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EDITORIAL

Genetically modified mouse models for the study of nonalcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is associated with obesity, insulin resistance, and type 2 diabetes. NAFLD represents a large spectrum of diseases ranging from (1) fatty liver (hepatic steatosis); (2) steatosis with inflammation and necrosis; to (3) cirrhosis. The animal models to study NAFLD/nonalcoholic steatohepatitis (NASH) are extremely useful, as there are still many events to be elucidated in the pathology of NASH. The study of the established animal models has provided many clues in the pathogenesis of steatosis and steatohepatitis, but these remain incompletely understood. The different mouse models can be classified in two large groups. The first one includes genetically modified (transgenic or knockout) mice that spontaneously develop liver disease, and the second one includes mice that acquire the disease after dietary or pharmacological manipulation. Although the molecular mechanism leading to the development of hepatic steatosis in the pathogenesis of NAFLD is complex, genetically modified animal models may be a key for the treatment of NAFLD. Ideal animal models for NASH should closely resemble the pathological characteristics observed in humans. To date, no single animal model has encompassed the full spectrum of human disease progression, but they can imitate particular characteristics of human disease. Therefore, it is important that the researchers choose the appropriate animal model. This review discusses various genetically modified animal models developed and used in research on NAFLD.

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Key words: Nonalcoholic fatty liver disease; Steatosis; Steatohepatitis; Knockout; Animal models

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) represents a histological spectrum of liver disease associated with obesity, diabetes and insulin resistance that extends from isolated steatosis to steatohepatitis and cirrhosis. Besides being a potential cause of progressive liver disease, steatosis has been shown to be an important cofactor in the pathogenesis of many other liver diseases. Mouse models have been developed and the different mouse models can be classified in two major groups. The first one includes genetically modified (transgenic or knockout) mice that spontaneously develop liver disease, and the second one

includes mice that acquire the disease after dietary or pharmacological manipulation. NAFLD and nonalcoholic steatohepatitis (NASH) are increasing due to the prevalence of the metabolic syndrome linked to visceral adiposity, insulin resistance, dyslipidemia and type two diabetes. In this context, research has been undertaken using animals models for human steatosis and NAFLD to NASH disease progression. Most of the animal models develop a fatty liver and many develop aspects of steatohepatitis. However, spontaneous development of fibrosis is very rare. Because it is highly unlikely that NAFLD in the human population is monogenic, study of animals with deletion or over-expression of a single gene may not mimic etiology of the human disease at the molecular level. Likewise, choice of experimental diet may not mimic the human diets associated with development of NAFLD in man. Although rodent models of hepatic steatosis and/or insulin resistance do not always perfectly reproduce the human pathology of NAFLD, the use of transgenic, knockout, and knockdown mouse models have helped over the past years to better our understanding of the molecular determinants of NAFLD. This literature review describes different genetically modified mouse models that exhibit histological evidence of hepatic steatosis or, more variably, steatohepatitis.

GENETIC MODELS FOR NAFLD

ob/ob mice

The *ob/ob* mice carry a spontaneous mutation in the leptin gene (leptin-deficient). These mice are hyperphagic, inactive, extremely obese and are severely diabetic, with marked hyperinsulinemia and hyperglycemia. *ob/ob* mice develop NASH spontaneously[1], but unlike human NAFLD, *ob/ob* mice do not spontaneously progress from steatosis to steatohepatitis. *ob/ob* mice require a 'second hit' to be administered in order to trigger progression to steatohepatitis. This may be provided by exposure to small doses of lipopolysaccharide (LPS) endotoxin, ethanol exposure or hepatic ischemia-reperfusion challenge which all provoke a severe steatohepatitis and frequently acute mortality[2-5]. *ob/ob* mice require other stimuli such as a methionine choline deficient (MCD) diet or a high fat diet to trigger progression to steatohepatitis. The effects of leptin deficiency on several aspects of physiology increase the complexity of studies while using this strain[6]. Similarly, the limited fibrotic capacity of a leptin-deficient model means that it is best suited to studies investigating the mechanisms behind the development of steatosis and the transition to steatohepatitis. Recent work demonstrates that the apparent flaws in this model can be turned to advantage, providing new insights into stellate cell function and the progression to fibrosis.

db/db mice

The *db/db* mice have a natural mutation in the leptin receptor (*Ob-Rb*) gene[7]. These mice are obese with insulin resistance, and are able to develop macrovesicular hepatic steatosis. These mice readily develop symptoms of NASH upon induction with a second hit, such as feeding with an MCD diet[8]. These mice have normal or elevated levels of leptin but are resistant to its effects. Studies have shown that the *db* gene encodes the leptin receptor (OB-R) which is structurally similar to a class I cytokine receptor[9,10]. There are two isoforms; the short OB-Ra isoform has not been shown to have any signaling activity. In contrast, the OB-Rb isoform has a long intracytoplasmic region that contains signal transduction motifs which activate the JAK/STAT protein kinase signal transduction cascade[11]. *db/db* mice carry a sequence insertion at the 3' end of the mRNA transcript exactly where the OB-Ra and OB-Rb transcripts diverge. This insertion contains a stop codon that leads to the premature termination of the OB-Rb long intracellular signaling domain, loss of function and consequently leptin resistance[12].

Yellow-obese agouti (Ay) mice

KK-A^y mice are a cross-strain of diabetic KK mice[13] and lethal yellow (A^y) mice, which carry mutation of the agouti(a) gene on mouse chromosome 2[14]. KK-A^y mice develop maturity-onset obesity, dyslipidemia, and insulin resistance, in part because of the antagonism of melanocortin receptor-4 by ectopic expression of the agouti protein[14]. Importantly, these mice present hyperleptinemia and leptin resistance without defects in the *Ob*R gene, and the expression of adiponectin is conversely down-regulated[15,16]. The phenotype of KK-A^y mice, including altered adipokine expression, quite resembles metabolic syndrome in humans indicating the potential usefulness of this strain as a model of metabolic syndrome NASH[17,18]. In fact, KK-A^y mice are more susceptible to experimental steatohepatitis induced by MCD diet.

CD36$^{-/-}$ mice

A valuable model for the study of the effects of alteration in fatty acid (FA) utilization on insulin responsiveness is the recently generated CD36-deficient mouse[19,20]. CD36, also known as fatty acid translocase (FAT)[21], is a multispecific, integral membrane glycoprotein[22,23] that has been identified as a facilitator of FA uptake. Its function in binding and transport of FA was documented *in vitro* by affinity labeling with FA derivatives and by cell transfection studies[23,24]. The CD36-deficient mouse exhibits greater than 60% decrease of FA uptake and utilization by heart, skeletal muscle, and adipose tissues and thereby increases FA delivery to liver and exhibits increased plasma free fatty acid (FFA) and triglyceride (TG) levels[20]. The pathogenic role of FAT/CD36 in hepatic steatosis in rodents is well-defined[25].

Phosphoenolpyruvate carboxykinase-sterol regulatory-element binding protein 1a-mice

Sterol regulatory-element binding protein (SREBP) family members have been established as transcription factors regulating the transcription of genes involved in cholesterol and FA synthesis. *In vivo* studies have demonstrated that

SREBP-1 plays a crucial role in the dietary regulation of most hepatic lipogenic genes[26,27]. Physiological changes of SREBP-1 protein in normal mice by dietary manipulation such as placement on high carbohydrate diets, polyunsaturated FA-enriched diets, and fasting-refeeding regimens has been reported[28,29]. SREBP-1a transgenic mice, under the control of liver specific Phosphoenolpyruvate carboxykinase promoter (TgSREBP-1a), show a massively enlarged liver and atrophic peripheral white adipose tissue, and develop steatosis[30].

aP2-NSREBP-1c mice

Leptin has similar effects in lipodystrophic rodents, most notably in aP2-nSREBP-1c transgenic mice. These animals express a truncated, constitutively active form of the SREBP-1c transcription factor under the control of the adipose tissue specific aP2 promoter and develop lipodystrophy with very low plasma leptin levels. These mice are also hyperphagic and have massive fat accumulation in peripheral tissues with hyperglycemia and hyperinsulinemia[31,32]. This mouse model has markedly reduced body fat and develops liver steatosis, profound insulin resistance, and increased levels of triglycerides[31,32].

aP2-diptheria toxin mice

The aP2/DTA mice have low serum leptin levels and are hyperphagic. These mice when fed a control diet are hyperlipidemic, hyperglycemic, and have hyperinsulinemia indicative of insulin-resistant diabetes. These mice are born normally and initially lack any distinguishing phenotypic features, but develop atrophy and necrosis of the adipose tissue at five to six months resulting in the complete absence of subcutaneous or intra-abdominal adipose tissue at eight to nine months of age[33]. This late onset of adipose tissue loss is associated with reduced leptin levels, increased food consumption, hyperlipidemia, hyperglycemia and insulin resistance. Monosodium glutamate-treated aP2/DTA mice develop gross hepatomegaly as a result of severe fatty changes in the liver[33].

A-ZIP/F-1 mice

The A-ZIP/F-1 mice express a dominant negative version of the C/EBPα leucine zipper domain that potently interferes with adipocyte differentiation[34]. The A-ZIP/F-1 mouse (*A-ZIPTg/+*) is a model of severe lipoatrophic diabetes and is insulin resistant, hypoleptinemic, hyperphagic, and shows severe hepatic steatosis. This mouse has essentially no white adipose tissue, reduced brown fat and severe metabolic phenotype with a reduced life span. These mice display massive hepatomegaly causing increased body weight, liver steatosis, severe diabetes (hyperglycemia, hyperinsulinemia, hyperphagic, polydipsia and polyuria), and are hypertensive[35]. They have increased triglycerides and FFA levels, alveolar foam cells and reduced leptin levels. These mice are unable to sustain glucose levels during fasting. The insulin resistance and much of the liver steatosis in the A-ZIP/F-1 mice can be reversed by transgenic over-expression of leptin[36] or by transplanting normal adipose tissue[37]. By contrast, transplantation of adipose tissue from *ob/ob* mice did not reverse the phenotype of the A-ZIP/F-1 mice indicating that leptin deficiency strongly contributes to the metabolic complications in lipodystrophy[38].

Peroxisome proliferator-activated receptor alpha$^{-/-}$ mice

Peroxisome proliferator-activated receptor alpha (PPARα) is expressed in the liver and other metabolically active tissues including striated muscle, kidney and pancreas[39,40]. Many of the genes encoding enzymes involved in the mitochondrial and peroxisomal FA beta-oxidation pathways are regulated by PPARα. In wild-type mice, peroxisome proliferators are compounds that induce lipid catabolism and an associated intracellular increase in peroxisome number and enzymatic activity. PPARα mutated mice exhibit alterations of intracellular lipid processing, particularly in response to peroxisome proliferators. Mice deficient in PPARα exhibit severe hepatic steatosis when subjected to fasting for 24-72 h, indicating that a defect in PPARα-inducible FA oxidation accounts for severe FA overload in liver, causing steatosis, in contrast to the wild-type mice[41,42].

Galactin-3 knockout mice

Galectin-3, a beta-galactoside-binding animal lectin, is a multifunctional protein. Galectin-3 plays a role in the regulation of hepatic stellate cell (HSC) activation *in vitro* and *in vivo,* thereby identifying galectin-3 as a potential therapeutic target in the treatment of liver fibrosis. This model plays a role in investigating liver carcinogenesis based on a natural history of NAFLD[43]. Previous studies have also suggested that galectin-3 may play an important role in inflammatory responses. The livers of *gal3(-/-)* male mice at six months of age displayed mild to severe fatty change. The liver weight per body weight ratio, serum alanine aminotransferase levels, liver triglyceride levels, and liver lipid peroxide in *gal3(-/-)* mice were significantly increased compared with those in *gal3(+/+)* mice. Furthermore, the hepatic protein levels of advanced glycation end-products (AGE), receptor for AGE, and PPARγ were increased in *gal3(-/-)* mice relative to *gal3(+/+)* mice[43,44].

Acetyl CoA oxidase$^{-/-}$ mice

Acyl-coenzyme A oxidase (AOX) is the rate-limiting enzyme in peroxisomal FA β-oxidation for the preferential metabolism of very long-chain FAs. AOX null (*AOX-/-*) mice have defective peroxisomal β-oxidation and exhibit steatohepatitis. Microvesicular fatty change in hepatocytes is evident at 7 d. At 2 mo of age, livers show extensive steatosis and they have clusters of hepatocytes at periportal areas with abundant granular eosinophilic cytoplasm rich in peroxisomes. At 4-5 mo there is increased PPARα, cytochrome P450, Cyp 4a10, and Cyp4a14 expression. By 6 to 7 mo, however, there is a compensatory increase in FA oxidation and reversal of hepatic steatosis resulting from hepatocellular regenera-

tion[45,46]. The *AOX*-/- mice proceed to develop adenomas and carcinomas by 15 mo of age[46,47].

Aromatase (CYP 19)-deficient mice

Aromatase P450 (CYP19) is an enzyme catalysing the conversion of androgens into estrogens[48]. These models present dyslipidemia, central obesity, hypercholesterolemia, hyperinsulinemia, hyperleptinemia, and hypertriglyceridemia[49], and importantly the male mice have hepatic steatosis. Aromatase knockout (ArKO) mice have a similar phenotype to that of estrogen receptor null mice with increased gonadal fat pad weight[50]. Only ArKO males have elevated hepatic triglyceride levels leading to hepatic steatosis partly due to an increase in expression of enzymes involved in *de novo* lipogenesis and transporters involved in FA uptake[51-53].

$MTP^{-/-}$ mice

Mitochondrial β-oxidation of FAs is the major source of energy for skeletal muscle and the heart, and it plays an essential role in intermediary metabolism in the liver and impairment of mitochondrial β-oxidation in pathogenesis of NAFLD. The fetuses of *Mtpa*-/- mice accumulate long chain FA metabolites and have low birth weight compared with the *Mtpa*+/- and *Mtpa*+/+ littermates. *Mtpa*-/- mice suffer neonatal hypoglycemia and sudden death 6-36 h after birth. Analysis of the histopathological changes in the *Mtpa*-/- pups revealed rapid development of hepatic steatosis after birth and, later, significant necrosis and acute degeneration of the cardiac and diaphragmatic myocytes. However, studies by Ibdah *et al*[54] indicated that aged but not young $MTPa^{+/-}$ mice developed hepatic steatosis with elevated alanine aminotransferase (ALT), basal hyperinsulinemia, and increased insulin compared with $MTPa^{+/+}$ littermates. Significant hepatic steatosis and insulin resistance developed concomitantly in the $MTPa^{+/-}$ mice at 9-10 mo of age. The cause resides in heterozygosity for β-oxidation defects that predisposes to NAFLD and insulin resistance in aging mice[55].

Phosphatase and tensin homologue -/- mice

Phosphatase and tensin homologue (PTEN) is a multifunctional phosphatase whose substrate is phosphatidylinositol-3,4,5-triphosphate and which acts as a tumor suppressor gene that downregulates phosphatidyl inositol kinases[56,57]. Hepatocyte-specific PTEN-deficient mice spontaneously develop steatosis, steatohepatitis, and hepatocellular carcinoma[58,59]. By 10 wk of age, these mice have increased concentrations of triglyceride and cholesterol esters, and a histological analysis displays micro- and macrovesicular lipid vacuoles. At 40 wk of age, they have macrovesicular steatosis, mallory bodies, ballooning degeneration, and sinusoidal fibrosis[59-60]. Mice that are homozygous for this allele are viable, fertile, and normal in size and do not display any gross physical or behavioral abnormalities. When crossed to a strain expressing Cre recombinase in liver, this mutant mouse strain may be useful in studies of fatty liver and insulin signaling. Piguet *et al*[61] have investigated the effects of hypoxia in the PTEN-deficient mouse, a mouse model that develops NAFLD. The authors also showed that a short period (7 d) of exposure to hypoxia aggravates the NAFLD phenotype, causing changes in the liver that are in keeping with NASH, with increased lipogenesis and inflammation.

Methionine adenosyl transferase 1A -/- mice

Mice deficient in methionine adenosyl transferase 1A (the enzyme responsible for SAM synthesis in the adult liver) have a decrease in hepatic SAM levels and spontaneously develop steatosis, NASH, and hepatocellular carcinoma (HCC)[62]. By three months of age, these mice have hepatomegaly with macrovesicular steatosis. These mice also have increased mRNA levels of CYP2E1 and UCP2, and levels of glutathione. Also, these mice have changes in the expression of genes involved in cell proliferation of lipid and carbohydrate metabolism[63]. These mice are predisposed to liver injury and have impaired liver regeneration after partial hepatectomy[64].

Adiponectin null mice

Adiponectin is an adipokine abundantly produced from adipocytes[65,66]. Adiponectin is an anti-inflammatory adipocyte-derived plasma protein known to alleviate steatosis and inflammation in NAFLD[65-67]. Two adiponectin receptors (adipoR1 and adipoR2) have been identified and found to be expressed in various tissues[68]. AdipoR1 is abundantly expressed in skeletal muscles, whereas adipoR2 is present predominantly in the liver, suggesting a role of adipoR2 in hepatic adiponectin signaling[68,69]. The physiological roles of adipoR1 and adipoR2 have recently been investigated by several laboratories in *adipoR1/2* knockout mice. Both *adipoR1* and *adipoR2* knockout mice exhibit mild insulin resistance[70]. In *adipoR1/R2* double knockout mice the binding and actions of adiponectin are abolished, resulting in increased tissue triglyceride content, inflammation oxidative stress[70-73] and mice exhibit impaired liver regeneration and increased hepatic steatosis.

Bid null mice

The protein Bid is a participant in the pathway that leads to cell death (apoptosis), mediating the release of cytochrome from mitochondria in response to signals from "death" receptors known as tumor necrosis factor (TNF) receptor 1/Fas on the cell surface. Genetic inactivation of Bid, a key pro-apoptotic molecule that serves as a link between these two cell death pathways, significantly reduced caspase activation, adipocyte apoptosis, prevented adipose tissue macrophage infiltration, and protected against the development of systemic insulin resistance and hepatic steatosis independent of body weight[74,75]. These mice can be used in research based on adipocyte apoptosis which is a key initial event that contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis associated with obesity.

Fas adipocyte-specific (AfasKO) null mice

Fas (CD95), a member of the TNF receptor super family, is a major contributor to apoptosis in many cells. Fas activation may contribute to obesity-induced insulin resistance, since mice lacking Fas in adipocytes were partly protected from developing insulin resistance. In particular, Fas activation led to increased release of pro-inflammatory cytokines, and reduced insulin-stimulated glucose uptake in 3T3-L1 adipocytes[75]. Fas-deficient (Fas-def) mice show increased insulin-stimulated glucose incorporation when compared to wild type (WT) with higher expression levels of Akt[76,77].

Interleukin-6 KO mice

Interleukin-6 (IL-6) is an adipocytokine associated with NALFD and obesity that is secreted in larger amounts by visceral fat compared to subcutaneous fat in obese adults[78]. Increased systemic IL-6 is associated with increased inflammation and fibrosis in NAFLD patients[79]. Expression of IL-6, a major proinflammatory cytokine, is increased in animal models of NAFLD. Hepatic IL-6 production may also play an important role in NASH development, as well as in systemic insulin resistance and diabetes. IL-6 is elevated in the plasma and peripheral blood monocytes of patients with fatty diseases, including alcoholic liver disease and non-alcoholic steatohepatitis, and elevation of IL-6 correlates with the progression and severity of liver disease, suggesting that IL-6 may be involved in the pathogenesis of fatty liver disease[80,81]. Studies using *Il6*-/- mice show these animals display obesity, hepatosteatosis, liver inflammation and insulin resistance when compared with control mice on a standard chow diet[82].

TNF alpha KO mice

TNF-α appears to play a central role in the development of hepatic steatosis. TNF-α, by mechanisms not completely defined, is over expressed in the liver of obese mice and is an important mediator of insulin resistance in both diet-induced and *ob/ob* models of obesity[83,84]. Data from animal and clinical studies indicate that TNF-α mediates not only the early stages of fatty liver disease but also the transition to more advanced stages of liver damage[85,86]. Mice homozygous for the TNF targeted mutation are viable and fertile. Further, male mutant mice at 28 wk old display lower insulin, triglyceride, and leptin levels compared to wild type controls.

NEMO^{LPC-KO} mice

The IκB kinase (IKK) subunit NEMO/IKKγ is essential for activation of the transcription factor nuclear factor kappa B (NF-κB), which regulates cellular responses to inflammation. NEMO-mediated NF-κB activation in hepatocytes has an essential physiological function to prevent the spontaneous development of steatohepatitis and hepatocellular carcinoma. These mice were generated with liver parenchymal cell-specific knockout of these subunits (*NEMO^{LPC-KO}*, *IKK2^{LPC-KO}*) by crossing mice carrying loxP-flanked *Nemo*[87] or *Ikk2*[88] alleles with Alfp-cre transgenic mice that mediate efficient Cre recombination in liver parenchymal cells, including hepatocytes and biliary epithelial cells, but not in endothelial or Kupffer cells[89]. *NEMO^{LPC-KO}* mice were born and reached weaning age at the expected Mendelian frequency[90]. These mice showed efficient ablation of the respective proteins in whole-liver extracts and NF-κB activity in the liver was completely abolished[91,92]. Hepatocytes are LPS sensitive. When fed a high-fat diet, mice had reduced β-oxidation and upregulated PPAR-γ, SReBP1 and FA synthase causing increased *de novo* lipid synthesis and macrovesicular steatosis with increased HCC occurrence[91,92].

Jun N-terminal kinase 1 null mice

Jun N-terminal kinase (JNK) 1 null mice have less hepatic inflammation and fibrosis when fed a choline-deficient, l-amino acid-defined (CDAA) diet due to the absence of JNK1 in immune cells. As JNK is activated by oxidants and cytokines and regulates hepatocellular injury and insulin resistance, this kinase may mediate the development of steatohepatitis. JNK promotes the development of steatohepatitis as MCD diet-fed *JNK* null mice have significantly reduced levels of hepatic triglyceride accumulation, inflammation, lipid peroxidation, liver injury, and apoptosis compared with wild-type and *JNK2* -/- mice[93,94]. Hence JNK1 is responsible for JNK activation that promotes the development of steatohepatitis in the MCD diet model[93]. JNK1 KO produces lean, male JNK1 KO mice which have decreased body weights, fasting blood glucose levels, and fasting blood insulin levels compared to their wild-type controls[94]. This model can be used to study a combination of genetic and dietary challenges that constitute the disease etiology for NASH development and mimic more closely the pathogenesis of human NAFLD/NASH.

