancers^[5]. The diag-

nostic criteria for HCC, the most common primary liver malignancy worldwide, rely primarily on imagistic methods^[3,4]. Current accepted guidelines worldwide are those proposed by the American Association for the Study of

Liver Disease (revised in 2010)^[3] and those of the association between the European Society for the Study of Liver and the European Organization for Research and Treatment of Cancer (EASL-EORTC, recently revised in April 2012)^[4]. The introduction of new generations of contrast agents marked the adoption of a generally accepted radiological hallmark for positive HCC diagnosis, namely contrast up-take in the arterial phase followed by washout in the venous/late phase. The American Association for the Study of Liver Diseases guidelines stipulate that only one imaging technique with contrast uptake, either CT or MRI, showing the radiological hallmark, is sufficient for positive diagnosis of tumors between 1 and 2 cm in diameter (this being the optimum tumor size for curative surgery)^[3,4]. Their European counterparts, however, are more cautious in applying a single imaging method, and recommend two coincidental techniques in suboptimal settings due to technical limitations^[4].

The use of CEUS is regarded as controversial in the latest EASL guideline^[4]. Second generation contrast agents use gas bubbles between 2 and 7 microns in diameter which resonate under the US probe, the amplified signal being registered by the US machine through the same probe^[5,6]. While the radiological hallmark can be clearly identified in this technique, the contrast microbubbles are bound to the intravascular space, as opposed to iodinated contrast-CT or gadolinium-based MR imaging in which the contrast agents are rapidly cleared from the bloodstream into the surrounding parenchymal space^[4,9,10]. According to some studies, intrahepatic cholangiocarcinoma or even some highly vascularized liver metastases can display uptake patterns similar to HCC during CEUS or gadolinium-based MRI, thus providing an important source of error^[9]. Currently, efforts are being made to overcome the inherited issues with CEUS investigations and diminish the rate of false interpretations^[11,12]. The use of TICs in the interpretation of CEUS movies seems a feasible method of increasing the specificity of this investigation^[12,13]. The method relies on plotting and comparing on a time scale the median intensities of two user-defined areas, one within the suspected tumor and one in a parenchymal area with no major vessels, thus producing two curves which depict contrast uptake during CEUS^[12]. The usual graphical representation show contrast uptake in the first 30-60 s, followed by tumor wash-out in portal and venous phases, when the maximum intensity are similar to those of the parenchymal-selected area of interest. While TIC quantification does add more precision to the investigation, several user-dependent and technique-dependent limitations have been identified, such as different depths of the analyzed tumor/parenchyma areas of interest, moving artifacts due to patient breathing or the interference of large blood vessels in the selected areas of interest^[12]. Our system showed good capabilities in selecting the appropriate ROIs, as it allowed for breathing compensation to be applied during post processing.

The neural network developed and tested consisted

of an input layer containing four classes of neurons corresponding to TIC-dependent parameters, one hidden layer for data processing and a variable output layer which classified the data into five categories. This model, the perceptron feed-forward multi-layered ANN with back-propagation algorithms is the preferred embodiment of a CAD system for medical diagnosis, as it provides rapid diagnosis with minimal over-fitting (the artificial increase of certain rules of inclusion, leading to incorrect classifications)^[13-19]. Automated quantitative image analysis techniques have been introduced in medical practice for a number of years^[21-25]. The use of ANNs or other adaptive, machine-based learning systems, can substantially improve the accuracy of any quantitative-based image analysis method^[25,26]. Moreover, current image analysis methods employed with US or CEUS are heavily dependent on the expertise of the medical operator^[22-26]. The neural network approach showed excellent training and validation characteristics, with fast and reliable cycles. The usage of ANNs proved adequate, having promising results compared to human interpretation of the data. Using an independent CAD system proved beneficial and can considerably reduce user-dependent bias from CEUS interpretation, perhaps improving its ability to correctly diagnose liver malignancy, further enhancing its role in current medical guidelines.

One possible limitation of our study is represented by the relatively low number of cases included in the analysis. This was resolved by applying specific training algorithms with a long history of success in small patient lots^[14,18,19]. Patients presented some of the most masses most commonly encountered by the physician in daily practice, therefore the system showed good promise in providing an independent diagnostic aid based solely on accurate quantification of imaging data. The system has promising telemedicine application, as it can be accessed from small tertiary referral centers, with limited experience in diagnosing liver malignancies. It can provide an independent expert diagnosis and verification system for physicians who do not routinely encounter liver-related pathology. Another indication for the system would be in medical training, as an independent help for training gastroenterologists. The self-improving model specific to the architecture of any ANN will only benefit from an extended number of cases, a greater training set further improving its diagnosis performances. Future directions should include other intelligent artificial systems, such as support vector machines, genetic algorithms, Bayesian classifiers and so on, with perhaps even better results.

We previously presented preliminary results of introducing an ANN analysis of CEUS parameters in a cascade ANN system for the diagnosis of HCC and other liver malignancies, with good prospects in terms of accuracy and efficiency^[27]. Therefore, we strongly believe that the merger between clinical data and imagistic parameters represent a necessary direction in future aiding clinicians in their therapeutic decisions.

In conclusion, neural network analysis of CEUS

TICs seems a promising field of development for future techniques, providing fast and reliable diagnostic aid for the clinician. A multi-layer perceptron ANN proved sufficient for the classification of liver masses based on TIC analysis data, producing the most rapid and accurate results. Future studies are needed in order to validate the system in larger cohorts and perhaps improving the architecture of the proposed ANN. The system can ultimately become an independent objective quantifier of clinical and imagistic data which can improve the diagnostic accuracy of CEUS regarding liver malignancies.

COMMENTS

Background

An early diagnosis of liver malignancies represent a major concern, as the therapeutic options become increasingly limited as the disease progresses. The imagistic diagnosis based on contrast agents is the preferred method for hepatocellular carcinomas (HCC), the most important primary malignant tumor of the liver. The role of contrast-enhanced ultrasonography (CEUS) in the differential diagnosis of liver tumors is currently subject to international debate, while time-intensity curve (TIC) analysis provides a rapid method to quantify perfusion parameters. Several computer-aided diagnosis (CAD) systems are currently employed in the diagnosis of various malignancies, specifically artificial neural network (ANN) systems being employed in image-recognition tasks.

Research frontiers

CEUS was found to provide limited data when differentiating hepatocellular carcinomas from other liver malignancies; however, the introduction of TIC analysis provides important parameters that can objectify the vascular particularities of liver masses. ANN systems designed for differential diagnosis of malignancies are employed in various areas of medicine, including gastroenterology, with various degrees of success.

Innovations and breakthroughs

This is, to the knowledge, the first report on an ANN-based diagnosis and classification system based on imagistic data, designed to differentiate between several types of liver tumors, both malignant and benign. The CAD system presented here relies on TIC parameters extracted from CEUS investigations which are imputed in a feed forward back propagation single-layer neural network trained to classify liver tumors.

Applications

This system can successfully be applied in telemedicine settings, where smaller referral centers or gastroenterologists with limited experience in CEUS-based diagnosis of liver tumors and especially HCC, may benefit from an independent, objective diagnosis suggestion. Another preferred application is in medical training of gastroenterologists who can fully benefit from the growing experience such a system can provide.

Terminology

CEUS: Ultrasonography (US) using 2nd generation contrast agents containing micro bubbles which resonate under the US probe, the amplified signal being registered by the US machine; TIC analysis: Method to visualize the parallel dispersion of the contrast agent between the tumor and an area selected from the surrounding parenchyma; ANN: Decision making artificial intelligent systems designed to mimic the operating principles of the human central nervous system and their key components - neurons and synapses.

Peer review

This is a high quality research in which authors analyze the role of TIC analysis parameters in a complex system of neural networks designed to classify liver tumors. The results are interesting and suggest that neural network analysis of CEUS-obtained TICs seems a promising field of development for future techniques, providing fast and reliable diagnostic aid for the clinician.

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Endoscopic ultrasound-guided choledochoduodenostomies with fully covered self-expandable metallic stents

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Abstract

AIM: To investigate the long-term outcomes of endoscopic ultrasound-guided choledochoduodenostomy (EUS-CDS) with a fully covered self-expandable metallic stent (FCSEMS).

METHODS: From April 2009 to August 2010, 15 patients with distal malignant biliary obstructions who were candidates for alternative techniques for biliary decompression due to a failed endoscopic retrograde cholangiopancreatography (ERCP) were included. These 15 patients consisted of 8 men and 7 women and had a median age of 61 years (range: 30-91 years). The un-

derlying causes of the distal malignant biliary obstruction were pancreatic cancer ($n = 9$), ampulla of Vater cancer ($n = 2$), renal cell carcinoma ($n = 1$), advanced gastric cancer ($n = 1$), lymphoma ($n = 1$), and duodenal cancer ($n = 1$).

RESULTS: The technical success rate of EUS-CDS with an FCSEMS was 86.7% (13/15), and functional success was achieved in 100% (13/13) of those cases. In two patients, the EUS-CDS failed because an FCSEMS with a delivery device could not be passed into the common bile duct. The mean duration of stent patency was 264 d. Early adverse events developed in three patients (3/13, 23.1%), including self-limited pneumoperitoneum in two patients and cholangitis requiring stent reposition in one patient. During the follow-up period (median: 186 d, range: 52-388 d), distal stent migration occurred in four patients (4/13, 30.8%). In 3 patients, the FCSEMS could be reinserted through the existing choledochoduodenal fistula tract.

CONCLUSION: EUS-CDS with an FCSEMS is technically feasible and can lead to effective palliation of distal malignant biliary obstructions after failed ERCP.

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Key words: Bile duct obstruction; Drainage; Endosonography; Self-expandable metallic stent; Neoplasms

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INTRODUCTION

A self-expandable metallic stent insertion is a well-established palliative treatment that has been verified in numerous studies for patients with inoperable malignant biliary obstructions^[1-4]. Although a self-expandable metallic stent can be inserted *via* the transpapillary route in most cases using endoscopic retrograde cholangiopancreatography (ERCP), a metallic stent insertion may be impossible due to duodenal obstruction, altered anatomy due to a previous operation (e.g., Roux-en-Y anastomosis), or tumor invasion of the major duodenal papilla^[5,6]. Until recently, percutaneous transhepatic biliary drainage (PTBD) has mainly been used in these cases. PTBD, however, is often accompanied by procedure-related adverse events and by many issues related to external drainage, such as pain, catheter dislodgement, and cosmetic problems. Thus, PTBD may cause a serious decline in the quality of life^[7,8].

Since the endoscopic ultrasound (EUS)-guided bile duct puncture was first reported in 1996, sporadic case reports on EUS-guided biliary drainage (EUS-BD) suggested that it was a feasible and effective alternative in patients with failed conventional ERCP stenting^[9-13]. Currently, three types of EUS-BD have been described, depending on the route of intervention [e.g., EUS-guided choledochoduodenostomy (EUS-CDS), EUS-guided hepaticogastrostomy, and EUS-guided gallbladder drainage]^[5,14,15]. Plastic stents usually have been used in the EUS-BD procedure, but several studies suggested that EUS-BD with a fully covered self-expandable metallic stent (FCSEMS) might be a feasible and useful alternative to PTBD^[6,16]. Although EUS-CDS with an FCSEMS is expected to show longer patency duration and fewer adverse events in patients with malignant biliary obstructions, to the best of our knowledge there have been no studies that address the long-term follow-up results of such a procedure. Therefore, we studied the long-term outcomes of EUS-CDS with an FCSEMS after failed conventional ERCP.

MATERIALS AND METHODS

Study population

From April 2009 to August 2010, a total of 2844 ERCPs were performed in a 2680-bed tertiary referral hospital; of these, 926 ERCPs were performed to relieve biliary obstruction. Endoscopic transpapillary biliary drainage failed in 115 (12.4%) patients, with 69 patients undergoing PTBD and 46 patients undergoing EUS-BD. EUS-CDS with an FCSEMS was attempted in 15 patients.

These 15 patients included 8 men and 7 women and had a median age of 61 years (range: 30-91 years). The causes of distal malignant biliary obstruction were 9 pancreatic cancers, 2 ampulla of Vater cancers, 1 renal cell carcinoma, 1 advanced gastric cancer, 1 lymphoma, and 1 duodenal cancer. The inclusion criteria were the presence of a distal malignant biliary obstruction and failed conventional ERCP stenting, and the exclusion criteria were

an inability to sedate the patient due to advanced heart or pulmonary diseases and a lack of informed consent. Finally, we excluded any patients who had been included in previous publications^[6].

Five experienced endoscopists (Lee SS, Park DH, Seo DW, Lee SK and Kim MH) performed the ERCP procedures, and two of them (Lee SS and Park DH) performed the EUS-CDS. These two endosonographers perform more than 500 EUS procedures for pancreaticobiliary diseases annually. This study was approved by the Institutional Review Board of our center. All patients provided written informed consent.

Techniques for EUS-CDS

We administered broad-spectrum, prophylactic antibiotics directed against gram-positive and gram-negative organisms before the procedure to minimize the risk of infection. Initially, ERCP was performed using a therapeutic duodenoscope (TJF-260, Olympus Optical, Tokyo, Japan). When the ERCP was unsuccessful, the EUS-CDS was performed using a linear-array echoendoscope (GF-UCT 240-AL 5) during the same endoscopy session or 1-2 d later.

The dilated extrahepatic duct was usually accessed with the echoendoscope placed at the duodenal bulb. The initial puncture was performed under real-time ultrasound and color Doppler guidance with a 19-gauge aspiration needle (EUSN-19-T, Cook Endoscopy, Winston-Salem, NC). After the puncture, the aspiration of bile and cholangiography was performed to confirm that there was an adequate puncture. Next, a 0.0889 cm guidewire was inserted through the needle and coiled in the bile duct lumen. The needle was exchanged for a 6F and a 7F tapered biliary dilator catheter (catheter tip, 4F; Cook Endoscopy, Winston-Salem, NC) to dilate the tract. If there was resistance to the advance of the dilator catheter, a triple-lumen needle-knife (Microtome, Boston Scientific, Natick, MA) with a 7F shaft diameter was gently inserted over the guidewire to dilate the tract using a brief burst of pure cutting current. Finally, an FCSEMS with an 8F deployment system (nitinol stent, 8 to 10 mm in diameter, 4 to 6 cm in length, and flared at both ends to prevent distal or proximal migration; BONASTENT, Standard Sci Tech, Seoul, South Korea) was inserted under echoendoscopic and fluoroscopic guidance (Figure 1).

Definition of events

Technical success was defined as the passage of a metallic stent across the duodenum, along with the flow of contrast medium and/or bile through the stent. Functional success was defined as a decrease in serum total bilirubin to < 75% of the pretreatment value within 4 wk. An early adverse event was defined as any stent-related adverse event within 4 wk, including pneumoperitoneum, bleeding, biloma, bile peritonitis, and stent migration. A late adverse event was defined as any stent-related adverse event occurring > 4 wk after the stent placement, such as stent migration or stent occlusion. Biliary re-intervention was defined as any type of endoscopic, percutaneous, or

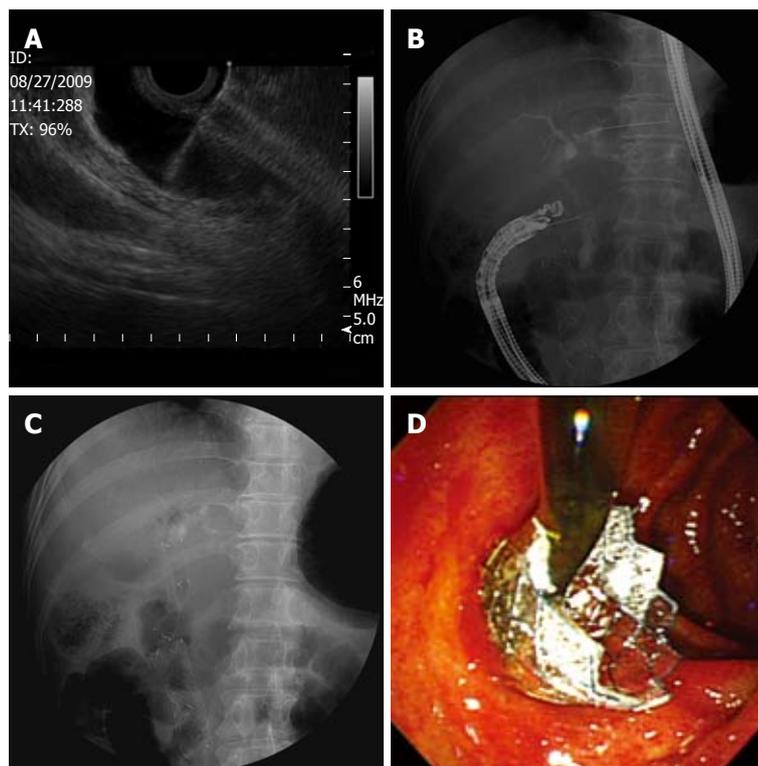


Figure 1 Technique for endoscopic ultrasound-guided choledochoduodenostomy with a fully covered self-expandable metallic stent. A: The bile duct was punctured with a 19-gauge needle under linear-array echoendoscopic guidance; B: A guidewire was introduced through the needle; C: A fully covered self-expandable metallic stent was inserted under fluoroscopic guidance; D: Illustration of a biliary stent extending from the first portion of the duodenum to the extrahepatic bile duct.

surgical procedure that was required to improve biliary drainage after the stent placement. The duration of stent patency was defined as the time between functionally successful EUS-CDS and the occurrence of stent occlusion, stent revision, or patient death.

Follow-up

Follow-up data were prospectively collected after the procedure until September 2011. Biochemical parameters and a simple abdominal X-ray were assessed on the day following the procedure, 1 wk after stent placement, and every month thereafter. The follow-up results of the patients were based on the findings from outpatient examinations.

Statistical analysis

The cumulative patency duration of the EUS-CD with an FCSEMS was estimated using the Kaplan-Meier technique. All statistical analyses were performed using SPSS software (version 12.0; SPSS Inc., Chicago, IL).

RESULTS

Technical and functional success

EUS-CDS with an FCSEMS was performed in 15 patients with a technical success rate of 86.7% (13/15). In two patients, the FCSEMS with a delivery device could not be passed into the CBD through the guidewire even after dilatation of the fistula tract with a needle knife and use of a 4-mm dilatation balloon because of the acute angulation of the scope. A 7F plastic stent, which is more flexible than an FCSEMS, was successfully inserted in one of these two patients, and PTBD was performed in

the other patient as a rescue method.

The functional success rate of EUS-CDS with an FCSEMS was 100% (13/13). The baseline demographic characteristics of the 13 patients who underwent successful EUS-CDS with an FCSEMS are shown in Table 1.

Adverse events

Early adverse events developed in 3 patients (3/13, 23.1%), including 2 cases of self-limited pneumoperitoneum and 1 case of cholangitis. In 1 patient, the proximal tip of the stent was placed in the left intrahepatic duct, impairing drainage of the right intrahepatic duct and leading to cholangitis of the right intrahepatic duct. The cholangitis improved after the tip of the stent was repositioned below the hilar region.

During the follow-up period, distal stent migration occurred in 4 patients (4/13, 30.8%) as a late adverse event. Among them, 2 patients presented with cholangitis, and 1 patient presented with jaundice. In these patients, an FCSEMS could be reinserted through the existing choledochoduodenal fistula tract (Figure 2). In another patient, distal stent migration was observed during a routine, follow-up X-ray without the patient experiencing adverse symptoms: additional stent insertion was not considered because his life expectancy was less than a month and bile was draining through his matured fistula tract.

Duration of stent patency

All patients were followed up until their time of death, with a median follow-up period of 186 d (range: 52-388 d). Stent patency was maintained until death in 9 patients, while distal stent migration occurred in 4 patients during

Table 1 Patients' characteristics and technical features of endoscopic ultrasound-guided choledochoduodenostomy with fully covered self-expandable metallic stents

No.	Age/sex	Diagnosis	Reason for failed ERCP	Diameter/length of FCSEMS (mm/mm)	Early adverse events	Late adverse events	Re-intervention
1	74/M	Pancreatic cancer	Periampullary tumor infiltration	8/40	None	None	None
2	63/F	Pancreatic cancer	Duodenal obstruction	10/50	None	Distal migration	Reinsertion of FCSEMS
3	56/M	Pancreatic cancer	Periampullary tumor infiltration	8/50	Pneumoperitoneum	None	None
4	78/F	Pancreatic cancer	Periampullary tumor infiltration	8/60	Pneumoperitoneum	None	None
5	75/F	Pancreatic cancer	Periampullary tumor infiltration	8/60	None	Distal migration	Reinsertion of FCSEMS
6	46/M	DLBL	Duodenal obstruction	8/50	None	None	None
7	91/M	AOV cancer	Periampullary tumor infiltration	8/60	None	Distal migration	Reinsertion of FCSEMS
8	59/F	RCC	Duodenal obstruction	8/60	None	Distal migration	None
9	30/M	Pancreatic cancer	Duodenal obstruction	8/50	None	None	None
10	68/M	AOV cancer	Periampullary tumor infiltration	8/50	None	None	None
11	54/F	Pancreatic cancer	Periampullary tumor infiltration	8/60	None	None	None
12	45/F	AGC	Periampullary tumor infiltration	8/60	Cholangitis	None	Repositioning of stent
13	58/M	Duodenal cancer	Duodenal obstruction	8/60	None	None	None

F: Female; M: Male; ERCP: Endoscopic retrograde cholangiopancreatography; FCSEMS: Fully covered self-expandable metallic stent; DLBL: Diffuse large B-cell lymphoma; AOV: Ampulla of Vater; RCC: Renal cell carcinoma; AGC: Advanced gastric cancer.