Toll-like receptor 9 KO mice

Development of NASH involves the innate immune system and is mediated by Kupffer cells and HSCs. Toll-like receptor 9 (TLR9) is a pattern recognition receptor that recognizes bacteria-derived cytosine phosphate guanine-containing DNA and activates innate immunity. Mice deficient in TLR9 have reduced steatohepatitis and fibrosis[95]. Hence this model can be used to study NAFLD involving innate immunity.

LDLR KO and farnesoid X receptor KO mice

Farnesoid X receptor (FXR) is essential for regulating bile-acid synthesis and transport. Mice with FXR deficiency have severe impairment of bile-acid homeostasis and manifest systemic abnormalities including altered lipid and cholesterol metabolism features known to be associated with the metabolic syndrome and NASH. Kong *et al*[96] studied LDL receptor knockout (*LDLr*-/-) mice fed with a high-fat diet for 5 mo, and checked whether FXR deficiency contributed to NASH development. Both high-fat diet and FXR deficiency increased

serum ALT activity, whereas only FXR deficiency increased bile-acid and ALP levels. FXR deficiency and high-fat feeding increased serum cholesterol and triglycerides. Although high-fat diet led to macrosteatosis and hepatocyte ballooning in livers of mice regardless of genotype, no inflammatory infiltrate was observed in the livers of *LDLr*-/- mice. In contrast, in the livers of *LDLr*-/-/*FXR*-/- mice, foci of inflammatory cells were observed when they were fed with control diet and were greatly increased when fed with the high-fat diet[96,97]. This model can be used to study a combination of genetic and dietary challenges that constitute the disease etiology for NASH development and mimic more closely the pathogenesis of human NAFLD/NASH.

Myd88 KO mice

Chemokines, strongly induced by TLR stimulation, play an important role in the development of metabolic syndrome including NAFLD. TLR4- and MyD88-deficient mice, which are resistant to metabolic syndrome, show reduced chemokine production compared with WT mice[98,99]. MyD88 is a key molecule in the development of metabolic syndrome including NAFLD[98,99]. MyD88, an adaptor protein for all TLRs except for TLR3, is required for the expression of various inflammatory cytokines and chemokines[100]. MyD88-deficient mice are protected from metabolic syndrome as well as atherosclerosis[98,99] and from liver injury induced by bile duct ligation or carbon tetrachloride[101,102]. Miura *et al*[100] demonstrated that MyD88-deficient mice on a CDAA diet show less steatohepatitis with less insulin resistance compared with wild type mice. Inflammatory cytokines and fibrogenic factors are also significantly suppressed in MyD88-deficient mice compared with wild type mice fed a CDAA diet[100].

Fatty liver dystrophy knockout mice

Fatty liver dystrophy (*fld*) is a spontaneous point mutation in *Lpin1* which occurred on C3H/HeJ in 1994. An unstable gait and tremor at 3 wk of age was initially observed in these mice. The pups from these mice have a fatty liver before reaching weaning age. Mice carrying mutations in the *fld* gene have features of human lipodystrophy, a genetically heterogeneous group of disorders characterized by loss of body fat, fatty liver, and hypertriglyceridemia and insulin resistance[103]. Homozygous *fld* mice have an enlarged, fatty liver and hypertriglyceridemia that resolve to normal during the weaning transition. However, decreased overall size, decreased lipid in the fat pads and a peripheral neuropathy persist throughout the lifespan. This peripheral neuropathy manifests as a tremor and an unsteady gait shortly after 10 d of age and worsens with age. As with the original mutation of *fld*, homozygous females will breed and raise their litters but homozygous males do not breed.

Platelet endothelial cell adhesion molecule-1 null mice

Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a 130-kDa transmembrane glycoprotein expressed on blood and vascular cells. Goel *et al*[104] demonstrated that genetic deficiency of PECAM-1 potentiates the development and progression of NASH. After 3 wk on an atherogenic diet, these mice developed mild microvesicular steatosis predominantly in hepatic parenchymal cells in the centrilobular region. At 9 and 18 wk on the atherogenic diet, more severe steatosis with lobular and sinusoidal inflammation developed in the livers, which are consistent with the typical histological features of steatohepatitis[104].

ApolipoproteinB 38.9 mutant mice

Fatty liver is prevalent in apolipoproteinB (apoB)-defective familial hypobetalipoproteinemia (FHBL). Similar to humans, mouse models of FHBL produced by gene targeting (*apoB+/38.9*) manifest low plasma cholesterol and increased hepatic TG even on a chow diet due to impaired hepatic VLDL-TG secretive capacity. These mice will be useful to study the genetic and molecular mechanism of apoB defects and lipid metabolism/liver fat accumulation, the relationship between hepatic steatosis and insulin resistance, and the progression of advanced NAFLD and atherosclerosis[105].

Cystathionine-synthase deficient mice

Cystathionine-synthase (CBS) deficiency causes severe hyperhomocysteinemia, which confers diverse clinical manifestations, notably liver disease. Robert *et al*[106] reported that CBS-deficient mice showed inflammation, fibrosis, and hepatic steatosis. These mice also had pathological resemblance to steatohepatitis and a pattern of perivenous and pericellular hepatic fibrosis around lipid-laden hepatocytes. CBS KO mice develop hepatic steatosis more tardily than inflammation and fibrosis at 8-32 wk old.

In addition to the above KO animals, Postic *et al*[107] has demonstrated a few animal models modulating enzymes in FA synthesis.

Acc2KO mice

Acetyl-CoA carboxylase (ACC) catalyzes the synthesis of malonyl-CoA, the metabolic intermediate between lipogenesis[108] and β-oxidation[109]; this lipogenic enzyme has garnered significant attention over recent years. In mammals, two ACC isoforms exist, each with distinct tissue distribution and physiological roles: ACC1 is highly expressed in liver and adipose tissue, whereas ACC2 is predominantly expressed in heart and skeletal muscle and, to a lesser extent, in liver[110]. It is believed that only ACC1, but not ACC2, is committed to *de novo* lipogenesis in liver. Targeting ACC has beneficial effects on both hepatic steatosis and insulin resistance. ACC1-knockout mice (*Acc1*$^{-/-}$ mice and *ACC2*$^{-/-}$ mice) have been developed to study the effect.

SCD KO mice

SCD1 has recently become a target of interest for the reversal of hepatic steatosis and insulin resistance[111].

Table 1 Potential candidate genes in fatty liver disease

Category of genes	Examples
Genes affecting insulin resistance	*ADIPOQ, AKT2, ENPP1, IRS1, PPARG, HFE, resistin*
Genes affecting hepatic lipid synthesis and uptake	*DGAT2, SLC25A13, ACC, ELOVL6, SCD1, GPAT, SREBP1*
Genes affecting hepatic lipid uptake	*APOC3*
Genes affecting hepatic triglyceride hydrolysis	*PNPLA2, CGI-58, LIPA*
Genes affecting hepatic lipid export	*APOB, MTTP, PEMT*
Genes affecting hepatic oxidative stress	*GCLC, NOS2, SOD2, HFE, UCP2, MAT1A, GST, GSH-Px*
Genes affecting immune regulation	*ADIPOQ, ADIPOR1, ADIPOR2, STAT3, TNFα, IL10, IL6, CTLA-4, IL-4, IL-18*
Genes influencing disease progression and fibrosis	*TGF-β1, 3, PPARα, DDX5, CPT1A, angiotensin Ⅱ*
Genes influencing response to endotoxin	*CD14, TLR4, NOD2*

ADIPOQ: Adiponectin; AKT: Beta serine/threonine-protein kinase; ENPP1: Ectonucleotide pyrophosphatase/phosphodiesterase 1; IRS-1: Insulin receptor substrate 1; PPARG: Peroxisome proliferator-activated receptor gamma; HFE: Hemochromatosis gene; DGAT2: Diacylglycerol acetyltransferase-2; SLC25A13: Solute carrier family 25 Member 13 (citrin); *ACC* gene: Acetyl-CoA carboxylase alpha; ELOVL6: Elongation of very long chain fatty acids; *SCD1* gene: Stearoyl-CoA desaturase gene; GPAT: Glycerol-3-phosphate acyltransferase; SREBP1: Sterol regulatory element-binding transcription factor 1; APOC3: Apolipoprotein C-Ⅲ; PNPLA2: Patatin-like phospholipase domain containing 2; CGI58: Comparative gene identification-58; LIPA: Lipase A; APOB: Apolipoprotein B; MTTP: Microsomal triglyceride transfer protein; PEMT: Phosphatidylethanolamine N-methyltransferase; GCLC: Glutamate-cysteine ligase, catalytic subunit; NOS2: Nitric oxide synthases2; SOD2: Superoxide dismutase-2; UCP2: Uncoupling protein 2; MAT1A: Methionine adenosyltransferase I alpha; GST: Glutathione S-transferase; ADH: Alcohol dehydrogenase; ALDH: Aldehyde dehydrogenase; CTGF: Connective tissue growth factor; CTLA-4: Cytotoxic T-cell associated antigen-4; GSH-Px: Glutathione peroxidase; STAT3: Signal transducer and activator of transcription 3; IL: Interleukin; PPAR: Peroxisomal proliferator activated receptor; SCD-1: Stearoyl CoA desaturase-1; TLR: Toll-like receptor; TNFR: TNF-α receptor; DDX5: DEAD box protein 5; CPT1A: Carnitine palmitoyltransferase 1A (liver); NOD2: Nucleotide-binding oligomerization domain containing 2.

SCD1 catalyzes the synthesis of monounsaturated FAs, particularly oleate (C18:1n-9) and palmitoleate (C16:1n-7), which are the major components of membrane phospholipids, TGs, and cholesterol esters. Mice with SCD-1KO (*Scd1*$^{-/-}$ mice) show decreased lipogenic gene expression and increased β-oxidation and are protected from diet-induced obesity and insulin resistance when fed a HC/HF diet[112,113]. Inhibition of SCD1 using an ASO strategy (targeting SCD1 in both liver and adipose tissues) prevents many of the HF/HC-diet metabolic complications, including hepatic steatosis and postprandial hyperglycemia[114,115].

ELOVL6 KO mice

Elovl6$^{-/-}$ mice are protected against the development of hepatic insulin resistance when fed a HF/HC diet, despite the accumulation of palmitate concentrations. Improvement in insulin signaling (as evidenced by the restoration in insulin-mediated Akt phosphorylation) occurred despite hepatic steatosis and marked obesity in *Elovl6*$^{-/-}$ mice[116]. While these results are somewhat surprising given the role of palmitate as a potent inducer of insulin resistance (at least in primary cultures of hepatocytes)[117], they are also interesting since they indicate that the hepatic FA composition, and particularly the conversion of palmitate to stearate, is crucial for insulin sensitivity. It should be noted that the reduced SCD1 expression observed in livers of *Elovl6*$^{-/-}$ mice could have also contributed to the amelioration of insulin resistance in these mice[116].

ChREBP knockdown mice

ChREBP knockdown led to the expected inhibition of L-PK, ACC, FAS and SCD1 as well as GPAT. While a carbohydrate-response element was previously identified in the promoter region of the *GPAT* gene[118], its expression was found to be unaffected in the liver of ChREBP-knockout mice upon refeeding[119]. It is possible that the nutritional regulation of GPAT may be more sensitive to insulin *via* SREBP-1c than to glucose *via* ChREBP. Nevertheless, following ChREBP knockdown, a resultant decrease in lipogenic rates was observed in shChREBP-RNA-treated *ob/ob* mice, leading to a 50% reduction in hepatic and circulating TG concentrations[120]. ChREBP knockdown not only affected the rate of *de novo* lipogenesis but also had consequences for β-oxidation. Therefore, similarly to the liver-specific knockout of SCD1 (LKO mice)[121], the coordinated modulation in FA synthesis and oxidation in liver led to an overall improvement of lipid homeostasis in ChREBP-deficient mice. The decrease in lipogenic rates observed in LKO mice was at least partially attributed to a decrease in ChREBP nuclear protein content[122]. Clearly, ChREBP needs now to be considered as a key determinant of the molecular regulation of the lipogenic pathway.

CLASSIFICATION OF SOME ANIMAL MODELS WITH DISRUPTION OF GENES INVOLVED IN NAFLD

Table 1 presents a number of candidate genes that are involved in the pathogenesis of NAFLD and a few are discussed below.

Genes affecting lipid metabolism

Pemt KO animals: *Pemt-/- mice* have two selectively disrupted alleles of the *Pemt-2* gene at exon 2[123], which encode PEMT, and do not express any PEMT activity in liver. Therefore these mice completely depend on dietary choline intake to meet daily choline requirements. When fed a diet deficient in choline and insufficient in methionine, *Pemt-/-* mice develop decreased PtdCho concentrations in hepatic membranes, leading to severe liver damage and death; a choline supplemented diet prevents this[124] and, if provided early enough, can reverse hepatic damage.

DGAT2 mice: DGAT2, an isoform of the enzyme acylCoA: diacylglycerol acyltransferase, catalyses the final stage of triglyceride synthesis in the liver[125]. Overexpression of DGAT2 in mice led to a 2.4-fold increase in hepatic triglyceride content, but no effect on production of VLDL triglyceride or apoB[126]. In addition, mice on a high-fat diet that overexpress DGAT develop fatty liver but not glucose or insulin intolerance[127], showing that hepatic steatosis can occur independently of insulin resistance. Interestingly, antisense therapy reducing DGAT improves hepatic steatosis, but not insulin sensitivity[128].

Apolipoprotein C-Ⅲ: Apolipoprotein C-Ⅲ (apoC-Ⅲ) is the most abundant C apolipoprotein in human plasma, where it is present as an 8.8-kDa mature protein on chylomicrons, VLDL and HDL. ApoC-Ⅲ is synthesized in the liver and in minor quantities by the intestine[129]. Several lines of evidence have implicated apoC-Ⅲ as contributing to the development of hypertriglyceridemia in the human population. Investigation in *apoC3-/-* mice supports the concept that apoC-Ⅲ is an effective inhibitor of VLDL TG hydrolysis and reveals a potential regulating role for apoC-Ⅲ with respect to the selective uptake of cholesteryl esters[130].

Genes affecting insulin resistance/sensitivity

IRS 1: Studies on mice with targeted disruption of the *Irs* genes lend some support to both situations. *Irs1* knockout ($Irs1^{-/-}$) mice show significant embryonic and postnatal growth retardation, suggesting that IRS-1 plays a key role in relaying the growth-stimulating effects of insulin and insulin-like growth factor. IRS-1-deficient mice also have insulin resistance and mild glucose intolerance, but do not develop diabetes[131,132].

Ecto-nucleotide pyrophosphate phosphodiesterase: Ecto-nucleotide pyrophosphate phosphodiesterase (ENPP1) has been shown to negatively modulate insulin receptor and to induce cellular insulin resistance when over-expressed in various cell types. Systemic insulin resistance has also been observed when ENPP1 is over-expressed in multiple tissues of transgenic models and is largely attributed to tissue insulin resistance induced in skeletal muscle and liver. In the presence of a high fat diet, ENPP1 over-expression in adipocytes induces fatty liver, hyperlipidemia and dysglycemia, thus recapitulating key manifestations of the metabolic syndrome[133].

Transcription factor 7-like 2: Transcription factor 7-like 2 (TCF7L2) is a receptor for β-catenin and regulates the expression of a multitude of genes involved in cellular metabolism and growth. Various studies[134-136] have linked TCF7L2 variation with impaired insulin secretion and risk of diabetes, possibly mediated by altered β-cell glucose response. In addition, it regulates adipokine secretion and triglyceride metabolism through effects on PPAR-γ, CCAAT/enhancer-binding protein, and lipoprotein lipase; TCF7L2 SNPs are associated with serum triglyceride concentrations in familial hyperlipidemia[137].

Genes affecting oxidative stress

Glutamate-cysteine ligase: Glutamate-cysteine ligase (GCLC) is the first and rate-limiting enzyme in the synthesis of glutathione, the major antioxidant in the liver. Liver-specific deletion of GCLC in mice rapidly leads to hepatic steatosis and progressive severe parenchymal damage[138].

Nitric oxide synthase: Yoneda *et al*[139], who studied associations of PPARγC1α, also examined the influence of SNPs in the inducible nitric oxide synthase (*NOS2*) gene on their NAFLD cohort. iNOS is expressed as part of the inflammatory response and in the presence of superoxide radicals forms peroxynitrite, which can cause endoplasmic reticulum stress and cell death[140]. iNOS-deficient mice develop NASH with high fat diets[141].

Superoxide dismutase-2: Elevated hepatic reactive oxygen species play an important role in pathogenesis of liver diseases, such as alcohol-induced liver injury, hepatitis C virus infection, and nonalcoholic steatohepatitis. Satoshi *et al*[142] observed significant increases in lipid peroxidation and TG in the liver of *Sod1* KO and double KO mice but not in the liver of *Sod2* KO mice.

Genes affecting immune regulation

Signal transducer and activator of transcription 3: Signal transducer and activator of transcription 3 (STAT3) is an acute-phase transcription factor; after hepatic necrosis it activates pathways associated with liver regeneration and acute inflammation[143]. STAT3 is also implicated in nutrient metabolism and developing metabolic syndrome. Transgenic mice with hepatic deficiency of STAT3 develop insulin resistance and disturbed glucose homeostasis; whereas the constitutive liver specific expression of STAT3 in diabetic mice reduces blood glucose and plasma insulin concentrations and downregulates gluconeogenic gene expression[144].

CONCLUSION

Inbred strains of mice provide convenient tools to study the pathogenesis of NAFLD because they provide the opportunity to control genetic and environmental factors that might influence the natural history of NAFLD. Various genetic alterations or environmental stressors producing a similar phenotype prove that many different immunological, neuronal and hormonal factors are involved in the pathogenesis of NAFLD[145,146]. Transgenic mouse models also represent gene mediation to NAFLD. Therefore, any one of these animal models could be used to clarify how altered cross talk among immune cells, neurons and endocrine cells promote NAFLD. In contrast to human genetic studies, animal studies have found genes that consistently produce disease-like phenotypes, and the underlying genetic basis for the phenotypes in these models have often been

elucidated. Animal studies on NAFLD frequently reveal significant single-locus effects that can be reproduced across species and/or strains. Such "disease genes" in animal models can be found relatively easily using linkage mapping techniques in crossed inbred lines. Similarly, transgenic animals or genetically manipulated animals for NAFLD can reveal significant effects of candidate alleles in well-defined genetic backgrounds. This review has explored some of the advantages and disadvantages of a few genetically modified mouse models of NAFLD that would be useful in understanding the connections between lipid metabolism, host defences, environmental triggers, genetic variability, inflammatory recruitment, and fibrogenesis. These models will also serve as important platforms for assessing therapeutic strategies, which is an essential area of study.

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TOPIC HIGHLIGHT

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Trends on gastrointestinal bleeding and mortality: Where are we standing?

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Abstract

Bleeding from the gastrointestinal tract and its management are associated with significant morbidity and mortality. The predisposing factors that led to the occurrence of these hemorrhagic instances are largely linked to the life style of the affected persons. Designing a new strategy aimed at educating the publics and improving their awareness of the problem could effectively help in eradicating this problem with no associated risks and in bringing the mortality rates down to almost zero.

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Key words: Gastrointestinal bleeding; Peptic ulcer; Esophageal varices; Helminthic infestation; Bowel cancer; Mortality; Morbidity; Predicting factors; Age; Sex

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INTRODUCTION

Gastrointestinal (GI) bleeding involves any bleeding in the GI tract from the mouth, oesophagus, stomach, small intestines, large intestines, to the anus. The degree of bleeding can range from microscopic levels detected only by lab tests, to perceptible amounts of bleeding that can be seen in the stool or vomit. However, any level of bleeding can lead to serious problems. Microscopic levels of bleeding can lead to anaemia over time, and more massive amounts of bleeding can lead to death.

How to manage these instances more effectively and to prevent the happening of the co-incident adverse would be discussed by colleagues whom I invited to carry out this task. But in this review, I am going to assess how practically these haemorrhagic instances could be avoided.

TRENDS ON GASTROINTESTINAL BLEEDING AND MORTALITY

Upper GI bleeding involves bleeding from the mouth to the duodenum (common causes of upper GI bleeding are listed in Table 1). But lower GI bleeding involves bleeding from the small intestines to the anus and can be caused by haemorrhoids, cancer, polyps and colitis, among other causes (Table 2). Upper GI bleeding has been estimated to account for up to 20 000 deaths annually in the United States (international records are not available). The overall incidence of acute upper GI haemorrhage has been estimated to be 50 to 100 per 100 000 persons per year. The trends of hospitalization for GI bleeding in the United States in 1998 and in 2006 have

Table 1 Causes of acute upper gastrointestinal bleeding

Common	Gastric ulcer
	Duodenal ulcer
	Esophageal varices
	Malory-Weiss tear
Less common	Gastric erosive/gastropathy
	Esophagitis
	Cameron lesions
	Dieulatory lesion
	Telangiectasias
	Portal hypertensive gastropathy
	Gastric antral vascular ectasia (watermelon stomach)
	Gastric varices
	Neoplasms
Rare	Esophageal ulcer
	Erosive duodenitis
	Aortoenteric fistula
	Hemobilia
	Pancreatic disease
	Crohn's disease

Table 2 Causes of acute lower gastrointestinal bleeding

Common	Colonic diverticula
	Angioectasia
Less common	Colonic neoplasms (including post polypectomy bleeding)
	Inflammatory bowel disease
	Colitis
	Ischemic
	Raditation
	Unspecified (infectious or non specific)
	Haemorrhoids
	Small bowel source
	Upper gastrointestinal source
Rare	Dieulatory lesion
	Colonic ulcerations
	Rectal varices

been summarised in Table 3.

And the hospitals' discharge rates of the admitted subjects for different causes of GI haemorrhage in 1998 and 2006 in the United States have also been listed (Table 4).

The incidence rate for upper GI bleeding appears to be, in general, decreasing (Table 3). This may be due to the prescription of proton pump inhibitors and the skilled efforts to eradicate *Helicobacter pylori* infections (Table 4). But, the risk of upper GI bleeding appears to be increasing in particular groups of patients, such as those with a history of oesophageal varices (Table 5).

Regarding bleeding from the lower GI tract, it appears that haemorrhage from rectum and anus and the incidences of diagnosis of occult blood in stool are increasing (Table 5).