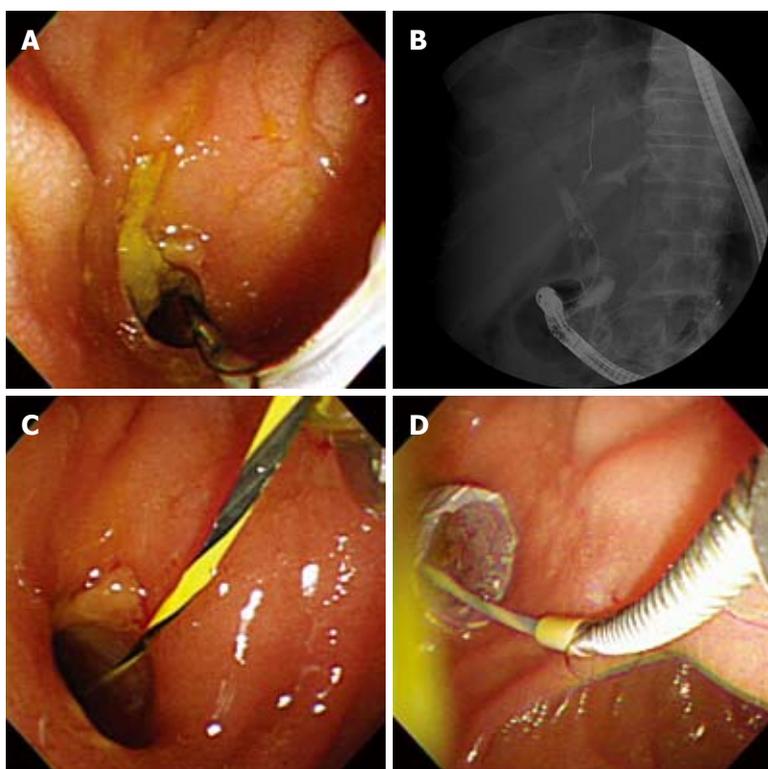


Figure 2 Endoscopic re-intervention for the distal migration of a fully covered self-expandable metallic stent. A: Endoscopic view showing the patent opening of a fistulous tract between the extrahepatic bile duct and duodenum; B: A fluoroscopic image demonstrated the distal stent migration; C: A guidewire was simply inserted through the existing choledochoduodenal fistula; D: Insertion of a new fully covered self-expandable metallic stent through a mature fistula with a large diameter.

the follow-up period (range: 68-185 d). The mean patency duration of the stents was 264 d (Figure 3).

DISCUSSION

Previous studies on EUS-CDS have shown favorable technical success rates, with resolution of obstructive jaundice observed in all patients after stent placement. To date, EUS-CDS with a metallic stent insertion has been reported in 11 patients, including covered metallic stents in 6 patients, partially covered metallic stents in 3, and uncovered metallic stents in 2^[6,17-20]. However, there have not yet been any long-term, follow-up results of stent

patency in patients who have undergone EUS-CDS with a metallic stent, making this study the first long-term follow-up evaluation of EUS-CDS with an FCSEMS.

Until recently, there has been only one study to our knowledge that has reported on the long-term results of EUS-CDS with plastic stents. Yamao *et al*^[11] reported that the mean duration of stent patency of EUS-CDS with plastic stents was 211.8 d. Usually, the diameter of metallic stents is 8-10 mm, which is larger than that of plastic stents (7-10F). Thus, theoretically, metallic stents have an advantage in terms of stent patency over plastic stents. In transpapillary drainage through the ERCP, metallic stents have been shown to have a longer

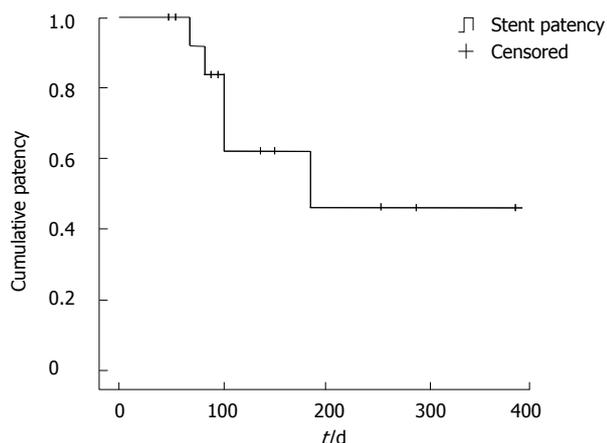


Figure 3 Kaplan-Meier survival curve showing stent patency.

patency duration compared with plastic stents, and the mean patency duration of a metallic stent inserted *via* the transpapillary route was 234–506 d^[4,21]. In our study, the mean duration of stent patency was 264 d, which is comparable with that observed in transpapillary drainage with an FCSEMS.

We found that the technical success rate of EUS-CDS with an FCSEMS was 86.7% (13/15). Although this rate is acceptable, it also indicates that EUS-CDS may not be simple to perform and that careful consideration and adequate experience in EUS-related intervention are mandatory. In this study, EUS-CDS with an FCSEMS was unsuccessful in 2 patients. Because of the acute angulation of the echoendoscope in the duodenal bulb in these patients, the force exerted on the FCSEMS resulted in the echoendoscope being pushed away from the duodenal wall, thereby leading to technical failure. This finding indicates that despite the creation of a fistula with an adequate diameter for FCSEMS insertion, stent insertion may fail if the echoendoscope has an acute angulation because an undeployed metallic stent is rigid.

In previous studies, early adverse events of EUS-CDS included bile peritonitis, pneumoperitoneum, and hemobilia, all of which are self-limiting^[10,14,17,22,23]. In EUS-CDS, the puncture site is selected to bypass the tumor around the distal bile duct, and thus, the puncture may be located close to the hepatic hilum. If the puncture is made near the hepatic hilum, then the proximal end of the stent can be inserted into the right or left intrahepatic duct. In this case, a fully covered metallic stent may block the opposite duct because its diameter is larger than that of a plastic stent. With uncovered metallic stents, although the drainage of the opposite duct is possible through the open mesh, its use is restricted because the risk of a bile leak through the fistula tract from the duodenum into the bile duct exists^[24]. In our study, 1 case showed cholangitis caused by the obstruction of the right intrahepatic duct, which resulted from the insertion of the proximal end of the stent into the left intrahepatic duct. Therefore, the puncture should be made while avoiding the hepatic hilum if possible, and the

insertion of a plastic stent is suggested to be better than a metallic stent in cases where the puncture site is close to the hepatic hilum. In addition, the development of a partially covered metallic stent-in which the proximal end is specially designed to lack a covered membrane-is required to overcome these disadvantages.

In this study, the stent patency of 9 patients was maintained until their death, but distal stent migration occurred in 4 patients during the follow-up period. These results indicate that the most important factor in maintaining stent patency in patients undergoing EUS-CDS with an FCSEMS may be the prevention of stent migration. Because the FCSEMS has a large bore diameter, it may theoretically have advantages over the conventional plastic stents in terms of stent revision. In patients with an FCSEMS insertion, stent revision was relatively easy because the opening of the fistula tract was large enough to be able to easily find, even after stent migration. In an FCSEMS inserted *via* the transpapillary method, tumor ingrowth due to a crack in the covered membrane or tumor overgrowth can be observed^[25,26]. EUS-CDS, however, has advantages in that it reduces the risk of tumor ingrowth or tumor overgrowth because it bypasses the tumor instead of directly passing through the tumor. Stent obstruction by tumor ingrowth or overgrowth was not found in this study.

This study has several limitations. First, the study population was not sufficiently large to allow for a decisive conclusion regarding our results. Second, standard techniques and devices for the EUS-guided drainage procedure have not yet been established. Each endosonographer used a slightly different technique, which may have affected the results of the study. A prospective multicenter evaluation may be valuable to overcome this limitation. Third, this study is an observational study. Thus, a prospective randomized study comparing EUS-CDS with an FCSEMS and other alternative drainage methods, such as percutaneous metallic stent insertion, is necessary.

In conclusion, we have prospectively assessed the long-term outcomes of EUS-CDS with an FCSEMS. EUS-CDS with an FCSEMS was a safe and effective method in patients with distal malignant biliary obstructions and had a comparatively long patency duration. Nevertheless, the significant rate of distal stent migration cannot be ignored, suggesting the need for a newly designed metallic stent for EUS-CDS.

COMMENTS

Background

Endoscopic ultrasound-guided choledochoduodenostomy (EUS-CDS) may be a feasible and useful alternative in patients with distal malignant biliary obstructions after failed endoscopic retrograde cholangiopancreatography (ERCP). Little is known, however, about the long-term outcomes of EUS-CDS with a fully covered self-expandable metallic stent (FCSEMS).

Research frontiers

This is the first study that addresses the long-term follow-up results of patients who underwent EUS-CDS with an FCSEMS.

Innovations and breakthroughs

The technical and functional success rates of EUS-CDS with an FCSEMS were 13 (86.7%) and 13 (100%), respectively. EUS-CDS with an FCSEMS is technically feasible and can lead to effective palliation of distal malignant biliary obstructions after failed ERCP. EUS-CDS with an FCSEMS showed a comparatively long patency duration (264 d).

Applications

Although EUS-CDS with an FCSEMS showed a high success rate and comparatively long patency duration, the significant rate (30.8%) of distal stent migration suggests the need for a newly designed metallic stent for EUS-CDS.

Terminology

EUS-CDS is a new technique for biliary drainage using EUS-guided puncture of the common bile duct from the duodenal bulb and is usually performed as a rescue drainage method when endoscopic transpapillary stenting fails.

Peer review

The authors examined the usefulness and long-term outcomes of EUS-CDS with an FCSEMS. It revealed that EUS-CDS with an FCSEMS is technically feasible and can lead to effective palliation of distal malignant biliary obstructions after failed ERCP. However, it also showed a significant rate of distal stent migration. Therefore, a prospective randomized study on EUS-CDS with newly developed stents is needed.

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Favorable surgical treatment outcomes for chronic constipation with features of colonic pseudo-obstruction

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Abstract

AIM: To determine long-term outcomes of surgical treatments for patients with constipation and features of colonic pseudo-obstruction.

METHODS: Consecutive 42 patients who underwent surgery for chronic constipation within the last 13 years were prospectively collected. We identified a subgroup with colonic pseudo-obstruction (CPO) features, with dilatation of the colon proximal to the narrowed transitional zone, in contrast to typical slow-transit constipation (STC), without any dilated colonic segments. The outcomes of surgical treatments for chronic constipation with features of CPO were analyzed and compared with outcomes for STC.

RESULTS: Of the 42 patients who underwent surgery for constipation, 33 patients had CPO with dilatation of the colon proximal to the narrowed transitional zone.

There were 16 males and 17 females with a mean age of 51.2 ± 16.1 years. All had symptoms of chronic intestinal obstruction, including abdominal distension, pain, nausea, or vomiting, and the mean duration of symptoms was 67 mo (range: 6-252 mo). Preoperative defecation frequency was 1.5 ± 0.6 times/wk (range: 1-2 times/wk). Thirty-two patients underwent total colectomy, and one patient underwent diverting transverse colostomy. There was no surgery-related mortality. Postoperative histologic examination showed hypoganglionosis or aganglionosis in 23 patients and hypoganglionosis combined with visceral neuropathy or myopathy in 10 patients. In contrast, histology of STC group revealed intestinal neuronal dysplasia type B ($n = 6$) and visceral myopathy ($n = 3$). Early postoperative complications developed in six patients with CPO; wound infection ($n = 3$), paralytic ileus ($n = 2$), and intraabdominal abscess ($n = 1$). Defecation frequencies 3 mo after surgery improved to 4.2 ± 3.2 times/d (range: 1-15 times/d). Long-term follow-up (median: 39.7 mo) was available in 32 patients; all patients had improvements in constipation symptoms, but two patients needed intermittent medication for management of diarrhea. All 32 patients had distinct improvements in constipation symptoms (with a mean bowel frequency of 3.3 ± 1.3 times/d), social activities, and body mass index (20.5 kg/m^2 to 22.1 kg/m^2) and were satisfied with the results of their surgical treatment. In comparison with nine patients who underwent colectomy for STC without colon dilatation, those in the CPO group had a lower incidence of small bowel obstructions (0% vs 55.6%, $P < 0.01$) and less difficulty with long-distance travel (6.7% vs 66.7%, $P = 0.007$) on long-term follow-up.