When the total number of discharges for cases of GI bleeding was investigated per age of the discharged patient, it appeared that incidences of GI bleeding are increasing in certain subgroups. The incidences of GI haemorrhage was, for example, found increasing in those who were less than 20 years old (Table 3).

Oesophageal varices form less than 10% of the all causes of GI haemorrhages. However, patients with variceal haemorrhage have a mortality rate of at least 30% during their initial hospitalization, with a one year mortality rate approaches 60%[1]. Patients who have bled once from oesophageal varices have a 70% chance of rebreeding, and approximately one third of further bleeding episodes are fatal[2]. The risk of death is maximal during the first few days after the bleeding episode and decreases slowly over the first 6 wk. Oesophageal varices are present in approximately 40% of patients with cirrhosis and in as many as 60% of patients with cirrhosis and ascites[3]. In cirrhotic patients who do not have oesophageal varices at initial endoscopy, new varices will develop at a rate of approximately 5% per year. In patients with small varices at initial endoscopy, progression to large varices occurs at a rate of 10%-15% per year and is related chiefly to the degree of liver dysfunction[4]. On the other hand, improvement in liver function in patients with alcoholic liver disease who abstain from alcohol is associated with a decreased risk, and sometimes even disappearance of the varices[5]. It has been estimated that up to 25% of the patients with newly diagnosed varices would bleed within two years[4]. The risk of bleeding in patients with varices less than 5 mm in diameter is 7% by two years, and in patients with varices greater than 5 mm in diameter is 30% by two years[4]. Mortality rates in the setting of surgical intervention for acute variceal bleeding are high[6]. Associated abnormalities in the renal[7], pulmonary[7], cardiovascular[8], and immune systems in patients with oesophageal varices contribute to 20%-65% of mortality[9]. In Western countries, alcoholic and viral cirrhosis are the leading causes of portal hypertension and oesophageal varices. Thirty percent of patients with compensated cirrhosis and 60%-70% of patients with decompensate cirrhosis have gastroesophageal varices at presentation[9]. The *de novo* rate of development of oesophageal varices in patients with chronic liver diseases is approximately 8% per year for the first 2 years and 30% by the sixth year[9]. A recently published survey[10] on consumption of alcohol by teenagers in the North West of England revealed that almost 90% of the participant school children (aged 15 and 16) drink alcohol at least occasionally. Of those, 38.0% usually binge drink (5+ drinks in one session), 24.4% are frequent drinkers (drinking two or more times a week) and 49.8% drinks in public settings (such as bars, clubs, streets and parks). It is worth to note that excessive drinking by young people, for example, has seen a 20% rise in hospital admissions in England over the last five years. The number of people taken to Accident and Emergency with alcohol-related injuries has also doubled to 148 477 a year. Alcohol-related conditions such as liver disease have doubled in less than a decade, to 262 844 a year as well.

But in developing countries, hepatitis B is endemic in the Far East and Southeast Asia, particularly, as well as South America, North Africa, Egypt and other countries in the Middle East. Schistosomiasis is an important cause of portal hypertension in Egypt, Sudan and other African countries[8]. Those that have been affected with bilharsiasis, they almost have additional complications from hepa-

Table 3 Trends of hospitalization for gastrointestinal bleeding in the United States in 1996 and 2006

	Total number of discharges per 100 000 persons (principal diagnosis)			Total number of discharges per 100 000 persons (all diagnosis)		
	1998	2006	Percent changes (%)	1998	2006	Percent changes (%)
	189	182	-3.8	390	375	-3.7
By bleeding site						
Upper	96	82	-14	170	146	-14
Lower	43	44	+2	75	82	+8
Unspecified	50	56	+11	156	158	+1
By age (yr)						
< 20	7.4	7.5	+1.5	23	25	+8.6
20-29	25	23	-7	55	59	+6.1
30-44	65	59	-8.3	139	140	+0.6
45-64	187	181	-3.4	399	396	-0.9
65-84	859	806	-5.6	1731	1596	-7.8
> 85	2207	1871	-15.2	4257	4375	-18.4
By sex (%)						
Female	259 808 (51)	276 663 (51)				
Male	252 060 (49)	268 589 (49)				

Table 4 Death rates for gastrointestinal bleeding inpatients

	1998	2006	Percentage change (%)
Inpatient death number	20 013	16 344	-18
Inpatient death number/100 000	7	5	-26
Inpatient death rate (%)			
By bleeding site			
Upper	3.5	2.7	-23
Lower	3.5	2.9	-17
Unspecified	5	3.6	-28
By sex			
Male	4	3	-25
Female	3.8	3	-21
By age (yr)			
< 20	-	-	-
20-29	-	-	-
30-44	1.6	1.1	-31
45-64	2.7	2.2	-19
65-84	4.1	3	-27
> 85	6.4	5.2	-19

titis B and C[11-14]. The instances of acute lower GI bleeding are mainly self-limited and affected patients do not require hospitalization care, approximately 21 per 100 000 adults in the United States require hospitalization for severe lower GI bleeding every year[15] (international records are not available). The hospitalization rate for lower GI bleeding is approximately one third of that for upper GI bleeding[16] and in a survey by the American College of Gastroenterology, lower GI haemorrhage accounted for 24% of all GI bleeding occasions[17]. It has been estimated that detection of occult blood in stools formed 7% of all instances of GI bleeding in the United States in 2007. But it is expected that the incidence rates of detection of occult blood in the stools of patients in developing countries exceed this figure by many times. Helminthic infestation is a common cause for occult blood in stools in developing countries (Table 6). It has been estimated that 80% of the population of most countries in Asia, Africa and south America are infected with helminths, such as Ascaris and widespread infection has been demonstrated throughout Europe, Particularly, Romania, Hungry, Portugal and Turkey[18]. When 312 children in the age group of 4-15 years were examined for different intestinal helminths in three schools located in rural areas in Kupwara, Kashmir, India[19], 222 of 312 (71.15%) tested positive for various intestinal helminths[20]. The various helminth parasites included Ascaris lumbricoides, Trichuris trichiura, Enterobius vermicularis and Taenia saginata. The highest frequency of 69.23% (216/312) was noted for Ascaris lumbricoides followed by Trichuris trichiura 30.76% (96/312), Enterobius vermicularis 7.69% (24/312) and Taenia saginata 7.69% (24/312). Single infection was found in 33.65% (105/312) and mixed infection was seen in 37.5% (117/312) children. Again, Chandrasekhar MR and others in 2003[21] collected faecal samples from 1000 children below 6 years of age. Six hundred and eighty children (68.0%) were detected to have intestinal helminthic infection. The incidence of intestinal helminthiasis in urban group of children was 56.8% (284 out of 500 tested) while in rural group of children was 79.2% (396 out of 500 tested) both in rural and urban population Ascaris lumbricoides was the single predominant species, whereas a combination of A. Lumbricoides and Trichuris trichiura was common multiple infection. All cultures of faecal samples were positive for hook worm ova. In Pakistan, out of 200 children examined, 132 (66%) were found positive for various intestinal helminths infestation[22]. There were 6 different types of helminths found in the specimens examined.

It has become visible from the above review that the main causes for the occurrence of haemorrhage from the GI tract are strongly linked to the life style of the affected persons. Educating the public is thus expected to solve this problem. However, when the effect of health education in the control of bilharzias is assessed the results were disappointing. In 2001, Garba *et al*[23] carried out a survey on two groups of endemic villages in the Niger. In one group of villages, there were health educa-

Table 5 Underlying conditions of gastrointestinal in 1998 and 2006 *n* (%)

Underlying condition	1998	2006	Discharge percentage change (principal diagnosis)	1998	2006	Discharge percentage change (all diagnosis)
Upper GI: oesophageal varices, ulcer, perforation and other haemorrhages	23 007 (4)	35 058 (6)	52% and 38% after population adjustment	84 382 (8)	103 381 (9)	23% and 11% after population adjustment
Gastric, duodenal ulcers, gastrojejinal uklcers or perforation	156 29 (31)	131 225 (24)	-16% and -24% after population adjustment	215 912 (20)	179 032 (16)	-17% and -25%after population adjustment
Gastritis or duiodenitis and haemorrhage	54 310 (11)	44 104 (8)	-19% and -27% after population adjustment	118 333 (11)	90 635 (8)	-23% and -31%after population adjustment
Angiodysplasia of stomach and dudodenum with haemorrhage	9237 (2)	14 679 (3)	59% and 43% after population adjustment	15 061 (1)	23 032 (2)	53% and 38% after population adjustment
Haematemesis	16 466 (3)	21 230 (4)	29% and 16% after population adjustment	58 955 (6)	72 655 (6)	23% and 11% after population adjustment
Perforation of the large intestine	9117 (2)	10 066 (2)	10% and -0.3% after population adjustment	26 200 (2)	33 246 (3)	27% and 15% after population adjustment
Haemorrhage of rectum and anus	12 084 (2)	21 456 (4)	78% and 60% after population adjustment	52 974 (5)	85 592 (7)	56% and 41% after population adjustment
Diverticulosis and diverticulitis of the colon and haemorrhage	80 007 (16)	83 927 (15)	5% and -5% after population adjustment	101 000 (10)	104 516 (9)	3% and -7% after population adjustment
Diverticulosis and diverticulitis of the small intestine and haemorrhage	15 369 (3)	16 259 (3)	6% and -5% after population adjustment	26 933 (3)	27 433 (2)	2% and -8% after population adjustment
Unspecified GI bleeding (blood in stool)	31 044 (6)	38 284 (7)	23% and 11% after population adjustment	283 440 (27)	325 035 (29)	15% and 4% after population adjustment
Haemorrhage of GI tract (unspecified)	104 991 (21)	129 164 (24)	23% and 11% after population adjustment	283 440 (27)	325 035 (29)	15% and 4% after population adjustment

GI: Gastrointestinal.

Table 6 Causes of occult gastrointestinal bleeding

Mass lesions	Carcinoma (any site)
	Large > 1.5 cm adenoma (any site)
Inflammatory lesions	Erosive oesophagitis ulcer (any site)
	Cameron lesion
	Erosive gastropathy
	Celiac sprue
	Ulcerative colitis
	Crohn's disease
	Non specific colitis
	Caecal ulcer
Vascular lesions	Angiodysplasia (any site)
	Portal hypertensive gastropathy (colonpathy)
	Gastric antral vascular ectasia
	Hemangioma
	Dieulatory lesion
Infection	Hockworm
	Whipworm
	Stronyloidosis
	Ascariosis
	Tuberculous enterocolitis
	Amoebiasis
	Cytomegalo virus

tion campaigns but there were no education campaigns in the second group. The people in the targeted areas received information on Bilharziasis and on how to fight against it. However, 46.6% of interviewed people in the project area couldn't mention any means for controlling bilharziasis. Behaviours that favour the illness were ignored by 1/3 of interrogated people in the project area. Yet, there was an increase in knowledge about the illness in the program zone in comparison with the control area. Despite this increase in knowledge level, changes in behaviour in relation to the illness remained low. Risky behaviour continued in about 2/3 of interrogated people. Only 33% of persons of the project area declared having adopted at least a single good behaviour. This means that changes of behaviour may take time to have effect.

In addition, areas endemic for helminthic infestation worldwide suffer from poor economic growth, poor sanitation and lack of appropriate toilet facilities.

Multiple studies demonstrated that in endemic areas re-infection is exceedingly common and mass chemotherapy alone is insufficient to prevent the spread of these diseases[24].

Relatively little attention has been focused on the impact of personal attitude on the development of haemorrhagic episodes from the GI tract. This review emphasizes on the importance of designing a new strategy aimed at preventing the happening of these episodes.

The occurrence of GI bleeding and its management are associated with significant harm; however, educating the publics through properly designed long term program and improving the general surrounding conditions are free from risk.

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TOPIC HIGHLIGHT

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Management of non-variceal upper gastrointestinal tract hemorrhage: Controversies and areas of uncertainty

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Abstract

Upper gastrointestinal tract hemorrhage (UGIH) remains a common presentation requiring urgent evaluation and treatment. Accurate assessment, appropriate intervention and apt clinical skills are needed for proper management from time of presentation to discharge. The advent of pharmacologic acid suppression, endoscopic hemostatic techniques, and recognition of *Helicobacter pylori* as an etiologic agent in peptic ulcer disease (PUD) has revolutionized the treatment of UGIH. Despite this, acute UGIH still carries considerable rates of morbidity and mortality. This review aims to discuss current areas of uncertainty and controversy in the management of UGIH. Neoadjuvant proton pump inhibitor (PPI) therapy has become standard empiric treatment for UGIH given that PUD is the leading cause of non-variceal UGIH, and PPIs are extremely effective at promoting ulcer healing. However, neoadjuvant PPI administration has not been shown to affect hard clinical outcomes such as rebleeding or mortality. The optimal timing of upper endoscopy in UGIH is often debated. Upon completion of volume resuscitation and hemodynamic stabilization, upper endoscopy should be performed within 24 h in all patients with evidence of UGIH for both diagnostic and therapeutic purposes. With rising healthcare cost paramount in today's medical landscape, the ability to appropriately triage UGIH patients is of increasing value. Upper endoscopy in conjunction with the clinical scenario allows for accurate decision making concerning early discharge home in low-risk lesions or admission for further monitoring and treatment in higher-risk lesions. Concomitant pharmacotherapy with non-steroidal anti-inflammatory drugs (NSAIDs) and antiplatelet agents, such as clopidogrel, has a major impact on the etiology, severity, and potential treatment of UGIH. Long-term PPI use in patients taking chronic NSAIDs or clopidogrel is discussed thoroughly in this review.

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Key words: Hemorrhage; Proton pump inhibitors; *Helicobacter pylori*; Prokinetic agents; Hemostasis; Thienopyridines

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INTRODUCTION

Fundamental changes have occurred over the past several decades in the management of upper gastrointestinal tract hemorrhage (UGIH). Pharmacologic gastric acid suppression, recognition of *Helicobacter pylori* (*H. pylori*) as a causative agent in peptic ulcer disease, and the wide-

Table 1 Sensitivity, specificity, positive predictive value and negative predictive value for death and rebleeding using 6 common upper gastrointestinal tract hemorrhage scoring systems

Scoring system	Death	Rebleeding
Pre-endoscopy Rockall risk score		
Sensitivity	100 (43.8-100)	69.6 (49.1-84.4)
Specificity	18.5 (14.8-22.9)	17.5 (13.8-22.0)
PPV	1.0 (0.4-3)	5.5 (3.4-8.8)
NPV	100 (94.4-100)	89.2 (79.4-94.7)
Post-endoscopy Rockall risk score		
Sensitivity	33.3 (6.1-79.2)	87.0 (67.9-95.5)
Specificity	29.6 (25.1-34.6)	31.1 (26.4-36.3)
PPV	0.4 (0.1-2.2)	8.1 (5.3-12.1)
NPV	98.1 (93.4-99.5)	97.2 (92.0-99.0)
Blatchford scoring system		
Sensitivity	100 (83.89-100)	94.29 (81.40-98.42)
Specificity	1.83 (0.71-4.61)	0.98 (0.27-3.50)
PPV	8.51 (5.58-12.79)	14.04 (10.17-19.06)
NPV	100 (51.01-100)	50.00 (15.00-85.00)
Forest classification		
Sensitivity	85.00 (63.96-94.76)	71.43 (54.95-83.67)
Specificity	50.23 (43.66-56.79)	50.49 (43.68-57.28)
PPV	13.49 (8.6-20.54)	19.84 (13.81-27.65)
NPV	97.35 (92.49-99.10)	91.15 (84.77-95.12)
Cedars-Sinai Medical Center predictive index		
Sensitivity	95.00 (76.39-99.11)	80.00 (64.11-89.96)
Specificity	41.55 (35.22-48.17)	41.67 (35.12-48.53)
PPV	12.93 (8.44-19.31)	19.05 (13.52-26.15)
NPV	98.91 (94.09-99.81)	92.39 (85.12-96.26)
Baylor College scoring system		
Sensitivity	87.50 (52.91-97.76)	30.77 (12.68-57.63)
Specificity	58.49 (45.09-76.74)	47.92 (34.47-61.67)
PPV	24.14 (12.22-42.11)	13.79 (5.50-30.56)
NPV	96.88 (84.26-99.45)	71.88 (54.63-84.44)

95% confidence intervals are recorded in parentheses. PPV: Positive predictive value; NPV: Negative predictive value.

spread dissemination of flexible endoscopy and endoscopic hemostatic techniques have contributed to a paradigm shift in the treatment of complicated peptic ulcer disease, in particular, from predominantly surgical to predominantly endoscopic management. At the same time, an increasing proportion of patients presenting with UGIH are older or elderly patients[1], and a significant number of patients with UGIH consume non-steroidal anti-inflammatory drugs (NSAIDs) and/or antiplatelet therapy to treat other medical comorbidities. Given the confluence of these factors, UGIH continues to have a considerable impact with respect to patient morbidity and mortality, as well as health care resource utilization. The annual incidence of UGIH has been estimated as low as 48 and as high as 165 cases per 100 000 population, and the mortality rate remains high somewhere between 7% and 14%[2-6]. UGIH accounts for > 300 000 annual hospitalizations in the United States, with an estimated cost of $2.5 billion[3,5]. This article aims to review the management of UGIH, with an emphasis on discussion of areas of controversy or uncertainty in current practice.

INITIAL ASSESSMENT

By definition, UGIH originates proximal to the ligament of Treitz. UGIH accounts for the preponderance of all gastrointestinal hemorrhage; estimated at 4-6 times more common than lower gastrointestinal (GI) hemorrhage[7]. Initial assessment of the pace and acuity of the bleeding episode has major bearing on the initial management of UGIH. For patients presenting with UGIH and evidence of hemodynamic compromise, the fundamental goal of initial management is adequate and appropriate volume resuscitation. Additional stratification of patients into high- and low-risk categories may be based on clinical and endoscopic criteria[2]. Predictors of poor prognosis include: age > 65 years, shock, poor overall health, comorbid conditions, low initial hemoglobin/hematocrit, active bleeding (red blood per rectum or hematemesis), sepsis, and elevated creatinine or serum transaminases[2,8,9]. Several scoring systems have been created and/or validated for this purpose, including APACHE Ⅱ, Forrest Classification, Blatchford, pre-endoscopic Rockall, Baylor College, Cedars-Sinai Medical Center and Rockall indexes (Table 1 compares 6 commonly used scoring systems)[10,11]. Some of these may be cumbersome (APACHE Ⅱ) or require data not immediately available based on initial clinical assessment (the Rockall Scoring System, for instance, requires endoscopic data) and therefore may be of limited utility in the acute setting[12].

The role of nasogastric tube placement and aspirate inspection in the initial assessment of UGIH has fallen out of favor in many emergency room and acute care settings. In theory, the presence of bright red blood *via* nasogastric aspirate suggests active UGIH and should prompt urgent esophagogastroduodenoscopy (EGD)[3]. The absence of blood on nasogastric aspirate, however, does not exclude the presence of a culprit UGIH source. In a study by Aljebreen *et al*[13], 15% of patients with UGIH and clear or bilious nasogastric aspirate were ultimately found to have an underlying high risk lesion during EGD.

PHARMACOLOGIC THERAPY PRIOR TO ENDOSCOPY

Pharmacologic gastric acid suppression has changed the face of peptic ulcer disease (PUD) treatment, both by favoring hemostasis in the short term (platelet aggregation and clot formation are impaired at acidic gastric pH[14]), and enabling ulcer healing and remission over the longer term[3]. Proton pump inhibitors (PPIs) covalently bind to and inhibit the H-K ATPase pump of the gastric parietal cells, thus inhibiting the final common pathway of acid secretion. PPIs offer more durable and sustained acid suppression than histamine receptor antagonists, which are prone to tachyphylaxis[15]. For these reasons, PPIs have become the dominant acid suppressive therapy used in the treatment of UGIH.

An emerging area of importance is the optimal dose, route of administration, and timing of PPI therapy in patients presenting with UGIH. Administration of neoadjuvant PPI, prior to diagnostic endoscopy or endoscopic therapy, has become widespread, and this practice recommendation has been supported by consensus guidelines[2]. This approach may be particularly reasonable in instances when access to prompt EGD and/or availability of endoscopic therapeutic expertise is limited. Otherwise, however, it is uncertain whether neoadjuvant PPIs confer benefit with respect to meaningful clinical outcomes. In a controlled prospective study of patients with UGIH randomized to intravenous PPI *vs* placebo prior to endoscopy[16], patients in the PPI arm were less likely to have active bleeding at the time of endoscopy and to require endoscopic hemostatic therapy. There was no difference in transfusion requirements, rates of rebleeding, requirement for surgery, or 30-d mortality when comparing patients receiving PPIs to those receiving placebo. A systematic meta-analysis, based on published controlled data in the medical literature, reported no benefit of neoadjuvant (pre-endoscopic) PPI therapy on rates of rebleeding, salvage surgery for failures of endoscopic hemostasis, or mortality[17].

Adjuvant PPI therapy, administered following diagnostic and/or therapeutic EGD, has been proven effective, as well, leading to a decrease in recurrent PUD bleeding, need for blood transfusion, need for surgery, and duration of hospital stay[3,18,19]. Despite these major impacts, studies have not demonstrated an impact of PPIs on mortality due to UGIH[3,20,21]. Furthermore, whether in the neoadjuvant or adjuvant setting, the optimal dosing, route of administration, and duration of post-endoscopy PPI has not been clearly established. Current guidelines recommend the use of high-dose intravenous PPI therapy for 3 d following successful endoscopic hemostasis[2]. In many studies, high-dose PPI therapy is defined as an initial bolus (omeprazole 80 mg) followed by continuous infusion (omeprazole 8 mg/h) for up to 72 h. However, there has been limited direct comparison of this high-dose intravenous regimen in comparison to alternative regimens. Whether continuous intravenous infusion of PPIs is clearly superior to intermittent bolus dose is uncertain; similarly, whether intravenous PPIs are clearly superior to highly bioavailable oral PPIs in patients able to take oral medications is uncertain.

TIMING OF ENDOSCOPIC EVALUATION

Endoscopic evaluation is an essential part of UGIH management. Urgent EGD has been proposed as the standard of care in patients with high-risk lesions, although the precise timing of urgent EGD has been variably defined. American Society for Gastrointestinal Endoscopy practice guidelines for the treatment of non-variceal UGIH suggest that early endoscopy (within 24 h) maximizes the impact on hospital length of stay and transfusion requirements, yet do not make formal recommendations regarding the optimal time for performing EGD within this 24-h window[22].

In theory, the availability both of on-call physicians proficient in endoscopic hemostasis and on-call support staff with technical expertise in usage of endoscopic devices enable performance of EGD on a 24-h/7-d basis. Several studies have examined the timing of upper endoscopy and resultant impact on both patient outcomes and resource utilization. In a study by Sarin *et al*[23], a retrospective review of > 500 patients who underwent upper endoscopy for non-variceal UGIH, the timing of endoscopy was stratified into three categories: < 6 h, 6-24 h and > 24 h. There was no significant difference in mortality or need for surgery between the < 6 h and 6-24 h groups. However, there was a difference between the two < 24 h groups and the > 24 h group. These findings were supported by a more recent retrospective review in 2007 that examined 169 patients with acute non-variceal UGIH with either systolic blood pressure < 100 mmHg or heart rate > 100 beats/min on presentation. Patients were divided into two groups: those who received endoscopy within 6 h *vs* 6-24 h. Again, there were no significant differences between the groups in any of the primary outcomes, including rebleeding, need for surgery, in-hospital mortality, or hospital readmission within 30 d[24].

In a variation on this theme, Dorn *et al*[25] have examined the difference in clinical outcomes among patients admitted for UGIH on either a weekday or a weekend. Those admitted on a weekend had a slight but significant increase in mortality (hazard ratio of 1.09), hospital length of stay, and hospitalization cost. Although the weekend patients waited longer for EGD than their weekday counterparts, the entire effect on patient outcomes could not be accounted for by the delay in endoscopy timing alone. Further study is needed to elicit other potential causes of this weekend effect. Our practice is to perform EGD as soon as is feasible following hemodynamic resuscitation and stabilization of the patient, and within 12 h of clinical presentation.

In addition to offering hemostatic techniques to prevent continued or recurrent bleeding, a major benefit of endoscopy is the ability to risk stratify the lesion and triage patients to those who require inpatient or more intensive monitoring, *vs* those who are candidates for early hospital discharge. With respect to peptic ulcer hemorrhage, lesions at high risk for recurrent bleeding include ulcers > 2 cm, or ulcers with active bleeding, a visible vessel, or adherent clot. Such lesions warrant both endoscopic therapy, when feasible, and inpatient monitoring post-endoscopy. Conversely, clean-based ulcers or ulcers with flat pigmented spots are considered low risk and do not require endoscopic therapy[26], and patients found to have such low-risk lesions may be candidates for early discharge, even from an emergency room setting.

Much improvement in current management of UGIH and resource allocation for treatment of UGIH could be achieved in this area. Numerous studies have shown that early endoscopy is not often followed by early discharge of appropriate low-risk patients. Bjorkman *et al*[27] have shown that EGD within 6 h of presentation to the emergency department versus 48 h did not change hospital utilization of resources, the study's primary endpoint. In

Table 2 Areas requiring further investigation

Pharmacologic therapy prior to endoscopy	Determine optimal route and dosage of PPI in UGIH (continuous infusion *vs* intermittent IV bolus *vs* oral dosing)
Timing of endoscopic evaluation	Defining optimal timing of initial endoscopy, implementation of early discharge in low risk patients
Prokinetic agents as endoscopic adjuncts	Clearly define the role for prokinetics in UGIH with randomized controlled trials, specifically define the optimal agent, dose and timing prior to endoscopy
Long-term PPI management	Clarification of potential long-term sequelae of PPI including: osteoporosis, c. difficile infection and community-acquired pneumonia
PPI and thienopyridines	Consensus on the clinical importance of this interaction, a complete randomized controlled trial to support the truncated COGENT trial data
H. pylori and UGIH	*H. pylori* testing that allows accurate test results in the setting of acute UGIH

PPI: Proton pump inhibitor; *H. pylori*: *Helicobacter pylori*; UGIH: Upper gastrointestinal tract hemorrhage; IV: Intravenous; COGENT: Clopidogrel and the optimization of gastrointestinal events trial.

a recently published study, Chaparro *et al*[28] have formulated an early discharge algorithm using retrospective data and then examined this algorithm in a prospective cohort. Even at their institution using their algorithm, only 13/29 (45%) low-risk patients were discharged early. In view of these data, there is much room for improvement in clinical practice surrounding implementation of practice guidelines concerning early hospital discharge of low-risk UGIH patients and achieving the potential health care cost savings. Table 2 illustrates other areas in need of further investigation and improved recommendations.

CHOICE OF ENDOSCOPIC HEMOSTATIC TECHNIQUE

Endoscopic therapies employed in the treatment of UGIH include: (1) pharmacologic therapies, including injection of epinephrine, sclerosants and even normal saline; (2) coagulation therapies, including monopolar or bipolar cautery and argon plasma coagulation; and (3) mechanical tamponade, including hemoclips and bands. Each of these therapies has demonstrated efficacy in different clinical settings. Often, the choice of which endoscopic therapy to employ is a clinical judgment based on provider preference and expertise.

Available data suggest that epinephrine injection plus a second endoscopic intervention is superior to epinephrine injection alone. Calvet *et al*[29] completed a systematic review and meta-analysis in 2004 which included 16 studies and > 1600 patients with UGIH secondary to PUD, and who underwent endoscopic therapy with epinephrine alone or epinephrine plus a second hemostatic modality. Adding an adjunct therapy reduced the rebleeding rate from 18.4% to 10.6% [odds ratio (OR): 0.53, 95% CI: 0.40-0.69], reduced the need for emergency surgery from 11.3% to 7.6% (OR: 0.64, 95% CI: 0.46-0.90), and reduced mortality from 5.1% to 2.6% (OR: 0.51, 95% CI: 0.31-0.84). Vergara *et al*[30] have confirmed these findings in a recent Cochrane review (2008). Rebleeding, need for surgical intervention, and mortality were all lower in groups receiving dual therapy. Additionally, there was no increased risk of significant complications or adverse events when dual therapy was used. In the above studies and reviews, endoscopic therapy is usually reserved for high-risk lesions (active bleeding and visible vessels). Jensen *et al*[31] have additionally defined a role for removal of adherent clots in the treatment of PUD bleeding. In one study, removal of adherent clot and treatment with a second endoscopic intervention resulted in lower rebleeding rates when compared to not removing the adherent clot (35% *vs* 0%).

Even when endoscopy fails to provide durable and definitive hemostasis, endoscopy may provide a beneficial role in localization of the bleeding lesion to target salvage non-endoscopic intervention. Repeat endoscopy can assist both interventional radiology and/or surgery in the event that bleeding continues or recurs and alternative management is necessitated. Second-look endoscopy has also been shown to improve rebleeding rates in certain clinical situations, but without improvement in the need for surgery or mortality[3]. Current guidelines do not support routine use of second-look endoscopy.

PROKINETIC AGENTS AS ENDOSCOPIC ADJUNCTS

The presence of retained blood in the UGI tract can limit the ability to identify definitively a bleeding source and/or deliver endoscopic hemostatic therapy. Prokinetic agents may promote UGI tract motility and facilitate gastric emptying of retained blood prior to endoscopy; however, the use of such agents may be highly physician dependent. American Society for Gastrointestinal Endoscopy guidelines[22] indicate that the use of erythromycin, when administered intravenously prior to EGD, may improve mucosal visibility. A recent meta-analysis published in *Gastrointestinal Endoscopy*[32] has shown that either erythromycin or metoclopramide given prior to endoscopy significantly reduced the need for repeat endoscopy to identify the bleeding source. However, use of a prokinetic agent led to no identifiable impact on total units of blood transfused, hospital stay, or need for surgical intervention[33]. As was germane to the discussion of pre-endoscopic PPI therapy, if there is a role of prokinetic agents in the endoscopic diagnosis and management of UGIH, the optimal agent,

dose and timing prior to endoscopy have not been defined. Metoclopramide has been assigned a "black box warning" by the United States Food and Drug Administration (FDA) due to risk of neurologic side effects, and caution should therefore be advised with use of this agent.

LONG-TERM PPI MANAGEMENT

PPIs are recommended for 6-8 wk following UGIH and/or endoscopic treatment of PUD to allow for full mucosal healing. Several studies have shown a therapeutic benefit of PPIs in patients using NSAIDs chronically and/or patients with confirmed *H. pylori* infection[34,35]. After initial mucosal healing has been achieved, is there a benefit to long-term PPI use for secondary prophylaxis? Studies have shown that in patients who have PUD complicated by bleeding, there is a 33% risk of rebleeding in 1-2 years. Furthermore, there is a 40%-50% rebleeding risk over the subsequent 10 years following the initial episode of bleeding[13]. Randomized prospective trials have demonstrated a benefit to long-term acid-suppression therapy in two settings: chronic NSAID users and *H. pylori*-infected patients. As demonstrated in a 2001 *New England Journal of Medicine* article by Chan *et al*[34], in patients taking NSAIDs other than acetylsalicylic acid (ASA) who were concomitantly infected with *H. pylori*, omeprazole provided added protection above bacterial eradication alone.

Although beneficial in both treatment and prevention of UGIH, PPI therapy is not without potential risks. Chronic PPI therapy has been associated with *Clostridium difficile* infection, community-acquired pneumonia, and calcium malabsorption resulting in osteoporosis and increased fracture risk[36]. These associations have originated largely from observational studies and no strong data exist to link PPI therapy directly as a causative factor in any of these outcomes. However, given these potential sequelae, long-term PPI therapy should only be used when justified by a clear medical indication[2].

PPIS AND THIENOPYRIDINES

Much recent attention has surrounded the concomitant use of PPIs and thienopyridines, particularly clopidogrel. This has included an FDA warning regarding combined use of these medications[36]. *In vitro* studies have suggested that omeprazole, which is metabolized predominately through the CYP2C19 isoenzyme of cytochrome P450, inhibits conversion of clopidogrel, a prodrug, to its active form, thus reducing its therapeutic margin[37]. Most of the original data were specific to omeprazole and subsequent experiments using pantoprazole, lansoprazole and esomeprazole have not shown an equal class effect[3,37,38]. Additionally, although a measurable change in platelet function occurred in patients concomitantly taking PPIs and clopidogrel, the data were mixed with regards to clinically significant cardiovascular outcomes.

Subsequent retrospective reviews and observational studies have attempted to define the true impact of PPIs on the therapeutic index of thienopyridines, but with mixed results. The French Omeprazole CLopidogrel Aspirin study measured vasodilator-stimulated phosphoprotein phosphorylation; a measure of the inhibitory effect of clopidogrel on platelets *in vivo*. Patients taking both 75 mg ASA and 75 mg clopidogrel daily were randomized to either 20 mg omeprazole or placebo daily. One hundred and twenty-four patients were included and the results showed an 11.6% decrease in platelet inhibition by clopidogrel among those randomized to omeprazole, but no clinical outcomes were measured[38]. The TIMI Study Group at Brigham and Women's Hospital in Boston have attempted to clarify the link between *in vitro* interactions, measures of platelet function, and clinical outcomes in patients on either of the thienopyridines and PPIs through the analysis of two trials: prasugrel in comparison to clopidogrel for inhibition of platelet activation and aggregation-TIMI 44, the primary endpoint of which was inhibition of platelet aggregation at 6 h, and trial to assess improvement in therapeutic outcomes by optimizing platelet inhibition with prasugrel-TIMI 38, with a primary composite endpoint of cardiovascular death, myocardial infarction or stroke. The first study demonstrated a reduction in the inhibitory effect of thienopyridines on platelet aggregation in patients taking PPIs after loading doses of either clopidogrel (12% decrease) or prasugrel (7.1% decrease). However, in the second trial, > 13 000 patients with acute coronary syndrome were randomized to prasugrel or clopidogrel, of which 33% of 4500 patients were on PPIs at randomization. PPI was continued without change in dosing or frequency. There was no association between PPI use and the primary endpoint, a composite cardiovascular event[39]. Additionally, when the data were assessed by specific PPI (omeprazole, lansoprazole, pantoprazole or esomeprazole), there was no correlation.

Most recently, the only prospective randomized controlled trial to address this important clinical question, the Clopidogrel and the optimization of gastrointestinal events trial (COGENT) Trial, was published in the *New England Journal of Medicine*. COGENT randomized patients with an indication for dual antiplatelet therapy to clopidogrel 75 mg plus omeprazole 20 mg daily or clopidogrel alone. In addition, every patient was given either 81 or 325 mg enteric coated aspirin. The primary cardiovascular endpoint was a composite of death from cardiovascular causes, non-fatal myocardial infarction, revascularization, or stroke. Over 3700 patients were included in the final analysis of events at 180 d after randomization. There were 109 cardiovascular events; 4.9% in the clopidogrel plus omeprazole arm, and 5.7% in the clopidogrel plus placebo arm (HR: 0.99; 95% CI: 0.68-1.44). The primary gastrointestinal endpoint was a composite of overt or occult bleeding, symptomatic gastroduodenal ulcers or erosions, obstruction or perforation. There were 51 gastrointestinal events; 1.1% in the clopidogrel plus omeprazole group, and 2.9% in the clopidogrel plus placebo arm (HR: 0.13; 95% CI: 0.03-0.56)[40]. These data suggest no association between omeprazole and clinically meaningful adverse cardiovascular outcomes when used in conjunction with clopidogrel. However, there was a significant reduction in adverse gastrointestinal events in patients receiving omeprazole. A significant limitation of this study

was premature study termination due to loss of funding, thereby attenuating the statistical power of the study.

The ambiguity of this matter is further demonstrated in the following consensus statements. The American College of Cardiology Foundation/American College of Gastroenterology (ACG)/American Heart Association (AHA) joint guidelines released in 2008 state, "In the interest of patient safety, the AHA/ACC and the ACG advise that patients who are currently taking these medications should not change their medication regimen unless advised by their healthcare provider". A revised joint statement was released in December 2010 stating, "Pharmacokinetic and pharmacodynamic studies, using platelet assays as surrogate endpoints, suggest that concomitant use of clopidogrel and a PPI reduces the antiplatelet effects of clopidogrel. The strongest evidence for an interaction is between omeprazole and clopidogrel. It is not established that changes in these surrogate endpoints translate into clinically meaningful differences. Observational studies and a single randomized clinical trial have shown inconsistent effects on (cardiovascular) outcomes of concomitant use of thienopyridines and PPIs. A clinically important interaction cannot be excluded, particularly in certain subgroups, such as poor metabolizers of clopidogrel. The roles of either pharmacogenomic testing or platelet function testing in managing therapy with thienopyridines and PPIs has not yet been established"[41].

NON-ACID DIRECTED PHARMACOLOGIC THERAPY

The role of *H. pylori* in PUD has been clearly elucidated over the years, beginning with Marshall and Warren's pioneering work. Since that time, many studies have demonstrated the beneficial effects of *H. pylori* eradication in the treatment of PUD. In a 1997 *Lancet* study, Chan *et al*[34] reported that clearance of *H. pylori* in patients taking naproxen for 8 wk, irrespective of PPI use, significantly reduced the presence of PUD at time of repeat endoscopy: 26% *vs* 7%. Current guidelines support the test and treatment mantra for *H. pylori* in the setting of UGIH[2]. However, the appropriate timing of *H. pylori* testing is unclear given the potential for false-negative test results in the setting of in the setting of UGIH and false negative results due to PPI use. In a 2001 study by Griñó *et al*[42], 78 patients with endoscopically documented UGIH secondary to PUD underwent diagnostic testing for *H. pylori* in the acute setting by one of four modalities: histology, rapid urease test, urea breath test and serology. The sensitivity/specificity for each respective diagnostic test was as follows: 48.5/100 for the rapid urease test, 91/77.8 for the breath test, 89.5/80 for serology and 86.3/100 for histology. Additional support for questioning immediate *H. Pylori* test results was provided by Guell *et al*[43], who have reported a 79% false-negative rate with rapid urease testing and a maximum sensitivity of only 86% if both a rapid urease test and histological examination were performed on each biopsy specimen. Based on these data, a recommendation to retest at a later date all patients with negative immediate *H. pylori* test in the setting of acute UGIH may be reasonable[42-44].

CONCLUSION

UGIH requires early and accurate assessment, triage and resuscitation, in addition to well-coordinated care between generalist and sub-specialist to maximize patient outcomes. All patients with UGIH need upper endoscopy within 24 h of presentation to characterize further and potentially treat the bleeding source. Epinephrine plus a second modality of endoscopic therapy has proven superior to epinephrine alone. *H. Pylori* infection should be confirmed and treated when present. Negative *H. pylori* test in the acute setting should be followed by repeat testing to avoid false-negative results and minimize the risk of recurrent bleeding. Appropriate duration of PPI therapy is of critical importance to allow mucosal healing and to prevent rebleeding in the high-risk patient. Current data do not support a meaningful clinical interaction between PPIs and thienopyridines. Future clinical and research attention to the issues addressed in this review may serve to resolve current areas of uncertainty and controversy, and optimize clinical outcomes for patients presenting with UGIH.

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TOPIC HIGHLIGHT

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Treatment of portal hypertension

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Abstract

Portal hypertension is the main complication of cirrhosis and is defined as an hepatic venous pressure gradient (HVPG) of more than 5 mmHg. Clinically significant portal hypertension is defined as HVPG of 10 mmHg or more. Development of gastroesophageal varices and variceal hemorrhage are the most direct consequence of portal hypertension. Over the last decades significant advancements in the field have led to standard treatment options. These clinical recommendations have evolved mostly as a result of randomized controlled trials and consensus conferences among experts where existing evidence has been reviewed and future goals for research and practice guidelines have been proposed. Management of varices/variceal hemorrhage is based on the clinical stage of portal hypertension. No specific treatment has shown to prevent the formation of varices. Prevention of first variceal hemorrhage depends on the size/characteristics of varices. In patients with small varices and high risk of bleeding, non-selective β-blockers are recommended, while patients with medium/large varices can be treated with either β-blockers or esophageal band ligation. Standard of care for acute variceal hemorrhage consists of vasoactive drugs, endoscopic band ligation and antibiotics prophylaxis. Transjugular intrahepatic portosystemic shunt (TIPS) is reserved for those who fail standard of care or for patients who are likely to fail ("early TIPS"). Prevention of recurrent variceal hemorrhage consists of the combination of β-blockers and endoscopic band ligation.

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Key words: Cirrhosis; Portal hypertension; Varices; Variceal hemorrhage; Primary prophylaxis; Secondary prophylaxis

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INTRODUCTION

Portal hypertension is the increase in porto-systemic pressure gradient in any portion of the portal venous system. Although portal hypertension could result from pre-hepatic abnormalities (e.g., portal or splenic vein thrombosis), post-hepatic abnormalities (e.g., Budd-Chiari syndrome) or intrahepatic non-cirrhotic causes (e.g., schistosomiasis, sinusoidal obstruction syndrome), cirrhosis is by far the most common cause of portal hypertension and, as such, has been the most widely investigated. In cirrhosis, the portosystemic gradient is assessed by measuring the wedged hepatic venous pressure (a measure of sinusoidal hepatic pressure) and subtracting the free hepatic venous pressure (systemic pressure) thus obtaining the hepatic

venous pressure gradient (HVPG). A normal HVPG is 3-5 mmHg. An HVPG above 5 mmHg defines portal hypertension, however an HVPG of 10 mmHg or greater defines clinically significant portal hypertension as this pressure gradient predicts clinical course in patients with cirrhosis including development of varices[1], clinical decompensation (i.e., development of ascites, variceal hemorrhage and encephalopathy)[2], decompensation or death after liver resection[3], and hepatocellular carcinoma[4].

The complications that most directly result from portal hypertension are the development of varices and variceal hemorrhage. This review summarizes the current standard management for varices and variceal hemorrhage in the context of cirrhotic portal hypertension.

Over the last decades, research on animal models and clinical trials have evolved and have led to our current management recommendations. The field has moved forward in large part through consensus conference among experts where events and endpoints have been defined and the existing evidence has been carefully reviewed leading to practice recommendations. The first such conference took place in 1986 in Groningen, the Netherlands and since then consensus conferences have been alternating between Europe (Baveno conference) and the United States [American association for the study of liver diseases (AASLD) or AASLD single topic conference (STC)], and are briefly summarized below (Table 1).

HISTORY OF CONSENSUS CONFERENCES ON PORTAL HYPERTENSION

Baveno is a small town in Northern Italy located on the west shore of Lake Maggiore. It has become the epicenter of the portal hypertension consensus workshops aimed to reach a consensus on the definitions of key events related to portal hypertension and variceal bleeding and to provide guidelines for future research as well as reviewing the evidence, eventually leading to clinical practice guidelines. The first Baveno consensus workshop was held in April 1990[5] in which significant advances in diagnosis and management of varices and variceal bleeding including vasoactive drugs and endoscopic sclerotherapy were reviewed. In addition to defining certain terms including size of varices, clinically significant bleeding and rebleeding; this workshop also provided recommendations on diagnostic modalities, imaging and directions for future clinical trials. The therapeutic recommendations included β-blockers for primary prophylaxis of large varices, sclerotherapy and vasoactive drugs for acute hemorrhage and endoscopic sclerotherapy, β-blockers or surgical shunt to prevent recurrent hemorrhage.

The Baveno Ⅱ workshop was held in April 1995[6]. Definitions of key clinical events were revised and new definitions were proposed. Based on multiple randomized controlled trials, non-selective β-blockers (NSBB) were recommended to be the treatment of choice for primary prophylaxis of variceal hemorrhage, while isosorbide-5 mononitrate (ISMN) was recommended in patients who did not tolerate β-blockers. Endoscopic sclerotherapy was not recommended in the prevention of first hemorrhage. Treatment of acute hemorrhage was mainly based on endoscopic therapy, terlipressin was deemed the most effective of the vasoactive agents, with somatostatin showing some efficacy. The transjugular intrahepatic portosystemic shunt (TIPS) was recommended in case of treatment failure of endoscopic and pharmacologic therapy. The recommendations to prevent recurrent hemorrhage included β-blockers or endoscopic variceal ligation (EVL) that had been shown to be better than sclerotherapy[7]. TIPS and surgical shunts were to be used only for patients with frequent repeated episodes of variceal hemorrhage.

In June 1996, the AASLD STC took place in Reston, Virginia, United States, with the objective of identifying important areas in the treatment of variceal hemorrhage and future research[8]. Guidelines for initial variceal screening and follow-up endoscopy were described in detail depending on severity of liver disease and the size of varices on first endoscopy. Areas of further research were identified as the role of sequential portal pressure measurements and their timing, and defining new predictors of first hemorrhage. Primary prophylaxis recommendations were the same as in the Baveno Ⅱ conference, with β-blockers as the mainstay of treatment and EVL requiring further studies. Vasoactive drugs in combination with endoscopic treatment (sclerotherapy or EVL) became the established treatment for acute hemorrhage, recognizing the advantage of initiating vasoactive therapy prior to diagnostic endoscopy[9]. For secondary prophylaxis EVL or β-blockers were recommended. TIPS or surgical shunts were considered acceptable therapies for failure to control acute hemorrhage or recurrent hemorrhage despite standard treatments.