CONCLUSION: Chronic constipation patients with features of CPO caused by narrowed transitional zone in the left colon had favorable outcomes after total colectomy.

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Key words: Constipation; Total colectomy; Pseudo-obstruction; Surgical outcome; Hypoganglionosis

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INTRODUCTION

Constipation is a common clinical problem with multiple etiologies, affecting approximately 16.5% of the population in South Korea^[1] and 5%-20% of the Western world^[2]. Most patients with constipation have various symptoms, representing different pathologic processes^[3,4] and including infrequent or difficult evacuation, abdominal pain, and bloating, which are often resistant to medical therapy or dietary manipulation. Constipation is believed to be frequently observed in women, elderly people, and those of low socioeconomic status^[5-7]. When chronic idiopathic constipation is diagnosed, a conservative treatment is generally conducted as a first-line treatment. If such a conservative treatment fails, surgical treatment is then considered^[8-12]. The British surgeon Lane^[13] first performed surgery for constipation in 1908. Since then, total colectomy (TC) with ileorectal anastomosis has been suggested as a standard option for the management of refractory chronic constipation. In our previous report, we demonstrated favorable surgical outcomes for patients with features of chronic pseudo-obstruction (CPO) with distinct transitional zone (TZ)^[14]. The purpose of this study was to analyze the long-term surgical outcomes of patients with chronic idiopathic constipation and features of CPO, and compare these results with treatment of patients with slow-transit constipation (STC).

MATERIALS AND METHODS

Patients

Between 1998 and 2011, 47 consecutive patients with chronic intractable constipation underwent surgery by a single surgeon at the Department of Surgery, Seoul National University Hospital. Two patients were excluded from this study because they had secondary constipation due to amyloidosis, and three patients were excluded because they had Hirschsprung's disease (HD). The remaining 42 patients with chronic idiopathic constipation were enrolled in our study. All patients were subjected to a full history taking and physical examination. Prior to surgery, 40 (95.2%) patients were treated with various medications, such as bulking agents, stimulant laxa-

tives, and probiotics, specifically, 24 patients (57.1%) were treated with prokinetic agents (e.g., mosapride and itopride) and eight patients (19.0%) were treated with pyridostigmine. Suppositories or enemas were used in 40 patients (95.2%). Anorectal manometry and colon transit-time studies were selectively conducted as preoperative functional tests. Plain abdominal X-ray, abdominal computed tomography scan, and colonoscopy were selectively conducted when necessary. The preoperative and postoperative clinical symptoms, postoperative complications, defecation frequency after surgery, results of postoperative histologic examination, postoperative satisfaction, and postoperative activities were retrospectively analyzed by a medical chart review and phone survey. Postoperative satisfaction after surgery was divided into five grades: poor, unhappy, good/satisfied, improved, and very good, and was scored from 1 to 5 points. Surgery was considered successful if the score was 3 points or higher^[15].

Statistical analysis

Analysis were performed using the SPSS statistical software package (version 18.0; SPSS Inc, Chicago, IL). Comparisons were made between groups using the χ^2 and Mann-Whitney tests. *P* values of less than 0.05 were considered statistically significant.

RESULTS

The patient group included 21 women and 21 men, with a mean age of 51.2 ± 16.1 years (range: 18-75 years) and a median follow-up duration of 39.7 mo (range: 3-147 mo). Thirty-three patients (78.6%) presented with features of CPO with distinct TZ (the CPO group), whereas the remaining nine patients (21.4%) had STC without colon dilatation (the STC group; Figure 1). The preoperative characteristics of the CPO and STC groups are shown in Table 1. The patients complained of symptoms including abdominal pain, abdominal distension, vomiting, and weight loss before surgery. The mean duration of symptoms was 67.1 ± 72.8 mo (range: 6-252 mo). Of the 42 patients, 26 (61.9%) experienced abdominal pain preoperatively, 21 (50.0%) complained of abdominal distention, and 15 (35.7%) experienced inadequate defecation. Nausea or vomiting was a chronic problem in six patients (14.3%), and three patients (7.1%) experienced weight loss preoperatively. Among the 42 patients, 40 patients (95.2%) received laxatives or enemas for treatment of chronic constipation. Three patients had a previous history of abdominal surgery in other hospitals; one patient underwent colostomy; one patient had ileostomy because of persistent intestinal obstruction after right hemicolectomy; and one patient underwent biopsy after exploration.

The surgical procedures performed in this study were as follows: 39 patients underwent TC with ileorectal anastomosis (92.9%); two patients underwent TC with end ileostomy (4.8%); and one patient underwent

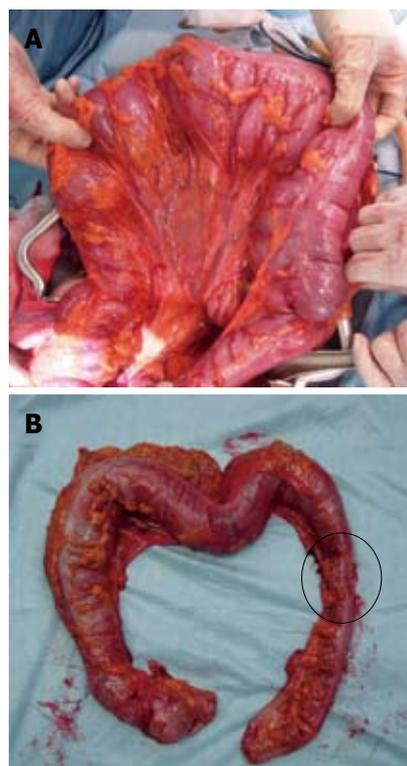


Figure 1 Operative findings and colectomy specimen from a 70-year-old female with chronic constipation. A: Operative findings; B: Colectomy specimen. A definite transitional zone (marked with a circle) with proximal dilatation and distal collapse can be seen.

transverse colostomy (2.3%). The patient who underwent transverse colostomy instead of TC was initially treated for severe bowel distension but refused further surgery. In 13 patients (30.9%), emergency surgery was performed because of impending perforation or toxic symptoms such as tachycardia, fever, hypotension, and marked dilatation of both the small intestine and colon.

In the entire CPO group, intraoperative findings manifested as marked colonic dilatation proximally within the TZ and a normal or collapsed distal bowel lumen. The location of the TZ was near the left side of the descending colon in 20 patients (60.6%) and of the sigmoid colon in nine patients (27.3%) patients; four patients (12.1%) had TZ in the distal transverse colon.

Postoperative histologic examination in the CPO group showed hypoganglionosis (HG) or agangliosis (AG) in 23 patients, and HG combined with visceral neuropathy or myopathy in 10 patients. In contrast, histology in STC group revealed visceral neuropathy [intestinal neuronal dysplasia (IND) type B, $n = 6$] and visceral myopathy ($n = 3$) (Table 2).

Postoperative results are shown in Table 1. The patients in the CPO group passed the first flatus significantly earlier than did patients in the STC group, and their hospital stay tended to be shorter. Early postoperative complications developed in seven patients (16.7%): paralytic ileus ($n = 3$), wound infection ($n = 3$), and intraabdominal abscess ($n = 1$). The incidence of early postoperative complications did not differ significantly

Table 1 Preoperative clinical characteristics and postoperative results of patients with constipation

	CPO ($n = 33$)	STC ($n = 9$)	<i>P</i> value	
Preoperative clinical characteristics				
Sex (male/female)	21/21	16/17	5/4	0.43
Age (yr)	51.2 ± 16.1	50.7 ± 16.2	53.2 ± 16.6	0.23
Constipation duration (mo)	67.1 ± 72.8	38.3 ± 49.4	69.7 ± 75.1	0.34
Defecation frequency (times/wk)	1.6 ± 0.5	1.5 ± 0.3	1.6 ± 0.6	0.15
Postoperative results				
Gas passing (d)	4.2 ± 1.5	4.1 ± 1.0	5.1 ± 0.8	< 0.01
Postoperative hospital stay (d)	11.4 ± 4.7	10.7 ± 4.9	13.3 ± 3.2	0.07
Defecation frequency (times/d) at 3 mo postop	5.1 ± 3.6	4.2 ± 3.2	5.2 ± 3.1	0.56

CPO: Colonic pseudo-obstruction; STC: Slow-transit constipation.

Table 2 Results of histologic examination in two groups

	CPO ($n = 33$)	STC ($n = 9$)
Hypogangliosis or agangliosis	23	0
Hypogangliosis with neuropathy or myopathy	10	0
Visceral neuropathy	0	6
Visceral myopathy	0	3

CPO: Colonic pseudo-obstruction; STC: Slow-transit constipation.

Table 3 Early and late postoperative complications

Complications	CPO	STC	<i>P</i> value
Early complications ($n = 7$)			
Wound infection	3	0	0.47
Ileus	1	2	0.33
Intra-abdominal abscess	1	0	0.78
Late complications ($n = 10$)			
Small-bowel obstruction	0	5	< 0.01
Diarrhea	2	2	0.15

CPO: Colonic pseudo-obstruction; STC: Slow-transit constipation.

between the CPO and STC groups (Table 3). All of these early complications resolved after conservative care, and there was neither anastomotic leakage nor mortality.

In long-term follow-up (median: 40 mo), recurrent small bowel obstruction requiring hospitalization occurred in five patients (11.9%). The incidence of small bowel obstruction was significantly higher in the STC group (0% in CPO *vs* 55.6% in STC, $P < 0.01$), and all patients in whom small bowel obstruction occurred had pathologic features of IND type B. These five patients in the STC group responded to conservative management. Postoperative diarrhea requiring intermittent medication occurred in four patients (9.5%); two in the CPO group and two in the STC group).

Preoperatively, the mean defecation frequency was 1.6 times/wk. The defecation frequencies were 1.5 ± 0.3 times/wk (range: 1-2 times/wk) and 1.6 ± 0.6 times/wk (range: 1-2 times/wk) in the CPO and STC groups, respectively (Table 1). At 3 mo after surgery, defecation

Table 4 Quality of life scoring (questionnaire) and postoperative satisfaction *n* (%)

Scores	Quality of life scoring	CPO	STC
1	Poor	0 (0)	0 (0)
2	Unhappy	0 (0)	1 (11.1)
3	Good/satisfied	5 (15.6)	2 (22.2)
4	Improved	25 (78.1)	5 (55.6)
5	Very good	2 (6.3)	1 (11.1)
Scores of ≥ 3		32/32 (100)	8/9 (89)

CPO: Colonic pseudo-obstruction; STC: Slow-transit constipation.

frequency increased in both groups (Table 1) but did not differ significantly between the two groups ($P = 0.56$).