The Baveno Ⅲ conference was held in April 2000[10], and introduced the concept of clinically significant portal hypertension (CSPH) which was defined as HVPG of 10 mmHg or more. The presence of varices, variceal hemorrhage or ascites is indicative of the presence of CSPH. Non-selective β-blockers remained the treatment of choice to prevent first hemorrhage from large/medium varices, while EVL required further assessment. The goals of therapy with β-blockers were defined (25% reduction in baseline heart rate or a heart rate of 55 beats/min). ISMN, previously recommended as an alternative to β-blockers, was no longer recommended[11]. For treatment of acute hemorrhage, the early administration of vasoactive drugs and continued use for up to 5 d along with endoscopic therapy (EVL or sclerotherapy) were considered standard. Additional measures included use of antibiotics to prevent bacterial infection[12], and lactulose to treat hepatic encephalopathy. With regard to prevention of rebleeding, β-blockers were considered first-line therapy[13] as was EVL, with TIPS reserved for treatment failures. The complications of treatment of portal hypertension were also defined for use in clinical settings and in research trials.

The Baveno Ⅳ conference was held in April 2005[14], and some of the key criteria (failure to control bleeding,

Table 1 Portal hypertension consensus conferences in the last two decades

Title	Year	Venue
21st meeting of the European association for the study of liver	1986	Groningen, The Netherlands
Definitions, methodology and therapeutic strategies in portal hypertension. A consensus development workshop	1990	Baveno, Italy
Developing consensus in portal hypertension	1995	Baveno, Italy
Portal hypertension and variceal bleeding. AASLD single topic symposium	1996	Virginia, United States
Updating consensus in portal hypertension. Reports of the Baveno Ⅲ consensus workshop on definitions, methodology and therapeutic strategies in portal hypertension	2000	Baveno, Italy
Evolving consensus in portal hypertension. Report of the Baveno Ⅳ consensus workshop on methodology of diagnosis and therapy in portal hypertension	2005	Baveno, Italy
Portal hypertension and variceal bleeding-unresolved issues. Summary of an AASLD and European association for the study of the liver single-topic conference	2007	Atlanta, United States
Revising consensus in portal hypertension: Report of the Baveno V consensus workshop on methodology of diagnosis and therapy in portal hypertension	2010	Baveno, Italy

AASLD: American association for the study of liver diseases.

failure of secondary prophylaxis) were revised. For primary prophylaxis, β-blockers remained the treatment of choice but endoscopic band ligation emerged as an excellent alternative for patients with medium or large varices, and contraindications or intolerance to β-blockers[15,16]. Isosorbide mononitrate as a single agent therapy was not recommended even in a combination of pharmacological therapies[17]. Primary prophylaxis of small varices could only be considered if they were high risk (red wale sign or Child C)[18]. There was no significant change in the recommendations of acute variceal hemorrhage from Baveno Ⅲ. Small changes included the use of vasoactive drugs for at least 5 d, and the use of balloon tamponade only in massive bleeding as a temporary bridge until definitive treatment could be instituted. EVL was declared superior to sclerotherapy and as the endoscopic procedure of choice in the control of acute hemorrhage[16,19]. Secondary prophylaxis should be initiated 6 d after the index variceal bleed, and included the combination of EVL and β-blockers[20,21]. TIPS or surgical shunts were reserved for patients with failure of secondary prophylaxis.

The second AASLD STC was held in 2007 in Atlanta, Georgia[22]. The objective of this conference was to make clinical recommendations in areas that did not require further investigation and to identify research directions for the remaining areas. Compensated and decompensated cirrhosis were identified as separate entities to be studied separately both in clinical practice and in research[23]. The main differences compared with Baveno Ⅳ included the emergence of capsule endoscopy as a non-invasive alternative to esophagogastroduodenoscopy (EGD) for assessment of varices, a firm recommendation regarding use of β-blockers for primary prophylaxis of small varices with high-risk features, and consideration of β-blockers for small varices and no high-risk features[24]. EVL was considered as effective and safe as β-blockers for primary prophylaxis of medium to large sized varices. Early TIPS emerged as an option in patients at high risk of rebleeding[25], but required further investigation.

The Baveno V conference in May 2010 revised the definitions of failure to control variceal bleeding, and failure of secondary prophylaxis[26]. Primary prophylaxis for small varices was the same as recommended in the 2007 AASLD STC. There was no significant change in the recommendations for primary prophylaxis of medium to large varices (β-blockers or EVL) with the choice of therapy dictated by local resources, expertise and patient preference[27]. The recommendations on the treatment of acute variceal bleeding were unchanged except that a stronger recommendation was made to consider early TIPS (within 72 h) in patients with high risk of treatment failure[28]. Recommendations for the prevention of recurrent hemorrhage, as in the AASLD STC, consisted of the combination of β-blockers and EVL.

Evidence-based guidelines endorsed by the AASLD[29] and the American College of Gastroenterology[30] as well as a recent comprehensive review[31] on the treatment of portal hypertension have been heavily based on these consensus conferences. These guidelines and review form the bases of the current recommendations that are described in the following section in which the advantages (pros) and disadvantages (cons) of these therapies are also discussed.

CURRENT STANDARD TREATMENT OF PORTAL HYPERTENSION IN ADULTS

Therapy of varices and variceal hemorrhage in the adult patient with cirrhosis needs to be stratified depending on the different clinical stages in the natural history of portal hypertension: (1) the patient with cirrhosis and portal hypertension who has not yet developed varices and in whom the goal is to prevent the formation of varices (pre-primary prophylaxis); (2) the patient with gastroesophageal varices who has never had bleeding from them, and in whom the goal is to prevent their rupture (primary prophylaxis); (3) the patient with acute variceal hemorrhage in whom the goal is to stop the hemorrhage and prevent its early recurrence; and (4) the patient who has survived an episode of acute variceal hemorrhage, in whom the goal of therapy is to prevent late recurrence of hemorrhage (secondary prophylaxis).

Prevention of formation of varices (pre-primary prophylaxis)

Every patient with a new diagnosis of cirrhosis should have an EGD to look for the presence and size of varices. In patients who do not have gastroesophageal varices, a large multicenter, randomized, controlled trial showed no differences between placebo and β-blockers in the prevention of varices[1]. Therefore, no specific treatment for portal hypertension is recommended in this setting. The main focus at this stage is to treat the underlying cause of cirrhosis which will reduce portal hypertension and may therefore prevent the development of clinical complications.

Prevention of first variceal hemorrhage (primary prophylaxis)

First variceal hemorrhage occurs at an annual rate of about 15% and although current mortality from an episode of variceal hemorrhage is lower than in the past two decades, it still carries a significant mortality of 7%-15%[32-34], and is still associated with significant morbidity and healthcare costs. Prevention of first hemorrhage, therefore, is an important part of treatment of portal hypertension. The size of varices, red wale signs on varices (visualized on EGD), and severity of liver disease (Child class C) identify the patients with highest risk of variceal hemorrhage[18]. Therefore, within this stage, patients need to be stratified by the risk of hemorrhage into (1) high-risk patients, i.e. ,those with medium/large varices or those with small varices that have red wale signs, or a Child C patient; and (2) low risk patients, i.e., those with small varices without red wale signs or occurring in a Child A or B patient.

In patients with medium/large varices, quality trials have shown that non-selective β-blockers (propranolol, nadolol) are as effective as EVL in preventing first variceal hemorrhage[35,36], and the recommendation is to use therapy based on local resources, expertise and patient preference.

In patients with high-risk small varices the mainstay of treatment is NSBB because technically performing EVL in these varices may be challenging (although there is no clear evidence for this).

In patients with low-risk small varices, there is limited evidence that shows that their growth may be slowed by the use of NSBB[24]. Therefore, the use of NSBB in this setting is considered optional and should be discussed with the patient.

The doses are shown in Table 2, with therapeutic goals and follow-up procedures for each of the recommended therapies.

Pros

NSBB decrease portal pressure through a reduction in portal blood flow. Their mechanism of action involves decreasing cardiac output *via* β-1 receptors and causing splanchnic vasoconstriction by blocking β-2 receptors, resulting in unopposed α-1 activity. The latter is the most important effect and therefore it is essential that NSBB (as opposed to selective β-blockers) be used. Advantages of NSBB include low cost, ease of administration and no requirement for specific expertise. As they act by decreasing portal pressure, NSBB may also reduce other complications of cirrhosis such as bleeding from portal gastropathy, ascites and spontaneous bacterial peritonitis[37,38]. In fact, a significant reduction in portal pressure has been related to an improvement in survival[38,39]. Additionally, once the patient is on NSBB there is no need for repeat EGD.

EVL has the advantage that the procedure can be done at the same time as screening endoscopy, although in some centers a screening EGD time slot will not allow for the performance of EVL, and a separate therapeutic EGD time slot is required. Also, there are relatively few contraindications to EVL and it has been associated with a lower incidence of side-effects compared with NSBB[15].

Cons

The main inconvenience of NSBB is that approximately 15% of patients may have absolute or relative contraindications to therapy, and that another 15% require dose-reduction or discontinuation due to its common side-effects (e.g., fatigue, weakness, shortness of breath) that resolve upon discontinuation but discourage patients from using these drugs[27].

EVL requires specific expertise. The risks include that of the endoscopic procedure and conscious sedation (bleeding, aspiration, perforation and reaction to medications), plus the risk of bleeding from ligation-induced ulcers. In fact, although the quantity of side-effects is greater with NSBB than with EVL[15], the severity of side-effects is greater with EVL. While no lethal side-effects have been reported with the use of NSBB[16], three deaths resulting from EVL-induced bleeding ulcers have been reported[15,16].

Recommendation

The issue of which is the best treatment for primary prophylaxis (NSBB or EVL) has not yet been settled, and there are centers that perform predominantly EVL while others prefer the more rational approach of starting with NSBB and switching to EVL if there is intolerance to NSBB. Carvedilol is a NSBB with an added vasodilatory effect through anti-α-1 adrenergic activity that has recently been shown to be more effective than EVL in preventing first variceal hemorrhage[40]. Although considered a promising alternative, further research is necessary before it can be widely recommended.

MANAGEMENT OF ACUTE VARICEAL HEMORRHAGE

Acute variceal hemorrhage is a medical emergency requiring intensive care. The basic medical principles of airway, breathing and circulation are followed to achieve hemodynamic stability. Blood transfusion is done conservative-

Table 2 Primary prophylaxis and secondary prophylaxis of variceal hemorrhage

Therapy	Starting dose	Therapy goals	Maintenance/follow-up
Propranolol	(1) 20 mg orally twice a day; (2) Adjust every 2-3 d until treatment goal is achieved; (3) Maximal daily dose should not exceed 320 mg	(1) Maximum tolerated dose; (2) Aim for resting heart rate of 50-55 beats per minute	(1) At every outpatient visit make sure that patientis appropriately β-blocked; (2) Continue indefinitely; (3) No need for follow-up EGD
Nadolol	(1) 40 mg orally once a day; (2) Adjust every 2-3 d until treatment goal is achieved; (3) Maximal daily dose should not exceed 160 mg	As for propranolol	As for propranolol
EVL	Every 2-4 wk until the obliteration of varices	Obliteration of varices; Eradication of new varices following initial obliteration	First EGD performed 1-3 mo after obliteration and every 6-12 mo thereafter
Propranolol	(1) 20 mg orally twice a day; (2) Adjust every 2-3 d until treatment goal is achieved; (3) Maximal daily dose should not exceed 320 mg	(1) Maximum tolerated dose; (2) Aim for resting heart rate of 50-55 beats per minute	(1) At every outpatient visit make sure that patient is appropriately β-blocked; (2) Continue indefinitely
Nadolol	(1) 40 mg orally once a day; (2) Adjust every 2-3 d until treatment goal is achieved; (3) Maximal daily dose should not exceed 160 mg	As for propranolol	As for propranolol
ISMN	(1) Only to be used in conjunction with propranolol or nadolol; (2) 10 mg orally at night every day; (3) Adjust every 2-3 d by adding 10 mg in am and then pm; (4) Maximal dose is 20 mg twice a day	(1) Maximal tolerated dose; (2) Systolic blood pressure remains over 95 mmHg	Continue indefinitely
EVL	Every 2-4 wk until the obliteration of varices	Obliteration of varices; Eradication of new varices following initial obliteration	First EGD performed 1-3 mo after obliteration and every 6-12 mo thereafter

Either one of the three therapies shown in the table are recommended. EGD: Esophagogastroduodenoscopy; EVL: Endoscopic variceal ligation; ISMN: Isosorbide-5-mononitrate.

ly for a target hemoglobin level between 7-8 g/dL[41], because excessive blood volume restitution can increase portal pressure[42,43]. There are no definite recommendations on management of coagulopathy and thrombocytopenia, as randomized controlled trials of recombinant factor VIIa have not shown any advantages[44,45]. Antibiotic prophylaxis is provided by quinolones with consideration of iv ceftriaxone in patients with advanced cirrhosis or previous therapy with quinolones[12,46]. Safe vasoactive drugs are started as soon as possible, prior to diagnostic endoscopy. Endoscopy is done as soon as possible and not more than 12 h after presentation. If a variceal source is confirmed, EVL is the procedure of choice, but sclerotherapy is an option when EVL is technically difficult. TIPS is recommended in patients who fail standard combination therapy with endoscopic and pharmacological therapy, however salvage TIPS is accompanied by a very high mortality. Predictors of failure are Child class C, HVPG > 20 mmHg and active bleeding at endoscopy[47]. The use of early (pre-emptive) TIPS (within about 48 h of admission) in patients at high risk of failing standard therapy has been shown to reduce mortality[28]. These patients are specifically those who are Child C (score of 10-13 points) or are Child B with active hemorrhage (at the time of diagnostic endoscopy), and constitute < 20% of the patients admitted for variceal hemorrhage. In these patients it is recommended to consider early preemptive TIPS. All others should continue standard therapy with vasoactive drugs continued for 2-5 d depending on control of bleeding and severity of liver disease. Vasoactive drugs can be discontinued once the patient has been free of bleeding for at least 24 h. Balloon tamponade is only used as a temporary measure (inflated for 12 h or less) to control bleeding while a definitive therapy (TIPS or endoscopic therapy) is planned. A new self-expanding esophageal stent is being tested that may replace balloon tamponade[48].

Although there are pros and cons for each of these first-line therapies (pharmacological and endoscopic), the current recommendation is to use them jointly in the control of acute hemorrhage.

Pros

Vasoactive agents improve the control of variceal hemorrhage when combined with endoscopic therapy and when compared to endoscopic therapy alone[49]. However there appears to be no significant difference among the different vasoactive agents regarding control of hemorrhage and early rebleeding. Vasopressin, a powerful vasoconstrictor, is associated with more adverse events[50], and should not be considered a first-line vasoactive drug. Terlipressin is the only agent that, in small studies and when compared to no treatment, improved survival[50]. In practice, the choice of pharmacological agent is usually based on availability and cost. Octreotide, a somatostatin analogue, is the only safe vasoactive drug available in the United States. Doses and schedules for the different vasoconstrictors are shown in Table 3. Except for vasopressin that must be administered with nitroglycerin, the administration of these agents does not require any special procedure or expertise and can be started in the emergency room setting.

Endoscopic therapy in the acute setting is very effective in controlling variceal hemorrhage, particularly when

Table 3 Vasoactive agents used in the management of acute hemorrhage

Drug	Standard dosing	Duration	Mechanism of action
Somatostatin	Initial iv bolus 250 μg (can be repeated in the first hour if ongoing bleeding); continuous iv infusion of 250 to 500 μg/h	Up to 5 d	Inhibits vasodilator hormones like glucagon causing splanchnic vasoconstriction and reduced portal blood flow
Octreotide (somatostatin analogue)	Initial iv bolus of 50 μg (can be repeated in first hour if ongoing bleeding); continuous iv infusion of 50 μg/h	Up to 5 d	Same as somatostatin, longer duration of action
Vapreotide (somatostatin analogue)	Bolus: 50 μg; continuous iv infusion of 50 μg/h	Up to 5 d	Similar to somatostatin with higher metabolic stability
Vasopressin + nitroglycerine	0.2-0.4 units/min continuous iv infusion intravenously, may titrate to a maximum of 0.8 units/min; always use in combination with nitroglycerine	Maximum of 24 h at lowest effective dose	Causes direct vasoconstriction on splanchnic circulation resulting in decreased portal blood flow
Terlipressin (vasopressin analogue)	Initial 48 h: 2 mg iv every 4 h until control of bleeding; maintenance: 1 mg iv every 4 h to prevent re-bleeding	Up to 5 d	Splanchnic vasoconstriction; the active metabolite lysine-vasopressin is gradually released over several hours thus decreasing typical vasopressin side effects

a spurting varix is observed. However, in a meta-analysis comparing sclerotherapy *vs* vasoactive drugs, no differences in efficacy were observed between treatments, with more side-effects with sclerotherapy[51]. EVL has replaced sclerotherapy as the endoscopic procedure of choice due to more effective control of bleeding, obliteration of varices in fewer treatment sessions, a lower rebleeding rate, and lower mortality[19,33]. How EVL compares with vasoactive drugs alone remains to be determined. There is no added benefit of a combination of EVL and sclerotherapy over band ligation alone.

Cons

Vasoactive drugs often require placement of central lines and require close monitoring for ischemic complications. Vasopressin is the most potent vasoconstrictor, but its use is limited by multiple side-effects related to splanchnic vasoconstriction (e.g., bowel ischemia) and systemic vasoconstriction (e.g., hypertension, myocardial ischemia). Terlipressin is an analogue of vasopressin that, although safer, is still accompanied by more side-effects than somatostatin[52]. The main side effects of the somatostatin analogs octreotide and vapreotide are sinus bradycardia, hypertension, arrhythmia, and abdominal pain.

Endoscopic therapy during acute hemorrhage carries the usual risks of endoscopic procedures, with increased risk of aspiration due to active bleeding and the emergency nature of the procedure. In the setting of active hemorrhage, the band ligator limits the visibility, and it becomes technically difficult to maneuver the endoscope back into the stomach. Elastic bands can slip or can cause ulcers that can result in rebleeding. As mentioned previously, EVL has less side-effects than sclerotherapy and is the endoscopic therapy of choice.

Recommendation

The specific treatment of choice for acute variceal hemorrhage is the combination of vasoactive drugs (started prior to EGD) and emergency endoscopic therapy (at the time of initial diagnostic EGD). The pharmacological therapy of choice is terlipressin (lower mortality in small placebo-controlled studies) or somatostatin (fewer side-effects), however the choice is dependent on availability and cost. Octreotide is the only vasoactive drug available in the United States. The endoscopic therapy of choice is EVL.

Recommendations may vary depending on the severity of liver disease. In patients who are Child C (or Child B with active hemorrhage), the risk of failing recommended treatment (vasoactive drugs and EVL) is high and therefore proceeding to a "rescue" therapy (i.e., TIPS) before failure occurs should be considered. In patients who are Child A, mortality with the treatment of choice is essentially nil[32,34], and these patients may respond to vasoactive therapy alone, although this requires further exploration.

PREVENTION OF RECURRENT VARICEAL HEMORRHAGE (SECONDARY PROPHYLAXIS)

The risk of rebleeding in patients who survive an episode of variceal hemorrhage is high (median rebleeding rate 60%), with a mortality of up to 33%. Prevention of rebleeding is therefore an essential part of the management of the patient with variceal hemorrhage. Patients who had a TIPS performed during the acute episode do not require specific therapy for portal hypertension or for varices but should be referred for transplant evaluation. TIPS patency should be checked through Doppler ultrasound every 6 mo. For the majority (patients who do not have a TIPS performed during the acute episode), secondary prophylaxis with NSBB should be started as soon as the intravenous vasoactive drug is discontinued. NSBB significantly reduce the risk of recurrent hemorrhage[13]. Although the addition of ISMN to NSBB has a greater portal pressure-reducing effect[53], in clinical trials the combination of NSBB and ISMN is no different from NSBB alone regarding the rate of overall rebleeding or mortality, but has a higher rate of side-effects[54]. Sclerotherapy decreases rebleeding rates and mortality, but is associated with serious complications (e.g., esophageal strictures, bleeding from ulcers). Sclerotherapy has been replaced by EVL, since it has significantly better outcomes

(rebleeding, mortality and side-effects) compared with sclerotherapy. Studies comparing pharmacological therapy (NSBB plus ISMN) *vs* EVL show no differences in recurrent hemorrhage, but there is a suggestion of a beneficial effect on survival with pharmacological therapy in the long term[54,55]. The combination of pharmacological (NSBB alone or NSBB + ISMN) plus EVL is associated with lower rebleeding rates than either therapy alone[31,56], and constitutes the treatment of choice.

In patients who experience recurrent variceal hemorrhage despite the combination of pharmacologic and endoscopic treatment, TIPS with polytetrafluoroethylene-covered stents[57] or, where expertise is available, surgical shunts[58] should be provided.

Table 2 presents the doses, therapeutic goals and follow-up procedures for the recommended therapies. The pros and cons of each of these first-line therapies (pharmacological and endoscopic) are the same as those described for primary prophylaxis, with some additional considerations described below.

Pros

Pharmacologic agents provide protection against rebleeding during the initial phase after index hemorrhage while esophageal varices are being obliterated by EVL. NSBB alone or in combination with ISMN should be used. The choice will depend on patient tolerability. Patients who are not candidates for EVL should receive combination NSBB + ISMN.

The lowest rates of recurrent variceal hemorrhage (approximately 10%) are observed in individuals who have a hemodynamic response to pharmacologic therapy, defined as a decrease in HVPG to < 12 mmHg or a decrease of > 20% from baseline levels[39,59]. The more rational approach would thus be to guide therapy based on hemodynamic response and, in those who achieve a hemodynamic response, endoscopic therapy would not be necessary. However there are cons (see below) to this approach.

Patients who are intolerant or have contraindications to pharmacological therapy should receive EVL alone.