Informed consent for the questionnaire on satisfaction and quality of life after surgery was received from 41 of the 42 patients (97.6%) who were alive at the time of the review. Overall, 97.6% of the patients (40/41) were satisfied with the results of their surgery (Table 4). All 32 patients in the CPO group responded that they were satisfied with the results of the surgery. In comparison, one patient in the STC group gave 2 points and was not satisfied with the surgery because she had treatments for intermittent obstructive symptoms. Thirty-three patients (80.5%) indicated that they had no problems in their daily or leisure activities, housework, travel, and social activities. However, eight patients (19.5%) complained of difficulty with long-distance travel because of increased bowel frequency. In the CPO group, only two patients (6.7%) had difficulty with long-distance travel in contrast to six patients (66.7%) in the STC group ($P = 0.007$). Body mass index increased from 20.5 kg/m² to 22.1 kg/m² in the CPO group, and from 19.9 kg/m² to 22.0 kg/m² in the STC group, which did not significantly differ between the two groups.

DISCUSSION

Surgical treatment may be considered in patients with intractable constipation who are poorly responsive to conventional medical treatments. TC with ileorectal anastomosis is the most commonly performed surgical procedure for constipation^[16-18]. The reasons for primarily performing TC in patients with chronic constipation are that it is difficult to precisely distinguish between normal bowel segments and pathologic lesions and that symptoms may recur if segmental resection alone is performed^[19-21]. In our series of patients, one of the patients in CPO group had undergone right hemicolectomy at another hospital for chronic constipation as well as two more operations because of persistent symptoms within the previous 2 years. Subsequently, the patient visited our hospital and underwent TC. As a result, the patient enjoys good nutritional status without any evidence of recurrence to date. This case illustrates the importance of appropriate resection with pathologic lesions, using TC as a favorable example.

When evaluating the results of surgical treatments

for chronic constipation, it is important to assess patient satisfaction after surgical treatment, complications, and improvement in quality of life. All the patients in this study experienced difficulty in their daily lives due to symptoms of intestinal obstruction preoperatively. In severe cases, these symptoms prevented the patient from sufficient nutritional intake. However, almost all of the patients were satisfied with their surgery and had no problems in their daily lives afterwards.

Our study suggests that defecation frequency improved significantly after surgery. Postoperative diarrhea, when it occurred, was present for only a short time and did not require long-term therapy. Constipation symptoms in the preoperative period, such as abdominal distention or pain, disappeared in both groups. There were no serious early postoperative complications and late complications, especially recurrent small bowel obstruction, were more common in the STC group. All five patients with small bowel obstruction had pathologic findings of IND type B and were successfully managed with conservative therapy. In the STC group of patients with pathologic features of IND type B, the pathology may not be localized to the colon; thus, the possibility of small bowel involvement exists. This may account for intestinal obstructions after the surgery in the STC group of patients with IND type B pathology. None of the three patients with visceral myopathy in the STC group had a small bowel obstruction episode. On the other hand, pathologic changes of AG or HG associated with features of CPO are limited to a relatively short segment of the colon and probably account for the low rate of small bowel obstruction after TC.

HG, a disease associated with a limited number of intestinal ganglion cells, is an intestinal neuronal dysganglionosis arising in the gastrointestinal tract^[17]. HG is typified by a decreased number of ganglion cells, a reduced size of ganglia, and a wider distance between myenteric ganglia. The diagnostic criteria used for diagnosis of HG and IND type B have been previously discussed^[14]. IND type B has also been described as one of the causes of chronic idiopathic constipation^[22]. In the CPO group, HG and AG were confined to a short segment of the colon in most patients, resulting in a failure of segment relaxation and dilatation of the proximal colon, as is the case for HD in which the region of the colon proximal to the aganglionic segment becomes dilated. Segmental HG or AG may be considered a variant of HD. A finding similar to our study was recently described by Do *et al.*^[23] in which the term adult-onset HD was used. However, clinically, radiologically, and histologically, there may be differences^[24,25]. Clinically, HG shows later symptom onset and a better prognosis than HD. In addition, in abdominal computed tomography and barium enema studies, TZ ratio (the transverse diameter ratio of the most dilated colonic segment proximal to the TZ to the narrowed colonic segment distal to the TZ) was higher in HD than in HG^[26]. Since our patients had symptom onset during adulthood, we suggest that our

patients with features of CPO have an acquired form of AG or HG, in contrast to typical HD, which manifests in early childhood. Additionally, various mechanisms for ganglion cell loss have been described^[27-32], and acquired loss of myenteric nerves may occur secondary to Chagas disease, multiple sclerosis, scleroderma, diabetes, amyloid disease, advanced malignancy, Crohn's disease, and in response to certain medications^[8,33].

In our STC patients, since there was no TZ, pathologic examinations were performed at random sites throughout the colon, thus reflecting the diffuse nature of the pathologic changes. As such, it is assumed that the CPO patients in our study have a zonal pathology that is responsible for their constipation compared to the diffuse pathologic changes observed in the STC patients. The persistent abdominal symptoms that include small bowel obstruction episodes after surgery may result from diffuse neurologic abnormalities of the gastrointestinal tract in IND type B patients. In the case of gastrointestinal dysmotility involving the small bowel, a careful approach is required for selecting surgical treatment because the role of surgery may be limited and recurrence is possible^[34,35].

In our study, the CPO group had better early postoperative results regarding gas passing and shortened hospital stays compared to the STC group. On long-term follow-up, all of the CPO patients had distinct improvements in constipation symptoms and were satisfied with the results of their surgical treatment. In comparison with the STC group, the CPO group had a lower incidence of small bowel obstructions and fewer difficulties with long-distance travel on long-term follow-up.

CPO in patients with chronic constipation is characterized by a narrowed TZ in the left side of the colon. Its pathologic features suggest that CPO is caused by acquired segmental HG or AG. Pathologic findings in the CPO group were quite different from patients in the STC group without a dilated colon, and the CPO group had outcomes that were more favorable after TC compared to those of STC patients.

COMMENTS

Background

Constipation is a common clinical problem with multiple etiologies and affects. When chronic idiopathic constipation is diagnosed, a conservative treatment is conducted as the first line treatment. If the conservative treatment has failed, surgical treatment is then considered

Research frontiers

Because of diagnostic difficulty, hypoganglionosis (HG) is often considered to be a variant of Hirschsprung's disease (HD) or a subtype of intestinal neuronal dysplasia type B. But clinically, radiologically and histologically, there are differences between them. Clinically, HG shows later onset of symptoms and a better prognosis than HD. In abdominal computed tomography and barium enema studies, transition zone ratio (the transverse diameter ratio of the most dilated colonic segment proximal to the transitional zone (TZ) to the narrowed colonic segment distal to the TZ) is higher in HD than in HG.

Innovations and breakthroughs

In the series of patients operated for intractable constipation, patients were divided into two groups; features of chronic pseudo-obstruction (CPO) with distinct transitional zone and slow-transit constipation (STC) without colonic dilata-

tion. Authors suggested that CPO group had more favorable outcome after total colectomy as compared to STC patients.

Applications

By understanding pathophysiology of CPO, this study may represent a future strategy for surgical procedure in the treatment of patients with chronic idiopathic constipation.

Terminology

HG has been associated with fewer intestinal ganglion cells. HG is one of an intestinal neuronal dysganglionosis arising in the gastrointestinal tract.

Peer review

The authors provide evidence that patients with chronic constipation with CPO do have a better outcome from surgical resection than patients with slow transit dysmotility. Distinguishing the two groups of patients is not difficult and the article suggests that if CPO is identified with a normal caliber distal colon, that these patients have better outcome. While that authors suggest the aganglionosis in this segment of bowel is acquired, others believe that this disease is still a variant of HD. Nevertheless, it seems reasonable that such patients tend to have better outcomes with surgery than patients who have dysmotility.

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***In vivo* detection of mucosal healing-involved histiocytes by confocal laser endomicroscopy**

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Abstract

Histiocytes have a pivotal role in wound repair and intestinal epithelial recovery - the most important goal to sustain gut functionality. Yet, an *in vivo* description of colonic histiocytes by confocal laser endomicroscopy (CLE) is missing. Here, we report the case of a 45-years-old male patient who was referred to our clinic with

weight loss and a history of two consecutive *Clostridium difficile* colitis episodes, the latter cured 3 wk before present admission. Stool microbiology was negative. Conventional colonoscopy showed atrophy and a light mucosal oedema in the distal colon. During on-going endoscopy, we performed a fluorescein-aided CLE which revealed large polygonal (histiocytes-like) cells with copious cytoplasm and large nuclei in the lamina propria of the sigmoid colon as well as regenerative epithelial changes. Histopathological assessment of biopsies from the same areas confirmed the endomicroscopical findings: Periodic acid-Schiff- and CD68-positive foamy histiocytes in the colonic lamina propria and an advanced epithelial recovery. Since stool microbiology was repeatedly negative and polymerase chain reaction-analysis from colonic biopsies could not detect any mRNA for *Thropheryma whipplei* and common pathogens, we interpreted this particular setting as a mucosal healing process after consecutive *Clostridium difficile* infections. In conclusion, by describing these colonic histiocytes, we highlight the clinical usefulness of CLE in describing the entity of histiocytes *in vivo* and in real-time during the process of post-infectious mucosal healing in the colon.

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Key words: Endomicroscopy; Mucosal healing; Advanced colonic imaging; Colonic histiocytes

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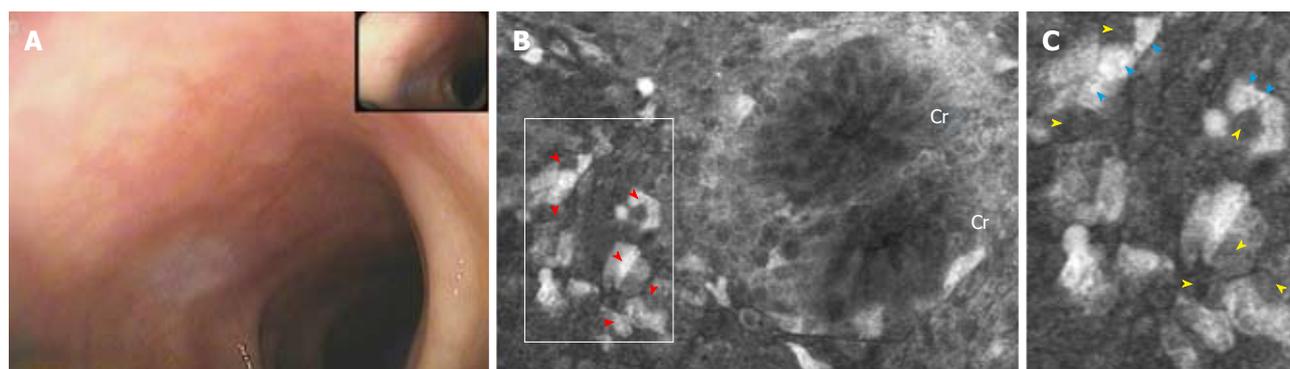


Figure 1 Endoscopic and endomicroscopic characterization of the sigmoid colon in the context of a post-infectious mucosal healing process. A: White light colonoscopy shows mild atrophy and reduced vascular pattern in the sigmoid colon; B: Fluorescence-guided confocal laser endomicroscopy reveals large polyclonal cells (red arrowheads) with copious cytoplasm in the lamina propria - near two crypts (Cr) - corresponding morphologically to foamy histiocytes; C: In an enlarged manner the aggregated histiocytes with large fluorescence-negative nuclei (yellow arrowheads) and foamy cytoplasm (blue arrowheads) defining their appearance and nomenclature.