Cons

A recent study suggests that NSBB are associated with a poorer survival in patients with refractory ascites[60], a condition that may be present in patients in this clinical stage. However, the study is retrospective and the groups were disparate at baseline, with patients on NSBB having more advanced disease as shown by a higher prevalence of varices and variceal hemorrhage, and there is evidence that indicates the contrary, that is, that NSBB may be beneficial for these patients[13,61]. Therefore, unless stronger evidence arises, the use of NSBB in patients with refractory ascites should not be contraindicated.

The combination of NSBB + ISMN has a higher incidence of side effects because of the added ones associated with ISMN, specifically headache and lightheadedness. As mentioned above, the lowest rebleeding rates are in patients who experience a hemodynamic response. Although HVPG-guided therapy would appear rational, a small trial showed that outcomes with HVPG-guided therapy are no different from those in patients treated with combined pharmacological and endoscopic therapy[62]. Until the best treatment for non-responders is settled, larger clinical trials are performed, and HVPG measurements are standardized across centers, HVPG-guided therapy cannot be currently recommended[63].

As mentioned above, EVL is associated with bleeding from EVL-induced ulcers. Treatment with proton pump inhibitors post ligation reduces the size of these ulcers, with a trend towards a lower risk of bleeding[64], and can be considered in this setting.

Recommendation

The treatment of choice to prevent rebleeding is the combination of pharmacological therapy (NSBB ± ISMN) and EVL. Contrary to other clinical stages, risk stratification has not been tested in this setting. The main predictor of recurrent bleeding and death is the Child classification. It is conceivable that patients who are Child A would only require one or other therapy, while patients who have more advanced disease require the combination therapy. Patients who fail this therapy should be considered for TIPS placement and, in centers where expertise is available, for a surgical shunt. Patients with recurrent variceal hemorrhage are in a category of "further decompensation" of cirrhosis and, as such, should be evaluated for liver transplantation.

CURRENT STANDARD TREATMENT OF PORTAL HYPERTENSION IN CHILDREN

The most common causes of portal hypertension in children are biliary atresia and portal vein thrombosis. Data regarding the prevalence of esophageal varices in children with portal hypertension is very limited and to date there have been no randomized controlled trials comparing different treatments for primary and secondary prophylaxis[65].

Regarding primary prophylaxis, there is currently no recommended treatment[22,66]. In a recent gathering of experts at the AASLD annual meeting, it was concluded that, before a randomized trial could be performed in children, pediatric research should focus on addressing questions of the natural history and diagnosis of varices, prediction of variceal bleeding, optimal approaches to β-blocker and ligation therapy, and alternative study designs to explore therapeutic efficacy in children[65].

Regarding acute variceal hemorrhage, management in children is based on limited data comparing EVL and sclerotherapy[67], and expert pediatric opinion based on adult Baveno Ⅳ guidelines[66]. These include vasoactive agents, antibiotic prophylaxis and endoscopic variceal ligation.

EVL is also recommended for secondary prophylaxis of variceal hemorrhage but it has not been compared with β-blockers[22,66]. In children with portal vein throm-

bosis, meso-rex bypass appears to be the best option for secondary prophylaxis[22,66].

CONCLUSION

In the last two decades significant advances in the field of portal hypertension have improved the clinical care and survival of patients with cirrhosis and portal hypertension. In addition to better treatment strategies and improved therapeutic options, the issue of risk stratification has become more important so that, within each clinical stage, different patient subpopulations have been identified that require a different management. Clearly, further research is necessary to explore new pharmacological options that would allow a majority of patients to be hemodynamic responders, thereby foregoing the need for HVPG measurements and even the need for endoscopic therapy. The identification of different risk populations within each stage also requires further definition. It is expected that future trials and Baveno and AASLD conferences will continue to advance the field.

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TOPIC HIGHLIGHT

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Management of portal hypertension in children

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Abstract

Portal hypertension can be caused by a wide variety of conditions. It frequently presents with bleeding from esophageal varices. The approach to acute variceal hemorrhage in children is a stepwise progression from least invasive to most invasive. Management of acute variceal bleeding is straightforward. But data on primary prophylaxis and long term management prevention of recurrent variceal bleeding in children is scarce, therefore prospective multicenter trials are needed to establish best practices.

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Key words: Portal hypertension; Variceal hemorrhage; Children

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INTRODUCTION

Normal portal pressure is between 5 and 10 mmHg. Once portal pressure rises to 12 mmHg or greater, complications such as varices and ascites may occur.

The portal system drains the capillaries of the mesenteric and splenic veins and ends in the hepatic capillaries. The portal vein supplies partially oxygenated blood to the liver, supplementing the highly oxygenated blood of the hepatic artery to the liver. Blood flow to the liver is finely tuned; any disturbance of the flow in one of these vessels can be offset to a certain degree by increased flow through the other vessel. This is known as the arterial buffer response. Blood from both the portal venous system and the hepatic arterial systems combine within the sinusoids.

Portal hypertension occurs when there is increased portal resistance and/or increased portal blood flow. Generally, the portal venous system has a low baseline portal pressure of 7-10 mmHg and the hepatic venous pressure gradient (HVPG) ranges from 1 to 4 mmHg. Portal hypertension is defined as a portal pressure greater than 10 mmHg or gradient greater than 4 mmHg. Pressure gradients above 10 mmHg have been associated with esophageal varices formation, and those above 12 mmHg are associated with ascites and variceal bleeding in adults[1]. To measure the portal pressure gradient, a catheter can be wedged into the hepatic vein *via* the femoral or transjugular approach and a wedged hepatic venous pressure (WHVP) measurement obtained. If the catheter is then retracted into a free flowing hepatic vein, a free hepatic venous pressure (FHVP) can be measured. The HVPG is the difference between the WHVP and the FHVP. The cause of portal hypertension can be suggested by the

HVPG value. In pre-sinusoidal obstruction, the HVPG is normal but the WHVP is raised, whereas in cirrhosis both HVPG and WHVP are increased.

HEMODYNAMIC CHANGES

Clinically, portal hypertension causes splenomegaly with resulting hypersplenism and the formation of a collateral circulation. Despite formation of a significant collateral network, portal hypertension persists. This is a result of an increase in cardiac output (result from increased venous return and diminished afterload); and a decrease in splanchnic arteriolar tone (mediated by several factors including glucagon and nitric oxide). Retention of sodium and water *via* a hepato-renal reflex increases the circulating blood volume. There is also production of vasodilatory factors that cause arterial vasodilation of the splanchnic circulation. Increases in the intrahepatic resistance are due to hepatocyte swelling, fibrosis and inflammation within the portal tracts. Clinical studies and animal models have demonstrated the hemodynamic events that occur; however, most of these investigations have not been performed in children or in pediatric models. The hyperdynamic circulatory state has not been well characterized in any cohorts of children.

GASTROINTESTINAL BLEEDING

The clinical presentation of portal hypertension can be dramatic because it may be the first symptom of long-standing silent liver disease. In several large series of children with portal hypertension, approximately two thirds presented with hematemesis or melena, usually from rupture of an esophageal varix[2]. Gastrointestinal hemorrhage also may be associated with bleeding from portal hypertensive gastropathy, gastric antral vascular ectasia, or gastric, duodenal, peristomal, or rectal varices. Variceal hemorrhage is the result of increased pressure within the varix, which leads to changes in the diameter of the varix and increased wall tension. When the wall tension exceeds the variceal wall strength, physical rupture of the varix occurs. The majority of patients reported in the series had splenomegaly at the time of hemorrhage; thus, the combination of gastrointestinal bleeding and splenomegaly suggests portal hypertension until proven otherwise. The sentinel bleeding episode in children may occur in a wide range of ages, starting as early as 2 mo of age[3]. The risk of first-time bleeding from studies in children with cirrhosis is 22%, but rises to 38% in children with known varices over a 5 year period[4]. Bleeding occurs in 15%-25% of patients with biliary atresia in long term follow up[5,6]. The age of bleeding is dependent on the underlying etiology of cirrhosis. Patients who have surgically corrected but progressive biliary atresia bleed for the first time at a mean age of 3 years while children with cirrhosis due to cystic fibrosis bleed at a mean age of 11.5 years[7].

Variceal bleeding in children often follows an acute upper respiratory infection, fever, or aspirin ingestion[8]. The combination of factors including increased abdominal pressure from coughing or sneezing, increased cardiac output from fever, and ulceration from medications such as nonsteroidal antiinflammatory drugs or aspirin contribute to the rupture of varices. Prolonged gastro-esophageal reflux can contribute to erosions over the varices that could result in bleeding.

Triger *et al*[9] followed 44 children aged 12 years for a mean follow-up of 8 years. At the time of portal venous obstruction diagnosis, no child had either abnormal liver enzymes or abnormal liver function. The actuarial probability of bleeding was 49% at age 16 years and 76% at 24 years of age. If the child bled before 12 years of age, the probability of bleeding was higher than in those who had not bled before aged 12. Further, there was no evidence of variceal regression over time. Instead, progression of varices occurred in the majority of children suggesting that the previous hypothesis that variceal bleeding decreased in adolescence due to development of spontaneous porto-systemic collaterals was incorrect.

SPLENOMEGALY

Splenomegaly is the second most common finding in children with portal hypertension after gastrointestinal bleeding. In many instances, an enlarged spleen is first discovered on routine physical examination. Many children will admit to a vague fullness in the left upper quadrant for many years prior to the diagnosis. Occasionally, manifestations of hypersplenism including thrombocytopenia, leukopenia, petechiae, or ecchymoses will prompt evaluation, leading to the discovery of portal hypertension. Hematologists should consider a biochemical liver profile and a Doppler ultrasonographic examination in the evaluation of any child with thrombocytopenia, especially if leukopenia is also present. Rarely will associated cytopenias lead to clinically relevant disease. Although splenomegaly is a common finding in patients with portal hypertension, splenic size does not correlate well with portal pressure[10,11]. Hypersplenism rarely requires surgical intervention. Exceptions include symptoms of symptomatic anemia and severe physical discomfort[12].

ABDOMINAL VENOUS PATTERNING

Specific cutaneous vascular patterns are observed with portal hypertension. Prominent vascular markings on the abdomen are the result of porto-collateral shunting through subcutaneous vessels. The direction of flow through these veins may be indicative of the site of obstruction. When the inferior vena cava is occluded, drainage is usually cephalad, but caudad below the umbilicus if the inferior vena cava is patent. Portal hypertension decompression through the umbilical vein results in prominent periumbilical collaterals, referred to as caput medusae. An audible venous hum (Cruveilhier-Baumgarten murmur) may occasionally be heard. Caput

Table 1 Initial manifestation of portal hypertension

Reference	Mitra *et al*[60] (1978)	Pinkerton *et al*[62] (1972)	Spence *et al*[33] (1984)	Howard *et al*[61] (1988)
Patients	70	33	27	152
% Presenting with				
Hemorrhage	80	97	85	46
Splenomegaly	99	24	100	94
Ascites	17	21	8	7

medusae are rare in children, partly because of the high prevalence of portal vein obstruction associated with umbilical vein obliteration. Rectal varices are more common in children[13]. In children with short bowel syndrome, stomal varices which are a site of low resistance, are often present and a common site for hemorrhage[14].

ASCITES

Ascites arises when the hydrostatic and osmotic pressures within the hepatic and mesenteric capillaries result in a net transfer from blood vessels to lymphatics at a rate that overcomes the drainage capacity of the lymphatics. It is the presenting sign of portal hypertension in 7%-21 % of children (Table 1).

In patients with portal hypertension, increased sodium retention and raised portal pressure may cause accumulation of fluid within the abdomen. Impaired lymphatic drainage compounds the situation. Treatment includes salt and fluid restriction and the use of diuretics. Albumin infusions can be used to increase intravascular osmotic pressure, followed by diuretic dosing to facilitate urination. Paracentesis has been used safely in children and is reserved for use when the ascites is difficult to control resulting in respiratory compromise or if peritonitis is suspected for cell count and culture[15,16].

PULMONARY COMPLICATIONS

Hepatopulmonary syndrome (HPS) and portopulmonary hypertension are undoubtedly underdiagnosed in children. Barbé *et al*[17] reported on the presence of HPS in 29 pediatric patients of which 26 had cirrhosis and 3 had an extrahepatic cause of portal hypertension. HPS progresses more rapidly in patients with biliary atresia associated with polysplenia[18]. Patients with HPS have a higher incidence of dyspnea, cyanosis, clubbing and spider nevi[19-21]. There are two forms of HPS. In type Ⅰ, the vessels enlarge such that the red blood cells traveling through the center of the vessel do not have significant contact time with the oxygen-rich alveoli. In type Ⅱ HPS, the diffusion-perfusion mismatch is presumed to be due to arteriovenous communications completely bypassing alveoli[22,23]. HPS is thought to occur as a result of shunting of vasodilatory mediators from the mesentery away from the liver in portal hypertension. Liver transplantation reverses HPS in greater than 80% of patients. If large shunts are present and the arterial partial pressure of oxygen is less than 50 mmHg on 100% oxygen, a poorer outcome may be expected.

Portopulmonary syndrome eventually leads to right-sided heart failure. Histologically there is pulmonary arteriopathy with concentric laminar intimal fibrosis consistent with a vasoconstrictive etiology. Pediatric cases have been reported[24,25]. The condition is defined by a pulmonary arterial pressure greater than 25 mmHg at rest and above 30 mmHg with exercise, raised pulmonary vascular resistance with pulmonary arterial occlusion pressure, or a left-ventricular end-diastolic pressure of less than 15 mmHg[26]. The most common symptom of pulmonary hypertension is exertional dyspnea. Other symptoms include fatigue, palpitations, and syncope or chest pains.

THERAPY

Therapy of portal hypertension is primarily directed at the management of its most dramatic manifestation, variceal hemorrhage. Variceal bleeding is a life threatening medical emergency, and patients with chronic liver disease should be instructed to seek immediate medical attention for any signs or symptoms of bleeding. The management can be divided into preprimary prophylaxis, prophylaxis (primary) of the first episode of bleeding, emergency therapy, and prophylaxis (secondary) of subsequent bleeding episodes. As with many other aspects of portal hypertension, almost all the modes of therapy are based on adult trials (Figure 1). Many of the trials are well-controlled randomized double-blinded studies, and comprehensive meta-analysis of these trials have been performed[27-30]. The literature on the management of variceal hemorrhage in children is predominantly descriptive and anecdotal. There have been few randomized trials of therapy for portal hypertension in children[31,32].

Preprimary prophylaxis

The concept is that early treatment of portal hypertension has the potential to delay or prevent the development of esophageal varices or other manifestations of portal hypertension. In a *S. mansoni* mouse model of portal hypertension, the administration of propranolol 5 wk into the infection resulted in a significant reduction in the development of portal hypertension, portosystemic shunting, and portal venous inflow[34]. A randomized controlled trial of timolol, a non-selective beta blocker, on the development of varices in adults did not show a significant benefit[35]. Currently, preprimary prophylaxis remains an interesting concept that is not applicable in clinical practice.

Primary prophylaxis

The issue of prophylaxis of the first episode of variceal bleeding in children is controversial and is predicated on experience with adults who primarily have alcoholic cirrhosis. Surveillance endoscopy in children with liver disease and stigmata of portal hypertension is justified if the clinician anticipates recommending a prophylactic

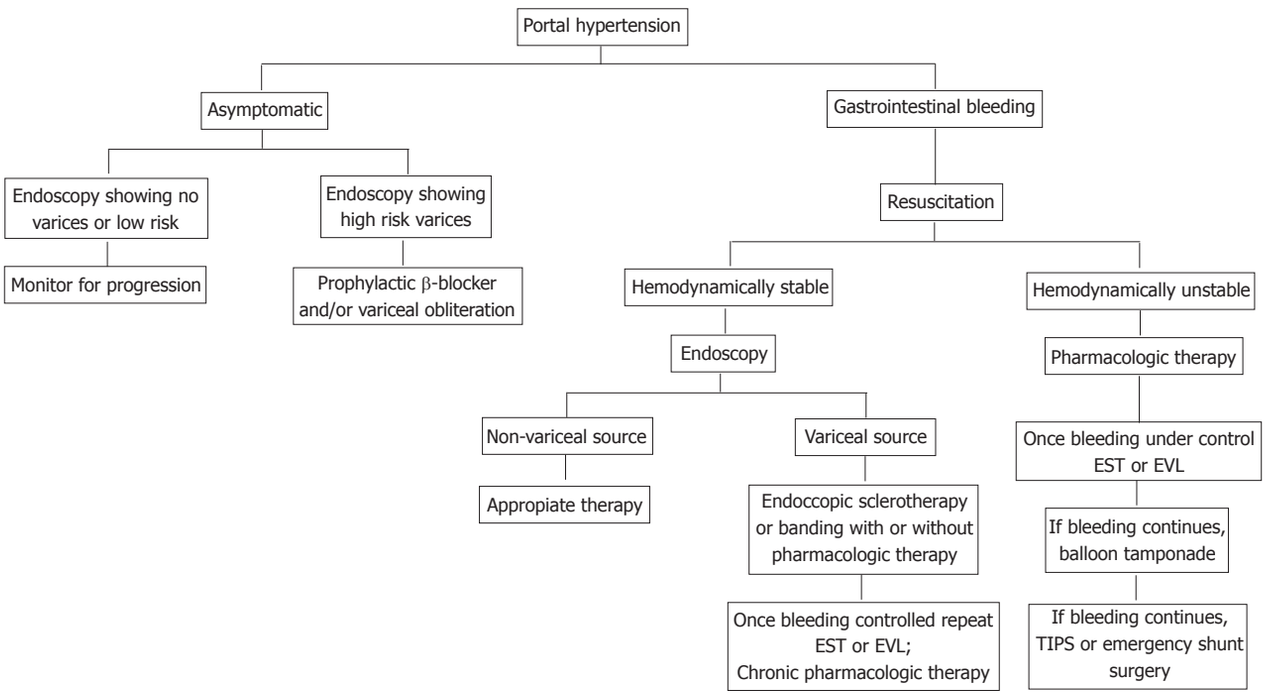

Figure 1 Portal hypertension. EST: Endoscopic sclerotherapy; EVL: Endoscopic variceal band ligation; TIPS: Transjugular intrahepatic portosystemic shunt.

regimen. Prophylaxis may also be valuable in patients who live in remote areas far from emergency medical care. Given the unpredictable timing of the first episode of variceal bleeding, primary prophylaxis regimens need to be associated with relatively low morbidity and mortality. As such, beta blockade has been more extensively used in this setting. The improved risk-benefit ratio of endoscopic ligation therapy relative to sclerotherapy has led to reassessment of its role in primary prophylaxis[36,37]. Uncontrolled preliminary pediatric experience of variceal hemorrhage using beta blockade has recently been reported[38-40]. Beta blocker use in children has reduced the frequency of bleeding episodes, and in some trials has improved long-term survival in patients with esophageal varices. Initial randomized trials demonstrated efficacy in patients who had a previous bleeding episode[41]. Subsequently, propranolol was shown to be effective in patients with varices who had never bled. In a study of 230 subjects randomized to propranolol or placebo, the incidence of bleeding and mortality over a 14 mo period was reduced by almost 50%[42]. Several meta-analyses have demonstrated the success of propranolol[43-50]. It is clear that a goal of at least 25% reduction in resting heart rate needs to be achieved to realize these effects. In patients in whom HVPG drops below 12 mmHg, subsequent variceal bleeding is unlikely. Achieving such a large reduction in resting heart rate and achieving a HVPG below 12 mmHg may be problematic in children in whom baseline measurements may be difficult. A wide range of dosing (0.6-0.8 mg/kg per day) divided into two to four doses of propranolol has been required in children in order to observe a "therapeutic effect".

Unfortunately, propranolol often does not reduce HVPG below 12 mmHg, therefore a combination of beta blockade and vasodilatation therapies are now under investigation. Isosorbide-5-mononitrate, a long-acting vasodilator, may potentiate the effects of propranolol on the HVPG[51]. Combination pharmacologic agents, such as carvedilol, may have enhanced efficacy[52]. Unfortunately, there is little if any prospective data in children on the safety and effectiveness of beta blockade with or without vasodilators in patients with portal hypertension.

Endoscopic band ligation therapy has been used with greater frequency in adults with high risk varices[53,54]. As with beta blockade, endoscopic band ligation therapy cannot be recommended for routine use in children with varices. In fact, a small randomized trial of prophylactic endoscopic sclerotherapy in children showed no survival benefit[55].

Emergency therapy of variceal bleeding

The initial management of variceal bleeding is stabilization of the patient. Vital signs, particularly tachycardia or hypotension, can be especially helpful in assessing blood loss. Patients on beta blocker therapy may not manifest the usual compensatory tachycardia and are at higher risk of developing significant hypotension. Fluid resuscitation in the form of crystalloid initially, followed by red blood cell transfusion, is critical. One needs to administer these carefully to avoid overfilling the intravascular space and increasing portal pressure. Optimal hemoglobin levels in adults with variceal hemorrhage are between 7 and 9 g/dL[56]. Nasogastric tube placement is safe and may be an essential part of the management of these patients. It allows documentation of the rate of ongoing bleeding and removal of blood, a protein source that

may precipitate encephalopathy. In addition, blood in the stomach increases splanchnic blood flow and could aggravate portal hypertension and ongoing bleeding. Platelets should be administered for levels less than 50×10^9/L, and coagulopathy corrected with vitamin K and fresh frozen plasma. There may be a value to the use of recombinant factor Ⅶa in severe coagulopathy as the fluid requirements may be diminished[57]. Intravenous antibiotic therapy should be considered for all patients with variceal bleeding in light of the high risk of potentially fatal infectious complications[58,59]. Once the patient is stabilized, endoscopy should be performed to document that hemorrhage is indeed from variceal rupture. Continued bleeding at the time of endoscopy is a finding that portends poor prognosis. A significant percentage of both adults and children with chronic liver disease will have a source of bleeding other that varices, including duodenal or gastric ulceration[58]. Pharmacotherapy of acute hemorrhage should not be withheld until endoscopy can be performed. In fact, it may facilitate the procedure. At the time of initial endoscopy, management can begin in the form of sclerotherapy or band ligation. Bleeding that lasts more than 6 h or requires more than one red blood cell transfusion necessitates further investigation. A wide variety of therapeutic options exist in adults. Documentation of their efficacy in adults is fairly convincing, but data in children is scarce

Pharmacology

The pharmacologic therapy of variceal bleeding usually consists of vasopressin or somatostatin (or their analogs) infusions. Vasopressin has the longest history of usage and acts by increasing splanchnic vascular tone and thus decreasing portal blood flow. Its use is often limited by the side effects of vasoconstriction, which include left ventricular failure,, bowel ischemia, angina, and chest/abdominal pain[53]. In a study of 215 children with acute variceal hemorrhage, 184 had bleeding arrested by the combined use of fluid support and vasopressin. Vasopressin has a half-life of 30 min and is usually given as a bolus followed by continuous infusion. The recommended dose for children is 0.33 U/kg as a bolus over 20 min, followed by an infusion of 0.2 U/1.73 m^2 per minute (may be increased up to 3 times the initial rate). These recommendations are empiric, based on clinical practice, and derived from extrapolation of adult dosages. Terlipressin, a long-acting synthetic analogue of vasopressin, has shown similar effects and does not require continuous infusion[57]. Side effects appear to be reduced compared to vasopressin, but prospective data in children are lacking.