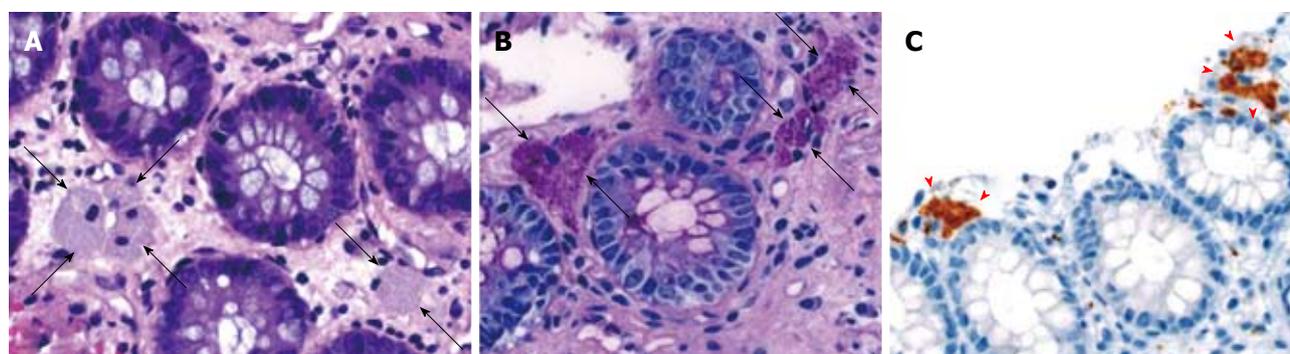


Figure 2 Histopathologic confirmation of the endomicroscopic findings. A-C: Large polyclonal histiocytes between mucosal crypts (arrows) are highlighted in the hematoxylin-eosin stain (A) as well as in Periodic acid-Schiff stain after diastase predigestion (B) and image (C) (CD68 immunostaining) with a CD68-positive cytosol, overall confirming the endomicroscopical findings.

INTRODUCTION

A subset of macrophages differentiating to histiocytes were proved to have a pivotal role in wound repair processes^[1] and intestinal epithelial recovery. This is the most important goal to sustain functionality of the gut and it was also defined as therapeutic goal in order to achieve mucosal healing in chronic inflammatory bowel disease^[2]. So far, two reports have described histiocytes in the duodenum^[3,4] using confocal laser endomicroscopy (CLE)^[5]. Yet, the *in vivo* description of histiocytes within the colon by confocal endomicroscopy has not been published so far.

CASE REPORT

Here, we report the case of a 45-year-old male patient who was referred to our endoscopy unit with anaemia and weight loss. In the last three months he had a history of 2 consecutive *Clostridium difficile* (*C. difficile*) colitis episodes, the latter resolved completely after appropriate treatment 3 wk before the present admission. Microbiological analysis of stool samples was repeatedly negative for *C. difficile* and other intestinal pathogens (Salmonella, Shigella, Yers-

inia, Campylobacter). White light colonoscopy showed signs of atrophy, mild mucosal oedema and reduced vasculature pattern in the distal colon (Figure 1A). During on-going endoscopy, we performed a fluorescein-aided confocal endomicroscopy of the colonic mucosa which revealed large polygonal (histiocytes-like) cells with copious cytoplasm and large nuclei in the lamina propria of the sigmoid colon (Figure 1B, C). By taking biopsies from the areas analysed by confocal imaging, we were able to correlate and verify the endomicroscopical findings with histopathology. These were Periodic acid-Schiff-positive and CD68-positive foamy histiocytes in the colonic lamina propria between basal mucosal crypts (Figure 2A-C).

DISCUSSION

Since stool microbiology was repeatedly negative and PCR-analysis could not detect any intestinal pathogens (incl. *Tropheryma whippelii* that causes Whipple's disease), we interpreted this particular setting as a mucosal healing process after two consecutive *C. difficile* infections^[6].

In conclusion, by describing these colonic histiocytes *in vivo* and real-time, we highlight the clinical usefulness of confocal laser endomicroscopy in characterizing the

cell entity of colonic histiocytes and the context of a post-infectious mucosal healing process in the colon, for the first time. Confocal laser endomicroscopy was used previously for the detection of architectural changes, vascularity changes like leakage but not for the differentiation of a specific cell entity. Our report provides the morphological criteria and exemplifies the differentiation and characterization of a particular cell entity, namely the foamy histiocytes, involved in the post-infectious mucosal healing.

In this histopathological and clinical setting, our report is also the first non-invasive and real-time description of human foamy histiocytes in the colon.

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Candida-associated gastric ulcer relapsing in a different position with a different appearance

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Abstract

An 87-year-old, Japanese woman was shown to have a submucosal tumor-like lesion with a deep, central ulceration covered with thick, whitish exudate in the stomach. Biopsy showed Candida tropicalis but not *Helicobacter pylori* (*H. pylori*). She had no predisposing factors or history of peptic ulcers nor had taken non-steroidal anti-inflammatory drugs (NSAIDs), diagnosed with Candida-associated gastric ulcer. Though cured of the lesion, she developed another ulcer in a different position, in which Candida was demonstrated but *H. pylori* was undetectable. This is the first case of recurrent Candida-associated gastric ulcer in the world. Detected in both the original and recurrent lesions in an *H. pylori*-negative patient with no antecedent ulcers who had not taken NSAIDs, Candida is considered, contrary to the prevailing opinion, to play an etiologic role in ulcer formation.

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Key words: Candida-associated gastric ulcer; Gastric candidiasis; *Helicobacter pylori*-negative gastric ulcer; Non-steroidal anti-inflammatory drugs-induced gastric ulcer; Recurrent gastric ulcer

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INTRODUCTION

Candida is ubiquitously indigenous to the normal human gastrointestinal tract throughout^[1] but infection with the fungus of the tract is more widespread than previously recognized^[2,3]. The most frequently involved organ is the esophagus followed by the stomach^[2]. Gastric candidiasis is classified into thrush, nodular and ulcerated types^[4]. Though usually seen in immunocompromized hosts^[2,3], Candida-associated gastric ulcer also occurs in apparently healthy individuals^[5-9] and its frequency is widely different contingent on authors^[8-12]. The clinical significance of the fungus in and the natural history of Candida-associated gastric ulcer remain to be clarified^[2] so that the treatment of the disease has not yet been established. The fungus has been reported to be no longer detected once the ulcers were healed and no recurrence of the disease has been described, so far^[9,11].

Presented is a hitherto unreported case of the disease relapsing in a different site with a different shape in a *Helicobacter pylori* (*H. pylori*)-negative patient with no antecedent peptic ulcers who had not taken non-steroidal anti-inflammatory drugs (NSAIDs).

CASE REPORT

Complaining of anorexia after succumbing to the summer heat, an 87-year-old, Japanese housewife was shown



Figure 1 Endoscopic photograph showing the deep ulcer with the submucosal tumor-like margin on the greater curvature of the upper gastric body.

to have a medium-sized, submucosal tumor (SMT)-like elevation covered with the erythematous mucosa with an oval, deep, central ulceration covered with thick whitish exudate on the greater curvature of the upper gastric body (Figure 1). Biopsy showed no malignancy or *H. pylori* but a large number of hyphae in the ulcer slough (Figure 2A). No signs of candidiasis were detected in the oropharynx through esophagus or in the duodenal bulb through descending part of the duodenum.

Though suffered from adhesion ileus 2 mo ago, when no such lesions or any scars in the upper or any signs of candidiasis in the lower gastrointestinal tract were endoscopically detected, in the wake of ovariectomy and concomitant appendectomy she underwent when younger and had been given steroid inhaler for bronchial asthma for the past several years, she had no past history of peptic ulcers. Neither had she taken NSAIDs or antibiotics then. She was not diabetic. The ulceration was found much diminished in size spontaneously 2 wk after the first endoscopic examination and the organism was proven to be *Candida tropicalis* by culture. No circulating *Candida* antigens or β -D-glucan was detected, however. Though diagnosed as having *Candida*-associated gastric ulcer, she refused to undergo further endoscopic examination, not given any proton pump inhibitors (PPIs), H_2 receptor antagonists, or antifungal agents.

She complained of heartburn 10 mo later, when the lesion was shown to have turned into a white scar (Figure 3A) but another large, oval, deep ulcer was detected covered with thick, whitish exudate surrounded by the markedly swollen margin on the lesser curvature of the lower gastric body (Figure 3B). Biopsy demonstrated no malignancy but numerous hyphae of *Candida* again (Figure 2B). No findings of candidiasis were recognized in the oropharynx through other parts of the upper gastrointestinal tract. *H. pylori* was not detected by histopathologic examination or rapid urease test. Though had not taken any NSAIDs or antibiotics then, either, she had been given risedronate by an orthopedist under the diagnosis of osteoporosis once a week for the past 9 mo. She was diagnosed with *Candida*-associated gastric ulcer recurrent in a different location with a different appearance.

The lesion was proven to have turned into a red scar in 6 wk with administration of a PPI without antifungal agents (Figure 3C). The original lesion was shown to have remained to be a white scar. The follow-up endoscopy performed 3 mo later showed the red scar to have turned into white (Figure 3D) and the original scar to have remained unchanged. She has kept an uneventful course ever since, even though she has kept taking risedronate. No further recurrence has been detected, so far.

DISCUSSION

Candida is a fungus indigenous to the entire human gastrointestinal tract^[1] and the demonstration of infiltration of the tissue or ulcer slough by the hyphae is proposed as the diagnostic criterion of gastrointestinal candidiasis^[5,8]. The present case was diagnosed according to the criterion. *Candida*-associated gastric ulcer is more widespread than previously considered, seen not only in patients with predisposing conditions but in apparently healthy individuals^[5-9]. The present case was also lacking in conspicuous predisposing factors.

While endoscopic features of *Candida*-associated gastric ulcer have been asserted to be nonspecific^[4,9,11], an SMT-like lesion with a deep, central ulceration has been regarded as specific by some Japanese authors^[13,14]. In the present case, the original lesion did indeed assume such a form but the recurrent one appeared to be an ordinary, large ulcer. Since an SMT-like lesion with a central ulceration has been detected only in Japanese patients^[13,14], it may be peculiar to the race and considered suggestive of *Candida*-associated gastric ulcer, though not all ulcers associated with the fungus take on such an appearance.

Whereas *Candida*-associated gastric ulcer has been reported to have low healing rate^[6,11], some cases of the disease have been reported to have spontaneously healed^[12]. In the present case, the original lesion was found to have spontaneously healed and the recurrent one was in no way intractable. The intractability of the disease may be affected by other factors than the fungus, such as *H. pylori* or NSAIDs because such factors were not inspected in the cases reported to be intractable^[6,11].

Candida has been reported to be no longer detected once the ulcers were healed even without antifungal treatment and no recurrence of the lesions has been described, so far^[9,11]. The natural history of *Candida*-associated gastric ulcer is yet to be elucidated and the disease was reported to engender no specific symptoms^[8]. Presenting no specific symptoms, either, the present patient developed the disease without any antecedent peptic ulcers or their vestiges, which healed spontaneously at the first time and recurred 10 mo later. This is the first case of *Candida*-associated gastric ulcer in the world, which was demonstrated to have recurred not only in a different site but with a different appearance and followed up from before development of the original ulcer till complete cicatrization of the recurrent, disclosing the natural history of the disease.

Bisphosphonates are known to be ulcerogenic, consid-

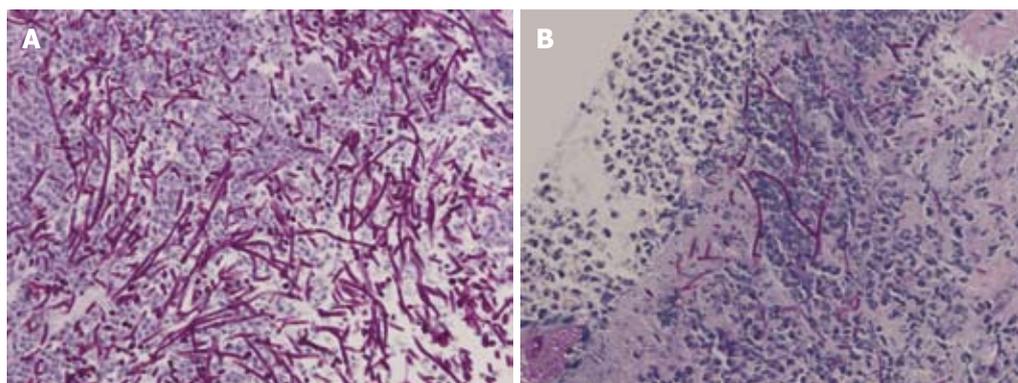


Figure 2 Biopsy demonstrated no malignancy but numerous hyphae. A: Light micrograph of the specimen biopsied from the original ulcer demonstrating hyphae in the ulcer slough [Periodic acid-Schiff (PAS)/diastase, original magnification $\times 400$]; B: Biopsy from the recurrent ulcer illustrating hyphae of *Candida* on the ulcer edge (PAS/diastase, original magnification $\times 400$).

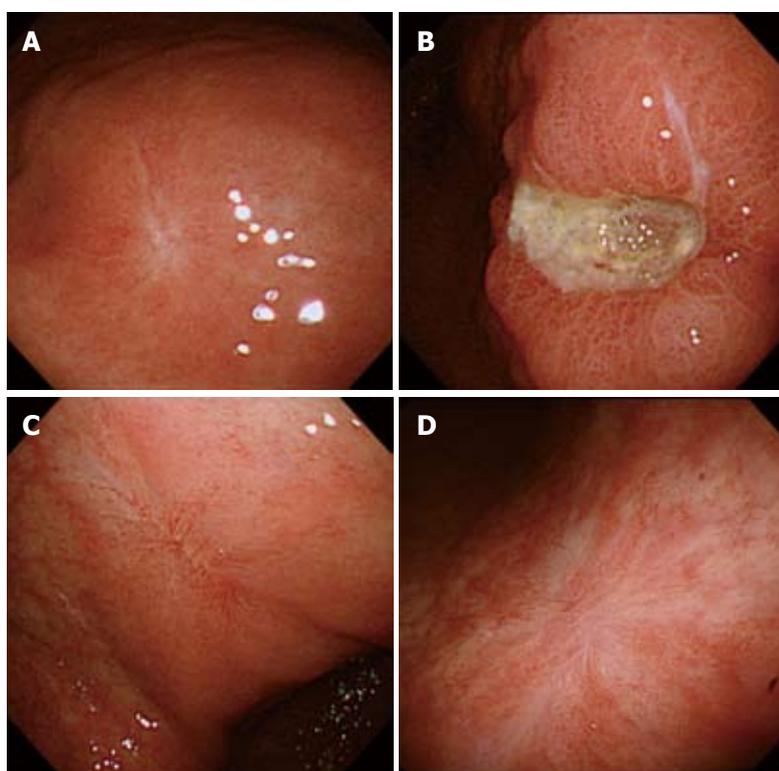


Figure 3 Endoscopic photographs. A: The white scar of the original ulcer; B: The recurrent ulcer on the lesser curvature of the lower gastric body; C: The red scar of the recurrent ulcer; D: The transition from the red to white scar in 3 mo.

ered to disrupt the protective hydrophobic phospholipid barrier of the gastric mucosa to trigger ulceration^[15,16]. But they are reported to greatly differ in potential to damage the mucosa^[15,16], risedronate having by far the weakest potential^[15]: the drug has been shown to have a gastrointestinal safety profile similar to that of placebo^[17-19]. It was not until 9 mo after the treatment with the drug was instituted when the recurrent ulcer was developed in the present case and, after it was healed, no recurrence has been detected even under the regimen. Sufficient ground is, therefore, lacking to suspect the drug to be the culprit in development of the recurrent ulcer. Though the clinical significance of the fungus has not yet been elucidated, it has not been regarded as directly etiologic in develop-

ment of ulcer, possibly as the presence of the fungus aggravates or perpetuates ulceration^[5,6,8]. Contrary to the prevailing opinion, the fungus is considered, by analogy with *H. pylori*, to play an etiologic role in ulcer formation in the present case on the basis of the circumstantial evidences, since it was detected not only in the original but also in recurrent lesion in an *H. pylori*-negative patient with no antecedent peptic ulcers who had not taken NSAIDs.

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"Passive-bending colonoscope" significantly improves cecal intubation in difficult cases

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Abstract

Colonoscopy sometimes causes pain during insertion, especially in difficult cases. Over-insufflation of air causes elongation or acute angulations of the colon, making passage of the scope difficult and causing pain. We previously reported a sedative-risk-free colonoscopy insertion technique, namely, "Water Navigation Colonoscopy". Complete air suction after water infusion not only improves the vision, but also makes water flow down to the descending colon, while the sigmoid colon collapses and shortens. While non-sedative colonoscopy can be carried out without pain in most cases, some patients do complain of pain. Most of these patients have abnormal colon morphology, and the pain is caused while negotiating the "hairpin" bends of the colon. The "hairpin" bends of the colon should be negotiated by gently pushing the full-angled colonoscope. The proximal 10-20 cm from the angulated part of the conventional colonoscope is stiff, with a wide turning radius, therefore, a conventional colonoscope cannot be negotiated through the "hairpin" bends of the colon without stretching them and causing pain. The "passive-bending colonoscope" has a flexible tip with a narrow turning radius, so that the scope can be

negotiated through the "hairpin" bends of the colon with a minimum turning radius and minimal discomfort. Therefore, the intubation and pain-reducing performance of the "passive-bending colonoscope" was assessed in difficult cases.

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Key words: Computed tomographic colonography; Water navigation colonoscopy; Passive-bending colonoscope; Cecal intubation

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INTRODUCTION

Colonoscopy sometimes causes pain during insertion, especially in difficult cases. Over-insufflation of air causes elongation or acute angulations of the colon, making passage of the scope difficult and causing pain.

Administration of sedatives masks pain, which is a warning sign of perforation. Therefore, we developed a sedative-risk-free colonoscopy insertion technique, namely, "Water Navigation Colonoscopy"^[1,2] (Figure 1). Complete air suction after water infusion not only improves the visibility, but also makes the water flow down to the descending colon, while the sigmoid colon collapses and shortens. The Water Methods were reported to reduce pain and make it possible to perform colonoscopy without the use of sedatives^[3]. While non-sedative

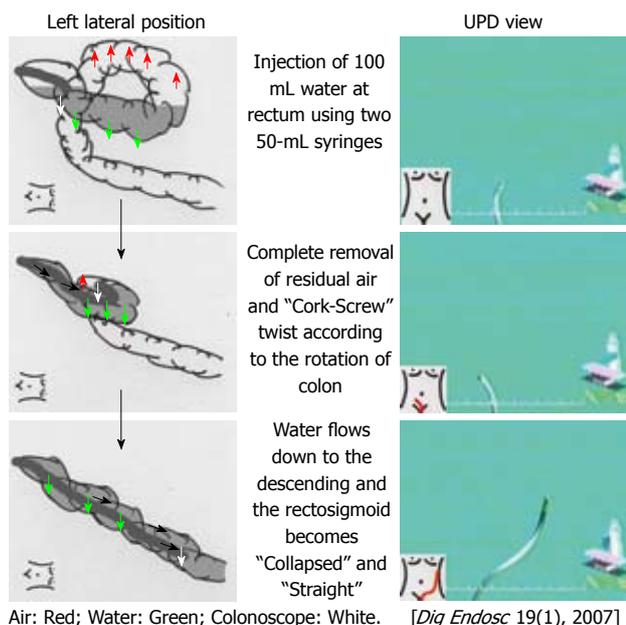


Figure 1 Water Navigation Colonoscopy. Red arrows mean buoyancy; Green arrows mean gravity of water; White arrows mean gravity of endoscope. UPD: Endoscope position detecting unit.

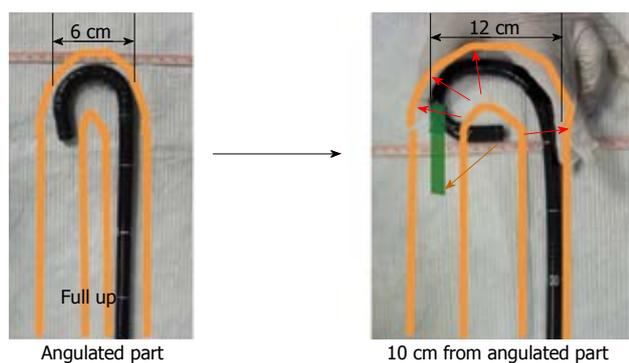


Figure 2 Conventional scope stretches the "hairpin" bend and causes pain. Red arrows mean the tension of colonoscope; Brown arrow shows that the tip of colonoscope stretches in the colonic lumen after passing the hairpin bend.

"Water Navigation Colonoscopy" can be carried out without pain in most cases, some patients do complain of pain. Most of these patients have abnormal colon morphology, and the pain is caused while negotiating the "hairpin" bends of the colon^[4,5]. The "hairpin" bends of the colon should be negotiated by gently pushing the full-angled colonoscope. The proximal 10-20 cm from the angulated part of the conventional colonoscope is stiff, with a wide turning radius, therefore, a conventional colonoscope cannot be negotiated easily through the "hairpin" bends of the colon without stretching them or causing pain (Figure 2).

"Passive-bending colonoscope," PCF-PQ260 (Olympus Co, Tokyo, Japan), has a flexible tip with a narrow turning radius, so that the scope can be easily negotiated through the "hairpin" bends of the colon, with minimal discomfort^[6,7] (Figure 3).

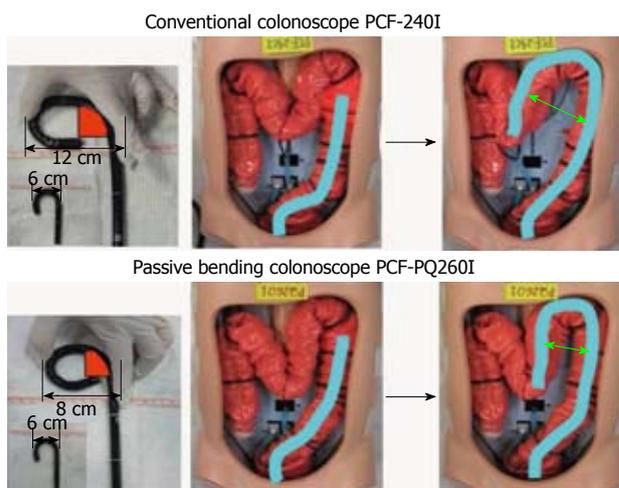


Figure 3 During negotiation of "hairpin" bend of the splenic flexure, "passive-bending colonoscope" does not stretch the bend. Green arrow shows the radius of colonoscope. Passive bending colonoscope has smaller one.