Alternatives to vasopressin have been investigated because of its poor side effect profile. Somatostatin and its synthetic homologue octreotide also have been shown to decrease splanchnic blood flow. Their effects on acute variceal hemorrhage appear to be similar to those of vasopressin, with fewer side effects[58,59]. Continuous infusion of 1-5 μg/kg per hour of octreotide appears to be effective but may need to be initiated by the administration of a bolus. New longer-acting somatostatin analogues are currently under investigation[59].

Endoscopy

Approximately 15% of children will have persistent hemorrhage despite conservative management plus some form of splanchnic vasoconstriction. The most common secondary approach is endoscopic sclerotherapy or endoscopic band ligation. Endoscopic therapy is very effective in controlling bleeding, although it may be technically challenging. An extensive experience with emergency sclerotherapy exists in children, and it's rare for additional therapy to be required. A variety of agents have been used (sclerosants, chemically irritating compounds such as ethanolamine/tetradecyl sulfate). These sclerosants are injected either intra-or para-variceal, until bleeding has stopped. In the setting of emergency sclerotherapy it is important to be aware of the significant incidence of associated bacteremia and to consider antibiotic prophylaxis in most patients.

Endoscopic band ligation of varices may be a preferable approach because it is easier and safer. A randomized trial of band ligation versus sclerotherapy in adults demonstrated similar control of active bleeding and recurrence of hemorrhage with significantly lower overall complications and mortality). A potential concern of this technique in children (whose esophageal wall is thinner than adults), is entrapment of the full thickness of the esophageal wall by the rubber band with subsequent ischemic necrosis and perforation.

Mechanical

The Sengstaken-Blackmore tube (SSBT) was designed to stop hemorrhage by mechanically compressing esophageal and gastric varices. The device consists of a rubber tube with at least two balloons. It is passed into the stomach, where the first balloon is inflated and pulled up snug against the gastroesophageal junction. Once the tube is secured in place, the second balloon is inflated in the esophagus at a pressure (60-70 mmHg) that compresses the varices without necrosing the esophagus. A channel in the rubber tube allows gastric contents to be sampled for evidence of bleeding. This therapy is very effective in controlling acute bleeding. Unfortunately, it is associated with significant number of complications and high incidence of re-bleeding when the tube is removed. Most patients find the treatment uncomfortable, and its use in children requires significant sedation. Use of the SSBT increases the risk of aspiration pneumonia, which can be a life threatening complication in a patient with liver failure. Re-bleeding has been reported in 33%-60% of patients. Given these problems it is reserved for severe uncontrollable hemorrhage and generally serves as a temporizing measure until a more definite procedure can be performed.

Surgical and interventional radiology

Surgical therapy is usually a last resort approach to acute

Table 2 Major complications of endoscopic sclerotherapy in children[61] (%)

Bleeding before treatment	39
Esophageal ulceration	29
Stricture formation	16
Recurrent varices	8

variceal hemorrhage. The reluctance to perform emergency surgery partly stems from its associated high mortality but also from concerns of an increased incidence of encephalopathy and greater difficulty for subsequent liver transplantation. The surgical procedures available can be divided into transection, devascularization, and portosystemic shunting. The first two techniques are rarely used and work by interrupting blood flow through the esophagus. Liver transplantation may be an effective means of treating esophageal variceal bleeding if an acceptable organ can be procured quickly enough. Variceal embolization *via* a percutaneous transhepatic or transsplenic approach has been advocated by some hepatologists as another method of controlling acute hemorrhage. Transjugular intrahepatic portosystemic shunt (TIPS) placement may be the optimal approach for intractable bleeding since it does not require surgery or puncture of an organ that is predisposed to hemorrhage. A catheter is inserted into the jugular vein and is advanced into the hepatic vein where a needle is used to form a tract between the portal vein and the hepatic vein. This tract is expanded with a balloon angioplasty catheter, and a stent is then placed forming a permanent portosystemic shunt. The experience in children is limited. Size limitations and local expertise may be limiting factors in some cases, but given the high risk associated with emergency surgery or the use of SSBT, TIPS may be the treatment of choice in the emergent setting, especially when liver transplantation is imminent.

Secondary prophylaxis: The long term management of portal hypertension in children with a previous episode of variceal bleeding is complex. One must take into consideration several factors; first the natural history; as discussed earlier, there are significant differences in the setting of minimal and inactive versus active and progressive hepatic disease. As a result, certain individuals may have the possibility of outgrowing their portal hypertension through the development of spontaneous portosystemic shunts, whereas other might be expected to develop end-stage liver disease and ultimately be candidates for liver transplantation. The second issue stems from the great diversity in therapeutic modalities. The physiologic goal of pharmacologic therapy varies from program to program (i.e, change in heart rate, hepatic portal venous gradient pressures, *etc*). Sclerotherapy may be administered with a wide variety of agents and by two different techniques (i.e, intra or para variceal). Endoscopic band ligation offers an important and generally safer alternative. Finally, at least six different portosystemic shunting procedures have been described, all with their own advantages and disadvantages.

Sclerotherapy and ligation therapy: Sclerotherapy and band ligation therapy work by physical obliteration of esophageal varices. Bleeding may occur during the several weeks required to complete the obliteration. Most importantly the principal problem of portal hypertension is not addressed. Despite these problems, endoscopic therapy has been a mainstay of the treatment of esophageal varices, and there is significant amount of clinical experience with these therapies in children.

The effectiveness of sclerotherapy has been studied for both prevention of initial and subsequent bleeding episodes. Scleroptherapy, which has in general been supplanted by band ligation, with the exception of very young or small children in which band ligation may not be feasible. Intravariceal, paravariceal, and some combination injection protocols have been used. A wide variety of sclerosing agents have been used without a clear cut difference in their efficacy or adverse side effects. A meta analysis of seven studies and 748 patients revealed mortality rates of 47% in the sclerotherapy group and 61% in the conservatively managed group. A variety of complications have been reported (Table 2).

Retrosternal pain, bacteremia, and fever post treatment are common. Esophageal ulceration may occur after sclerotherapy, and the associated symptoms may be ameliorated with sucralfate slurry therapy.

The range of complications associated with sclerotherapy has prompted the development of alternative endoscopic methods such as band ligation. This technique involves suctioning of a varix into the end of an endoscope so that a rubber band can be placed around the varix leading to thrombosis. Direct comparisons of endoscopic sclerotherapy and variceal ligation in adult patients have yielded results in favor of ligation. Similar results have been reported in children by Zargar *et al*. The major advantage of variceal ligation is avoidance of needle injection of varices, which appears to reduce the rate of complications. In addition, variceal ligation appears to lead to obliteration in fewer sessions and is associated with lower rate of rebleeding.

Portosystemic Shunting: A variety of procedures have been used to divert portal blood flow and decrease portal blood pressure: (1) Mesocaval Shunts: formed with the insertion of a graft between the superior mesenteric vein and the inferior vena cava; (2) Portacaval Shunts: formed by side-to-side anastomosis of the portal vein and the inferior vena cava; and (3) Distal Splenorenal Shunt: formed by end-to-side anastomosis of the splenic vein and the left renal vein.

The portacaval shunt diverts nearly all the portal blood flow into the subhepatic inferior vena cava. This is very effective decompressing the portal system, but also diverts a significant amount of blood from its normal hepatic metabolism, predisposing to the development

Table 3 Results of portosystemic shunting in children[63,64]

Type of portal hypertension	No. of patients	Rebleeding	Mortality
Extrahepatic	292	45%	5%
Intrahepatic	76	50%	53%

of hepatic encephalopathy. Decreased hepatic blood flow theoretically also may lead to worsening of underlying liver disease. An intermediate shunt can be made by placing a graft between the mesenteric or portal vein and the vena cava. This decompresses the portal system while allowing a greater amount of portal blood flow into the liver. The use of grafts unfortunately is associated with increased risk of thrombosis and many times with worsening retrograde flow.

Another approach involves diversion of splenic blood flow into the left renal vein, which can be done nonselectively (central) or semiselectively (distal splenorenal shunt).

A substantial pediatric experience with surgical portosystemic shunting has been accumulated over the past 20 years. The results are clearly different in patients with extra-or intrahepatic portal hypertension (Table 3).

An alternative shunting procedure for children with extrahepatic portal vein thrombosis is the meso-Rex bypass, this procedure involves the placement of an autologous venous graft from the mesenteric vasculature to the left intrahepatic portal vein. One of the major advantages of this approach is the restoration of normal portal blood flow, which eliminates the risk of hepatic encephalopathy and should preserve hepatic function. The selection of patients for this procedure is not clear both from a clinical indication and surgical feasibility, and some have advocated that this procedure be considered in all children with portal vein thrombosis. Standard diagnostic imaging may not clearly indicate whether there is patency of the intrahepatic portal vein, and the potential in this group of children for hypercoagulable states must be kept in mind.

In stark contrast to the excellent results in portal vein thrombosis, there are generally poor results of portosystemic shunts in children with decompensated liver disease. The incidence of recurrent bleeding and death approaches 50%. Hepatic encephalopathy is a frequent and serious complication of portosystemic shunting in decompensated liver disease, and studies have failed to show improvement in long-term survival in patients with intrahepatic disease.

Overall, surgical portosystemic shunting is an excellent approach to the long-term management of children with intractable variceal bleeding in the setting of compensated cirrhosis. In addition, significant gastric variceal hemorrhage in children may be an indication to consider surgical shunting. TIPS may be an alternative shunting procedure for children with refractory bleeding and serves as an effective bridge to transplantation. The procedure is typically feasible, with published success in children as small as 14 kg, although special procedural modifications must be undertaken in small children. Long term shunt occlusions limit the overall application of this efficacious therapy, although newer data with coated stents may improve long-term patency rates for TIPS.

CONCLUSION

The approach to acute variceal hemorrhage in children is a stepwise progression from least invasive to most invasive. Surveillance endoscopy is predicated on the availability of an efficacious primary prophylactic therapy. Beta-blocker therapy is accepted primary prophylactic therapy in adults, and endoscopic ligation therapy is also gaining acceptance. Preliminary data in children appear to indicate that this approach is feasible, but further studies are needed before a wide-spread recommendation for children can be endorsed. Therefore, surveillance endoscopy and primary prophylaxis are not generally be indicated in children with portal hypertension who have not had a variceal bleed. Special medical and or social circumstances in which an initial bleeding episode may be particularly dangerous could justify this approach.

Management of acute variceal bleeding is more straightforward. Initial interventions should include stabilization of the patient, placement of a nasogastric tube, and institution of antibiotic therapy. Diagnostic and/or therapeutic endoscopy should be scheduled as soon as it is safe and feasible. In the interim, pharmacologic treatment with either a vasodilator or octreotide is indicated and may facilitate endoscopic therapy. Intractable and severe hemorrhage should be treated by TIPS.

The long term approach to prevention of recurrent variceal bleeding in children must be adapted for the etiology of portal hypertension, the needs of the specific patient, and the particular skills of the institution. The approach to extrahepatic portal vein obstruction is evolving. In general, the unpredictability of the timing of the sentinel bleeding episode and the low incidence of mortality associated with that episode make prophylactic therapy inadvisable. Enthusiasm for the utilization of the meso-Rex shunt is increasing because of the physiologic nature of the procedure, and should be considered for children with extrahepatic portal vein thrombosis and cavernous transformation and a normal liver. The long term management of portal hypertension in the child with biliary atresia is more complex. In patients with incomplete bile drainage, liver transplantation appears to be inevitable and should be the major focus of therapeutic intervention. Temporizing measures for these children may include band ligation therapy and TIPS. Biliary atresia patients who have a more successful response to Kasai portoenterostomy, have a more favorable long term outlook. Variceal hemorrhage may be followed by a relatively long term survival with medical intervention. Recurrent bleeding might be amenable to portosystemic shunting as opposed to transplantation.

The approach to patients with more slowly progressive intrahepatic disease is more difficult to generalize.

Well-conceived multicenter trials are required to determine whether the principals that have been developed in adults can be extrapolated to children.

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TOPIC HIGHLIGHT

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Pros and cons of colonoscopy in management of acute lower gastrointestinal bleeding

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Abstract

Acute lower gastrointestinal bleeding (LGIB) is a frequent gastrointestinal cause of hospitalization, particularly in the elderly, and its incidence appears to be on the rise. Endoscopic and radiographic measures are available for the evaluation and treatment of LGIB including flexible sigmoidoscopy, colonoscopy, angiography, radionuclide scintigraphy and multi-detector row computed tomography. Although no modality has emerged as the gold standard in the management of LGIB, colonoscopy is the current preferred initial test for the majority of the patients presenting with hematochezia felt to be from a colon source. Colonoscopy has the ability to diagnose all sources of bleeding from the colon and, unlike the radiologic modalities, does not require active bleeding at the time of the examination. In addition, therapeutic interventions such as cautery and endoclips can be applied to achieve hemostasis and prevent recurrent bleeding. Studies suggest that colonoscopy, particularly when performed early in the hospitalization, can decrease hospital length of stay, rebleeding and the need for surgery. However, results from available small trials are conflicting and larger, multicenter studies are needed. Compared to other management options, colonoscopy is a safe procedure with complications reported in less than 2% of patients, including those undergoing urgent examinations. The requirement of bowel preparation (typically 4 or more liters of polyethylene glycol), the logistical complexity of coordinating after-hours colonoscopy, and the low prevalence of stigmata of hemorrhage complicate the use of colonoscopy for LGIB, particularly in urgent situations. This review discusses the above advantages and disadvantages of colonoscopy in the management of acute lower gastrointestinal bleeding in further detail.

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Key words: Colonoscopy; Acute lower gastrointestinal bleeding; Management; Diagnostic yield; Therapeutic intervention; Outcomes; Colon preparation; Stigmata of hemorrhage

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INTRODUCTION

Acute lower gastrointestinal bleeding (LGIB) is a disorder frequently encountered by gastroenterologists, surgeons and internists. Hospitalization for LGIB in the 1990s was estimated to occur at an incidence of 20.5 per 100 000 person-years[1]. The incidence of LGIB may be rising concomitant with the aging of our population and the increasing use of aspirin/non-steroidal anti-inflammatory drugs and anticoagulants[2]. LGIB is associated with significant mortality, health care costs and increased length of hospitalization, emphasizing the need for effective evaluation and treatment[2-4].

Currently, physicians managing LGIB have a number of different diagnostic and therapeutic options to choose from, ranging from radiographic interventions such as radionuclide scintigraphy and angiography to colonoscopy and flexible sigmoidoscopy. Although no modality has emerged as the gold standard in the management of LGIB, colonoscopy has several advantages and is generally regarded as the preferred initial test in the majority of cases. The advantages are as follows: (1) ability to identify bleeding source regardless of rate or presence of bleeding; (2) multiple therapeutic possibilities; (3) efficiency given diagnostic and therapeutic potential; (4) irrespective of initial testing, colonoscopy is required for definitive diagnosis; and (5) safety. Disadvantages are as follow: (1) requirement for colon preparation; (2) need for sedation, experienced staff and endoscopy facilities; (3) low prevalence of stigmata of hemorrhage; (4) invasive nature; and (5) rare but serious complications.

This article will explore the advantages and disadvantages of colonoscopy in the management of LGIB.

PROS OF COLONOSCOPY

Potential for diagnosis and therapeutic intervention

A major advantage of colonoscopy over other management options is the potential for diagnosis and therapeutic intervention even in the absence of ongoing bleeding. This is important given the often intermittent nature of LGIB and slow bleeding from diffuse mucosal sources such as colitis. In contrast, the radiographic alternatives including angiography, radionuclide scintigraphy and computed tomography (CT) require active bleeding at the time of the examination in order to identify and treat the source of bleeding, and are useful only in a subset of patients with severe ongoing bleeding. It is therefore not surprising that the diagnostic yield of colonoscopy exceeds that of the radiographic modalities.

Studies have shown a diagnosis is made in 74%-100% of patients with LGIB undergoing colonoscopy (Table 1)[5-11]. A pooled analysis by Strate *et al*[12] of six recent studies of colonoscopies following bowel preparation for LGIB found a composite diagnostic yield of 91% for colonoscopy. In comparison, in radionuclide scanning, a commonly used radiological diagnostic modality in LGIB, scans were positive in 40%-70% of patients with accuracy ranging from 35%-100%[13-17]. In small case series, multidetector row CT scanning (MDCT) has shown promising results with localization of bleeding in 50%-100% of select cases[16,18,19]. Angiography is less sensitive than radionuclide or MDCT scan and therefore is infrequently utilized as a front line test. In a collected series of 247 patients reported by Browder *et al*[20], angiography demonstrated a bleeding site in 72% of patients whereas a retrospective chart review study by Cohn *et al*[21] yielded only 35% positive angiographic findings. In addition, it is important to note that colonoscopy is generally required following radiographic tests, whether positive or negative, to confirm the source of bleeding and to exclude colorectal malignancy or other serious diagnoses. Therefore, colonoscopy is the most efficient initial test for the majority of patients with LGIB who can be stabilized and can undergo colon preparation.

Few studies have directly compared colonoscopy to radiographic interventions. In a study by Jensen *et al*[22], emergency colonoscopy and upper endoscopy as well as emergency angiography were performed in 22 patients with severe bleeding. In the 17 patients with lower intestinal sources of bleeding, the diagnostic yield of colonoscopy was 82% *vs* 12% for angiography[22]. Similarly, in a retrospective study by Strate *et al*[23], initial colonoscopy within 24 h of admission offered a diagnostic yield of 85% *vs* 45% for initial scintigraphy and angiography.

The timing of colonoscopy also may have an effect on diagnostic and therapeutic outcomes. Several studies indicate that urgent colonoscopy, or colonoscopy within 12-24 h of presentation, may improve the diagnostic and therapeutic yield of colonoscopy in LGIB. In a prospective study, Jensen *et al*[6] identified stigmata of hemorrhage in approximately 20% of patients with diverticular bleeding undergoing urgent colonoscopy. Subsequent endoscopic treatment significantly reduced the rate of rebleeding and surgery compared to historical controls. In a trial by Green *et al*[7] of colonoscopy following bowel preparation within 12 h *vs* elective colonoscopy (within 74 h), a definitive bleeding source was identified in 42% of patients undergoing urgent colonoscopy *vs* 22% in those undergoing elective colonoscopy. Most recently, in a trial of colonoscopy within 12 h *vs* colonoscopy in 36-60 h, Laine *et al*[24] found no differences in outcomes including the number of diagnoses. However, 78% of colonoscopies were diagnostic in the urgent group compared to 67% in the elective group, and the only 2 stigmata of hemorrhage were both identified in patients with diverticulosis undergoing urgent colonoscopy[24].

For patients undergoing colonoscopy, once a diagnosis is made, endoscopic hemostasis can be carried out depending on the source. Epinephrine or saline injection, thermal contact, argon plasma coagulation, clipping and band ligation are available therapeutic modalities. A review of published series by Strate *et al*[12] showed that endoscopic therapy was applied to 10% to 40% of patients undergoing colonoscopy for LGIB and the most common intervention was thermal contact plus injection. Their review of 71 cases of diverticular bleeding treated with endoscopically placed hemoclips showed

Table 1 Comparison of management options for lower gastrointestinal bleeding (%)

Procedure	Diagnosis	Therapy	Early rebleeding	Major complications	Colon preparation	Requires active bleed
Colonoscopy[5-8]	74-100	8-37	0-24[1]	0-2	Yes	No
Sigmoidoscopy[9-11]	About 10	0-20	0	Rare	Minimal	No
Angiography[20,21,26-29][2]	23-72	14-100	1-57	0-60	No	Yes
Radionuclide scan[13-15]	40-73	N/A	N/A	Rare	No	Yes
Multi-detector CT scan[16,18,19]	24-94	N/A	N/A	0%-11%	No	Yes

N/A: Not available; CT: Computed tomography. [1]Early rebleeding in patients who had undergone endoscopic therapy for diverticular bleeding; [2]Therapy, rebleeding and complications refer to superselective embolization only. It is difficult to determine the frequency of superselective embolization use because most series only report patients who receive therapy.

particular promise with a homeostasis success rate of 100% and with no complications.

Improved outcomes

It is important to note that in most cases, diagnosis needs to be followed by therapeutic maneuvers in order to alter outcomes in patients with LGIB. Early colonoscopy has shown particular promise in this regard in patients with diverticular bleeding. In a study by Jensen *et al*[6], patients undergoing endoscopic hemostasis for diverticular stigmata with epinephrine plus/minus thermocautery had significantly lower rates of rebleeding and surgery when compared to historical controls who did not receive endoscopic treatment. Indeed, none of the endoscopically treated patients experienced re-bleeding. In addition, length of hospital stay was significantly shorter in the colonoscopy treatment group ($P < 0.001$).

Subsequent randomized trials for all sources of severe LGIB have yielded less positive results. However, these trials were small and the lack of significant findings may have been the result of inadequate power or other methodological issues. In the Green *et al*[7] trial, patients were randomized to urgent colonoscopy within 8 h or standard of care which was elective colonoscopy within 4 d of admission. As noted above, the diagnostic yield was superior in the urgent colonoscopy group. Other outcomes such as rebleeding, surgery and number of blood transfusions showed a trend in the favor of urgent colonoscopy but did not reach statistical significance. Also of note, only 36% of colon preparations were noted to be "excellent", which may also have affected the efficacy of urgent colonoscopy. Laine *et al*[24] also did not find that urgent colonoscopy improved major outcomes compared to delayed colonoscopy. In addition to the small number of patients in their study (the study was stopped early with only 36 patients in each arm *vs* the planned 134 patients), patients in the urgent colonoscopy arm also appeared to have more severe bleeding which may have made it more difficult to detect a favorable difference.