CASE REPORT

The subjects were 11 patients, including 2 cases of cecal intubation failure and 9 cases of difficult colonoscopy with the "conventional colonoscope," PCF-240Z Olympus, who underwent a second colonoscopy with the "passive-bending colonoscope," PCF-PQ260, more than 7 d after the first procedure. All colonoscopies were performed by the same physician, Mizukami T. Colonoscopy was performed according to the method of "Water Navigation Colonoscopy". The cecal intubation time was measured and the patients were asked to report their level of discomfort after the colonoscopy on a visual analog scale of 1 to 5, as follows: grade 1, no discomfort; grade 2, strange feeling; grade 3, distension of the abdomen; grade 4, tolerable pain; grade 5, intolerable pain. Computed tomographic colonography (CTC) was performed for morphological evaluation. Cecal intubation was accomplished successfully in all cases and the intubation time was significantly shortened with the use of the "passive-bending colonoscope" ($n = 9$) (10.1 ± 3.2 min *vs* 5.1 ± 2.8 min, $P < 0.05$). The average self-reported pain score was also significantly lower in the "passive-bending colonoscope" group (3.7 ± 0.6 *vs* 2.3 ± 0.9 , $P < 0.01$). CTC showed that every patient had "hairpin" bends of the colon. PCF-PQ260 significantly shortened the cecal intubation time and reduced the pain score in difficult cases, and CTC showed that every case had "hairpin" bends of the colon. This showed that the "passive-bending colonoscope" can be negotiated more easily through the "hairpin" bends of the colon, with its minimum turning radius. Use of the "passive-bending colonoscope" shortens the cecal intubation time and reduces pain in difficult cases.

Case 1

This patient (78-year-old female) had sigmoid malrotation

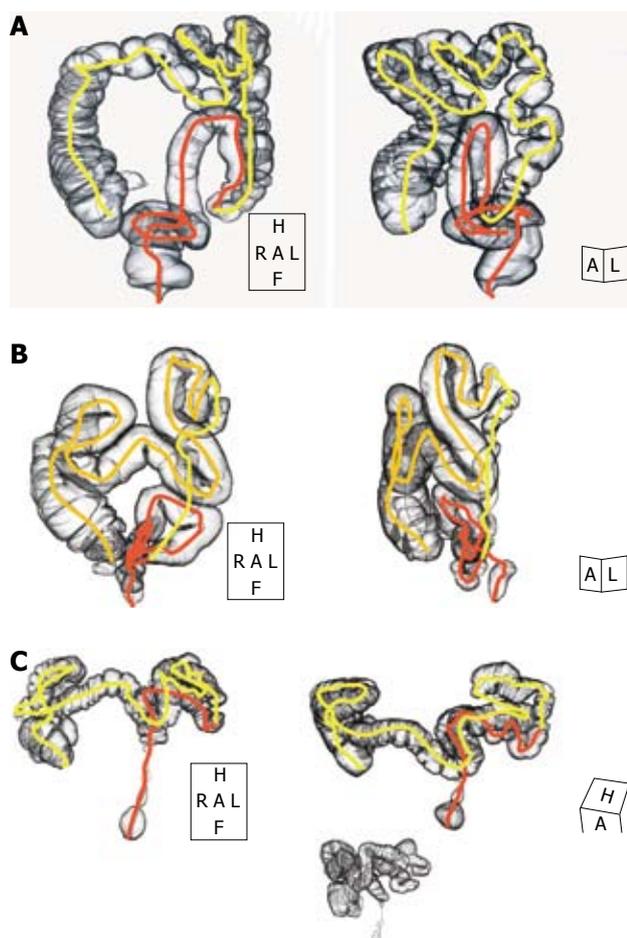


Figure 4 Sigmoid malrotation and loops, malrotation and mesocolon descendens, ascendens of patients. A: 78 years old female has sigmoid malrotation and loops at splenic flexure; B: 70 years old female has sigmoid malrotation and mesocolon descendens and ascendens; C: 70 years old male has mesocolon descendens and ascendens.

and loops at the splenic flexure. The cecal intubation time was 11 min (conventional), and 6 min (passive bending). The level of discomfort was 4 (conventional), and 2 (passive bending, Figure 4A).

Case 2

This patient (70-year-old female) had sigmoid malrotation and mesocolon descendens. The 1st cecal intubation (conventional) failed because of the "hairpin" bends at the splenic flexure. The 2nd cecal intubation (passive bending) was accomplished in 11 min. The level of discomfort was 4 (conventional), and 2 (passive bending, Figure 4B).

Case 3

This patient (70-year-old female) had mesocolon descendens and ascendens. The 1st cecal intubation (conventional) failed because of the "hairpin" bends in the descend-

ing colon. The 2nd cecal intubation (passive bending) was accomplished in 11 min. The level of discomfort was 4 (conventional), and 2 (passive bending) (Figure 4C).

DISCUSSION

Cases with difficult cecal intubation have looping and "hairpin" bends of the colon^[4,5]. There are some reports suggesting that the use of a variable-stiffness colonoscope or a pediatric colonoscope can reduce pain and facilitate intubation^[8,9].

In conventional colonoscopes, the 10-20 cm segment proximal to the angulated part is stiff, which causes stretching and pain during negotiation of the scope through the "hairpin" bends of the colon. The "passive-bending colonoscope" has a flexible tip with a narrow turning radius and can be more easily negotiated through the "hairpin" bends of the colon, with its minimum turning radius. Use of this scope in difficult cases was associated with a significantly shortened cecal intubation time and reduced pain score. CTC showed that every case had "hairpin" bends and loops of the colon, indicating that the "passive-bending colonoscope" could be negotiated through the "hairpin" bends of the colon relatively easily, with its minimum turning radius. In conclusion, use of the "passive-bending colonoscope" with "Water Navigation Colonoscopy" significantly shortens the cecal intubation time and reduces pain in difficult cases.

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Events Calendar 2012

January 13-15, 2012
 Asian Pacific *Helicobacter pylori*
 Meeting 2012
 Kuala Lumpur, Malaysia

January 19-21, 2012
 American Society of Clinical
 Oncology 2012 Gastrointestinal
 Cancers Symposium
 San Francisco, CA 3000,
 United States

January 19-21, 2012
 2012 Gastrointestinal Cancers
 Symposium
 San Francisco, CA 94103,
 United States

January 20-21, 2012
 American Gastroenterological
 Association Clinical Congress of
 Gastroenterology and Hepatology
 Miami Beach, FL 33141,
 United States

February 3, 2012
 The Future of Obesity Treatment
 London, United Kingdom

February 16-17, 2012
 4th United Kingdom Swallowing
 Research Group Conference
 London, United Kingdom

February 23, 2012
 Management of Barretts
 Oesophagus: Everything you need
 to know
 Cambridge, United Kingdom

February 24-27, 2012
 Canadian Digestive Diseases Week
 2012
 Montreal, Canada

March 1-3, 2012
 International Conference on
 Nutrition and Growth 2012
 Paris, France

March 7-10, 2012
 Society of American Gastrointestinal
 and Endoscopic Surgeons Annual
 Meeting
 San Diego, CA 92121, United States

March 12-14, 2012
 World Congress on
 Gastroenterology and Urology
 Omaha, NE 68197, United States

March 17-20, 2012
 Mayo Clinic Gastroenterology and
 Hepatology
 Orlando, FL 32808, United States

March 26-27, 2012
 26th Annual New Treatments in
 Chronic Liver Disease
 San Diego, CA 92121, United States

March 30-April 2, 2012
 Mayo Clinic Gastroenterology and
 Hepatology
 San Antonio, TX 78249,
 United States

March 31-April 1, 2012
 27th Annual New Treatments in
 Chronic Liver Disease
 San Diego, CA 92121, United States

April 8-10, 2012
 9th International Symposium on
 Functional GI Disorders
 Milwaukee, WI 53202, United States

April 13-15, 2012
 Asian Oncology Summit 2012
 Singapore, Singapore

April 15-17, 2012
 European Multidisciplinary
 Colorectal Cancer Congress 2012
 Prague, Czech

April 18-20, 2012
 The International Liver Congress
 2012
 Barcelona, Spain

April 19-21, 2012
 Internal Medicine 2012
 New Orleans, LA 70166,
 United States

April 20-22, 2012
 Diffuse Small Bowel and Liver
 Diseases
 Melbourne, Australia

April 22-24, 2012
 EUROSON 2012 EFSUMB Annual

Meeting
 Madrid, Spain

April 28, 2012
 Issues in Pediatric Oncology
 Kiev, Ukraine

May 3-5, 2012
 9th Congress of The Jordanian
 Society of Gastroenterology
 Amman, Jordan

May 7-10, 2012
 Digestive Diseases Week
 Chicago, IL 60601, United States

May 17-21, 2012
 2012 ASCRS Annual Meeting-
 American Society of Colon and
 Rectal Surgeons
 Hollywood, FL 1300, United States

May 18-19, 2012
 Pancreas Club Meeting
 San Diego, CA 92101, United States

May 18-23, 2012
 SGNA: Society of Gastroenterology
 Nurses and Associates Annual
 Course
 Phoenix, AZ 85001, United States

May 19-22, 2012
 2012-Digestive Disease Week
 San Diego, CA 92121, United States

June 2-6, 2012
 American Society of Colon and
 Rectal Surgeons Annual Meeting
 San Antonio, TX 78249,
 United States

June 18-21, 2012
 Pancreatic Cancer: Progress and
 Challenges
 Lake Tahoe, NV 89101, United States

July 25-26, 2012
 PancreasFest 2012
 Pittsburgh, PA 15260, United States

September 1-4, 2012
 OESO 11th World Conference
 Como, Italy

September 6-8, 2012
 2012 Joint International

Neurogastroenterology and Motility
 Meeting
 Bologna, Italy

September 7-9, 2012
 The Viral Hepatitis Congress
 Frankfurt, Germany

September 8-9, 2012
 New Advances in Inflammatory
 Bowel Disease
 La Jolla, CA 92093, United States

September 8-9, 2012
 Florida Gastroenterologic Society
 2012 Annual Meeting
 Boca Raton, FL 33498, United States

September 15-16, 2012
 Current Problems of
 Gastroenterology and Abdominal
 Surgery
 Kiev, Ukraine

September 20-22, 2012
 1st World Congress on Controversies
 in the Management of Viral Hepatitis
 Prague, Czech

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

November 3-4, 2012
 Modern Technologies in
 Diagnosis and Treatment of
 Gastroenterological Patients
 Dnepropetrovsk, Ukraine

November 4-8, 2012
 The Liver Meeting
 San Francisco, CA 94101,
 United States

November 9-13, 2012
 American Association for the Study
 of Liver Diseases
 Boston, MA 02298, United States

December 1-4, 2012
 Advances in Inflammatory Bowel
 Diseases
 Hollywood, FL 33028, United States

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The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systematically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensus and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

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Statistical review is performed after peer review. We invite an expert in Biomedical Statistics to evaluate the statistical method used in the paper, including *t*-test (group or paired comparisons), chi-squared test, Redit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc.* The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homoge-

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

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

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

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

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

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- 15 Morse SS. Factors in the emergence of infectious dis-

eases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

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- 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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