A number of observational studies indicate that urgent colonoscopy reduces hospital length of stay. In a retrospective study of patients admitted with LGIB, Strate *et al*[25] looked at the impact of time to colonoscopy on hospital length of stay in patients presenting with LGIB. They found that earlier performance of colonoscopy was associated with a shorter length of stay [hazard ratio (HR) 2.02, 95% CI: 1.5-2.6, $P < 0.0001$] even when adjusted for other factors. Similarly, Schmulewitz *et al*[30] also showed that having a colonoscopy was associated with reduced hospital length of stay (HR 1.54, 95% CI: of 1.2-1.8), and mean length of hospital stay was significantly shorter in patients undergoing colonoscopy within 24 h compared to more than 24 h (5.4 d *vs* 7.2 d, $P < 0.008$). Since hospital days are a major source of charges in LGIB this could have a significant economic impact. In a cost analysis study, Jensen *et al*[31] estimated that urgent colonoscopy was associated with an average savings of $10 065 per patient compared with medical, angiography and surgical management; although this number likely overestimates the degree of cost savings due to other trends in care such as shorter hospital stay, and a recent randomized trial found no difference in hospital charges in patients undergoing urgent *vs* elective colonoscopy[24]. Overall, colonoscopy, particularly when performed early in the hospital course, has the potential to improve outcomes such as rebleeding, surgery and length of stay, but larger randomized trials are needed to better define its efficacy.

Safety

Colonoscopy with or without intervention for LGIB is considered a safe procedure. In a review of 4 studies with 664 patients, there were 2 perforations for an overall complication rate of 0.3% for colonoscopy and 0.6% for colonoscopy performed urgently[12]. Other potential risks in addition to bowel perforation include congestive heart failure secondary to volume overload, electrolyte abnormalities and aspiration pneumonia. Also of note, in a review of 137 cases of endoscopic treatment for diverticular hemorrhage using a variety of modalities, there were no reported complications[12]. Complications are noted more frequently in patients undergoing angiography. The risks of bowel ischemia and cardiac arrhythmia seen with vasopressin infusions have been ameliorated with newer superselective embolization techniques. However, in a review of 20 studies utilizing superselective embolization for control of LGIB, minor complications were seen in 26% of patients and major complications requiring surgery or resulting in death were seen in 17%[12]. Another significant concern for both angiography and MDCT is renal compromise

secondary to contrast dye load. In the small amount of literature on MDCT there have been 2 reports of renal failure in patients with diabetes who also underwent angiography[19].

CONS OF COLONOSCOPY

Colon preparation

Cleansing the colon of stool and blood prior to colonoscopy for management of LGIB is imperative, difficult to accomplish, and unique to endoscopic interventions for LGIB. Colonoscopy preparation is necessary for complete examination to the cecum. Studies of unprepped colonoscopy in LGIB report examinations to the cecum in only 55%-70% of cases[32-34]. Unprepped colonoscopy may increase the risk of perforation due to poor visualization. The identification of subtle bleeding sites, which are often among multiple lesions as is the case in diverticular hemorrhage, the most common source of LGIB, is contingent on removal of excess debris. In a prospective study by Jensen *et al*[6] on urgent colonoscopy in severe diverticular bleeding, active bleeding was detected in 21% of cases in the urgent colonoscopy arm and endoscopic hemostasis was achieved in 100% of the cases. The excellent results may have been due, in part, to their aggressive bowel preparation regimen. This regimen required 5-6 liters of sulfate purge over 3-4 h with 33% receiving the purge through a nasogastric tube. However, achieving consistently excellent bowel preparations, particularly in the urgent setting for LGIB, is difficult. The 4 or more hour time frame required for preparation may delay colonoscopy examinations until after-hours when nursing support and endoscopic facilities are not available. Thus, colon preparation makes urgent colonoscopy logistically complicated. Even under highly regulated study situations, colon preparations have been suboptimal in a high percentage of patients. In Green *et al*[7]'s study, endoscopic view was rated poor to fair in 62%-64% of patients (62% in elective arm and 64% in urgent arm). Laine *et al*[24] did not comment on the quality of the colon prep in their study but did report that 7% of their patients required a second colonoscopy due to poor bowel prep (2 in the urgent arm and 3 in the elective arm). In these two studies poor colon preparation has been cited as a reason for the lack of significant findings in favor of the colonoscopy arm[35,36].

As mentioned above, aggressive bowel preparation requires a large volume of bowel preparation, often up to 6-8 liters. Rarely, this can lead to volume overload and electrolyte abnormalities such as hyponatremia[37]. There is also the risk for aspiration, especially when the prep is rapid or the patient is at risk for aspiration. The need for colon preparation makes colonoscopy for LGIB more complicated than esophagogastroduodenoscopy (EGD) for upper gastrointestinal bleeding, and is likely one of the main reasons it has not been equally embraced. Also, the need for colon prep clearly sets colonoscopy for management of LGIB apart from radiological interventions for LGIB.

Sedation, experienced staff and procedure facilities

Colonoscopy generally requires sedation given by trained professionals in a monitored location such as an intensive care unit or an endoscopy suite. Specialized support staff is also helpful to aid the gastroenterologist with the colonoscopy. These factors add to the complexity of coordinating the procedure, particularly on nights and weekends. However, angiography also requires sedation, an interventional radiologist, support staff and a specialized procedure suite. Indeed, in their study of utilization of early colonoscopy *vs* radiography in severe lower intestinal bleeding, Strate and Syngal found that the median times from admission to colonoscopy or angiography were similar (median of 17 h for colonoscopy *vs* 14 h for angiography)[23].

Urgent vs delayed performance of colonoscopy

The optimal timing of colonoscopy remains an area of controversy. As noted above, some studies suggest that performing colonoscopy within the first 12-24 h of hospital admission aids in the identification and treatment of the bleeding source. However, the two published randomized controlled trials of LGIB showed no difference in important clinical outcomes such as rebleeding and surgery[7,24]. Unfortunately, none of these studies was adequately powered to detect a statistically significant difference in important clinical outcomes, and the utility of urgent colonoscopy remains uncertain. The conflicting, albeit flawed, evidence paired with the practical issue of colon preparation make it difficult for clinicians to embrace urgent colonoscopy. Based on available literature, there is no specific time threshold for colonoscopy, but rather colonoscopy should be performed after a thorough bowel preparation, and more urgently in patients with signs of significant or ongoing bleeding as stigmata are rarely identified in delayed examinations[7,24].

Low prevalence of stigmata of hemorrhage

The identification and treatment of the bleeding source is the goal of urgent interventions for GI bleeding particularly when severe. Diagnostic interventions alone are unlikely to alter significant outcomes such as rebleeding and need for surgery. However, stigmata are infrequently identified in the colon. Studies report stigmata of hemorrhage in 7.7% to 43% of cases[5,30]. The colon has a large and complex surface area, often with multiple potential sources. In addition, bleeding tends to be intermittent in nature and it can be difficult to differentiate fresh blood from old blood and stool. Therefore, the effort entailed in orchestrating an urgent colonoscopy may be perceived as great in relation to the gain. Decision aids and predictive models have the potential to improve the utility of urgent colonoscopy for LGIB by identifying patients who are most likely to have ongoing bleeding and stigmata of hemorrhage and hence benefit from intervention. Three studies to date have aimed at

identifying high risk patients with LGIB with reasonable accuracy[38-40]. However, the use of these tools in routine practice has not been studied and they have not been widely embraced.

In addition to the low prevalence of stigmata, a substantial subset of patients (up to 20%) presenting with hematochezia and presumed LGIB will ultimately be found to have bleeding from the upper or small bowel[22,24]. An EGD or nasogastric lavage is necessary in briskly bleeding patients to begin to exclude these possibilities. In small bowel bleeding, colonoscopy and upper endoscopy serve only to exclude these areas of the bowel as bleeding sources, and these patients generally require a number of procedures for diagnosis[41].

Complications

As mentioned earlier, the complication rate of colonoscopy is low, 0.3% and 1.3% in two comprehensive reviews[12,42]. Safety is generally thought of as a relative strength of colonoscopy over other modalities. Radionuclide scanning and CT scanning are the only noninvasive option and these modalities do not provide therapeutic opportunities. However, complications of colonoscopy when they occur can be severe. Colon perforation is the most common complication and generally requires urgent surgical intervention. Other complications are mainly due to colon preparation and can be minimized with proper and careful administration of colon purge. For example, rapid preps should be avoided in patients with altered mental status, difficulty swallowing, delayed gastric emptying or bowel obstruction. Fleets Phosposoda has been associated with renal compromise and is no longer available. Balanced electrolyte solutions are considered safer and less likely to result in fluid and electrolyte shifts[43]. However, there have been case reports of patients with decreased left ventricular systolic function experiencing exacerbation of their symptoms, thought to be secondary to fluid shifts in the setting of bowel preparation with polyethylene glycol (Golytely)[44]. These patients should hence be monitored closely while undergoing their prep.

CONCLUSION

Colonoscopy offers many advantages in the management of acute lower gastrointestinal bleeding. All sources and severities of LGIB can be diagnosed with colonoscopy and the overall diagnostic yield of this procedure is high. Endoscopic hemostasis can be utilized to stop or prevent bleeding, and therefore colonoscopy offers the potential to improve important clinical outcomes such as rebleeding, although the data from existing small studies are conflicting. The need for bowel preparation, the logistical difficulty of coordinating the procedure after-hours and the infrequent identification of stigmata of hemorrhage deter the widespread use of urgent colonoscopy in LGIB. However, most of these difficulties also apply to other modalities such as angiography and radionuclide scan. Albeit imperfect, current data indicate that colonoscopy offers more advantages than other management options and should be the initial modality in the majority of patients with LGIB. Further prospective randomized studies are needed to more clearly define the optimal timing of colonoscopy in LGIB and its role relative to other available options including radionuclide scanning, angiography and MDCT scanning.

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TOPIC HIGHLIGHT

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Angiographic evaluation and management of acute gastrointestinal hemorrhage

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Abstract

Although most cases of acute nonvariceal gastrointestinal hemorrhage either spontaneously resolve or respond to medical management or endoscopic treatment, there are still a significant number of patients who require emergency angiography and transcatheter treatment. Evaluation with noninvasive imaging such as nuclear scintigraphy or computed tomography may localize the bleeding source and/or confirm active hemorrhage prior to angiography. Any angiographic evaluation should begin with selective catheterization of the artery supplying the most likely site of bleeding, as determined by the available clinical, endoscopic and imaging data. If a hemorrhage source is identified, superselective catheterization followed by transcatheter microcoil embolization is usually the most effective means of successfully controlling hemorrhage while minimizing potential complications. This is now well-recognized as a viable and safe alternative to emergency surgery. In selected situations transcatheter intra-arterial infusion of vasopressin may also be useful in controlling acute gastrointestinal bleeding. One must be aware of the various side effects and potential complications associated with this treatment, however, and recognize the high re-bleeding rate. In this article we review the current role of angiography, transcatheter arterial embolization and infusion therapy in the evaluation and management of nonvariceal gastrointestinal hemorrhage.

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Key words: Angiodysplasia; Aneurysm; Digital subtraction angiography; Contrast media; Hemorrhage; Radionuclide angiography; Therapeutic embolization

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INTRODUCTION

Most cases of acute nonvariceal gastrointestinal hemorrhage resolve spontaneously and of those that do not, the majority respond to conservative medical management measures such as fluid resuscitation, correction of any coagulopathy and administration of blood products[1]. In those cases that are refractory to medical management, endoscopy is the mainstay for diagnosis and treatment. However, there is a subset of patients in whom endoscopic management is ineffective, and imaging evaluation with an accompanying alternative intervention is necessary for bleeding control because

of recurrence or massive hemorrhage[1]. Localization and characterization of the bleeding source are important in determining the appropriate intervention, as treatment options range from minimally invasive catheter-directed therapy to extensive surgical resection. Although there has generally been a decline in the number of patients who present with acute gastrointestinal hemorrhage requiring angiography and/or transcatheter intervention, there are still patients who are unresponsive to either medical or endoscopic management and thus require emergency angiographic evaluation and possible transcatheter treatment.

The alimentary system is subdivided into the upper and lower gastrointestinal tracts and thus gastrointestinal hemorrhage is subcategorized according to the location of bleeding. The upper gastrointestinal system extends from the esophagus to the ligament of Treitz, while the latter includes the small bowel, colon and rectum. The distinction is important, as there are some characteristics that are relatively unique to each location and these may affect and determine the therapeutic approach to the particular bleeding source. Thus establishing the specific location of the bleeding as well as the etiology is critical to the treatment of patients with massive or recurrent gastrointestinal hemorrhage. Unfortunately the diagnosis can often be challenging because of the intermittent nature of gastrointestinal bleeding.

PATIENT EVALUATION AND MANAGEMENT

The initial management of a patient with acute gastrointestinal hemorrhage should be directed at stabilization through administration of fluids and blood products, correction of coagulation abnormalities, placement of appropriate intravenous access lines and insertion of a nasogastric tube if needed. The vital signs should be closely monitored for signs of active bleeding that may manifest as tachycardia, hypotension and potential hypoxemia. Although persistent melena, hematochezia or hematemesis may provide direct clinical evidence of active hemorrhage, hemodynamic instability despite vigorous resuscitation is the best indicator of active bleeding that may be angiographically demonstrable.

Acute gastrointestinal hemorrhage far more frequently involves an upper than a lower gastrointestinal source, with the former most commonly due to either peptic ulcer disease or gastritis. The most common etiology of lower gastrointestinal hemorrhage, in young adults, is inflammatory bowel disease, while in patients older than 50 years, the cause is diverticulitis (Figure 1) and to a lesser extent, angiodysplasia. The colon is the source of lower gastrointestinal hemorrhage in 80% of individuals, with the bleeding site originating in the ascending colon in one-third, the transverse colon in another one-third, and the remainder in the descending colon and rectosigmoid. A small bowel source of lower gastrointestinal hemorrhage occurs in only 20% of patients.

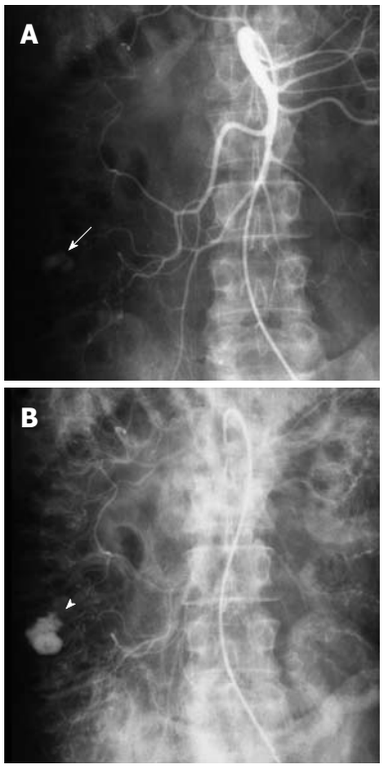

Figure 1 Example of a bleeding colonic diverticulum. A: Arterial phase of a superior mesenteric artery arteriogram, obtained in a patient with acute lower gastrointestinal (GI) bleeding and a prior history of diverticulitis shows a rounded contrast collection (white arrow) arising from a branch of the right colic artery; B: In the later arterial phase the collection (white arrowhead) has increased in size but maintains the rounded configuration. The extravasated contrast medium is pooling in a colonic diverticulum, indicating that diverticular hemorrhage is the etiology of the lower GI bleeding.

If an upper gastrointestinal bleeding source seems most likely, endoscopy should be the initial diagnostic study, as the source can usually be identified and treated by the endoscopist. Unfortunately the endoscopic detection and treatment success rates in patients with lower gastrointestinal hemorrhage are generally less than with an upper source, particularly if rapid and significant bleeding is present.

IMAGING PRIOR TO ANGIOGRAPHY

If a bleeding site cannot be identified endoscopically, or if a catheter-based intervention is being considered as a treatment option, obtaining imaging studies may be helpful. In order to successfully demonstrate bleeding by angiography, there must be active ongoing hemorrhage at the time of the examination. Bleeding rates of 0.5-1.0 mL/min have traditionally been considered necessary in order to angiographically demonstrate contrast extravasation[2], but digital subtraction angiography (DSA) may be far more sensitive in detecting active extravasation than was previously thought[3]. Given the well-recognized intermittent nature of gastrointestinal bleeding, many advocate obtaining other imaging before proceeding directly to angiography unless there is massive ongoing

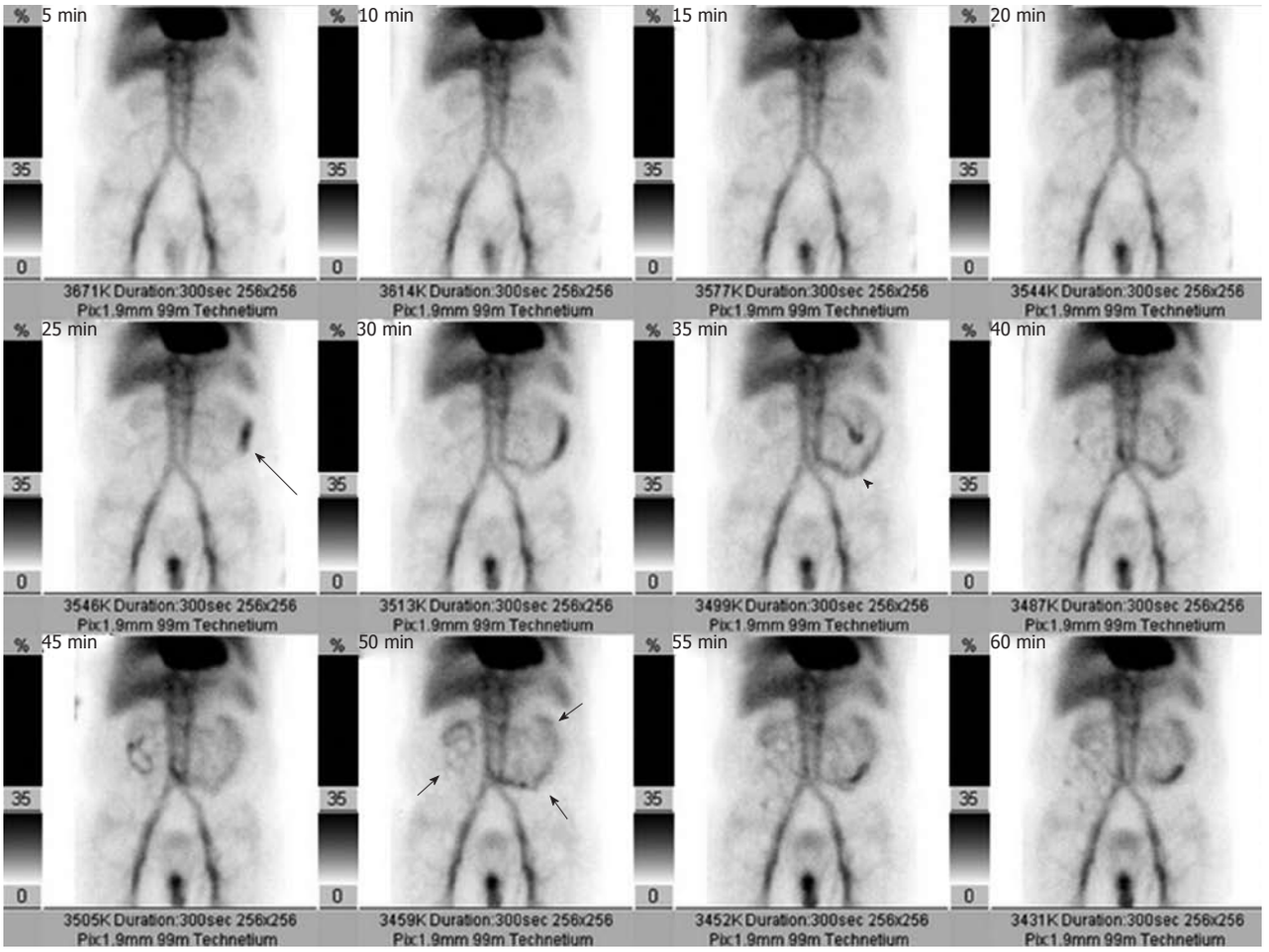

Figure 2 A radionuclide Tc^{99m}-labeled red blood cell scan obtained in a patient with acute lower gastrointestinal hemorrhage shows radioisotope accumulation in the left abdomen at 25 min (black arrow), which by 35 min has a small bowel location (black arrowhead) and by 50 min has distributed throughout much of the small intestine (short black arrows). The study demonstrates that the bleeding source is in the small bowel, and thus serves to appropriately direct either angiography or surgery.

hemorrhage or the patient is hemodynamically unstable. Thus a patient may be first evaluated with a radionuclide technetium^{99m}-tagged red blood cell scan, as the nuclear scintigraphy study can demonstrate active bleeding at rates as low as 0.1 mL/min[4-6]. An arteriogram can be obtained following a positive bleeding scan (Figure 2), as a positive scintigram increases the likelihood of a positive angiogram from 22% to 53%[7]. The potential disadvantage of first obtaining a radionuclide study while there is clinical evidence of active bleeding is that prior to angiography the bleeding may cease during the delay necessitated by the nuclear medicine scan. A potentially useful algorithm to consider is to immediately perform angiography in hemodynamically unstable patients, and to first obtain nuclear medicine imaging in hemodynamically stable patients. This approach may potentially decrease the negative angiography examination rate.

ANGIOGRAPHIC EVALUATION OF ACUTE GASTROINTESTINAL HEMORRHAGE

Any angiographic evaluation of a patient with acute gastrointestinal hemorrhage should begin with the selective catheterization of the artery supplying the most likely site of bleeding, as determined by the available clinical, endoscopic and imaging data. Thus for suspected upper gastrointestinal hemorrhage, the celiac artery should first be evaluated (Figure 3), followed by the superior mesenteric artery (SMA), as the latter may contribute to a site of upper gastrointestinal hemorrhage through the pancreaticoduodenal arcade. The lower gastrointestinal tract, however, is the primary territory within the distribution of the SMA. It supplies the small bowel and the ascending and transverse portions of the colon, while the inferior mesenteric artery (IMA) supplies the splenic flexure, descending and sigmoid colonic segments as well as the rectum and anus. An additional arterial supply to the rectosigmoid and anus arises from the internal iliac arteries and this may become a dominant vascular pathway if there is IMA occlusion. The SMA becomes another collateral pathway in the presence of IMA occlusion and may provide the entire arterial supply to the descending and sigmoid colon *via* either the Arc or Riolan or the marginal artery of Drummond. One should be aware of the numerous mesenteric circulatory variations that may

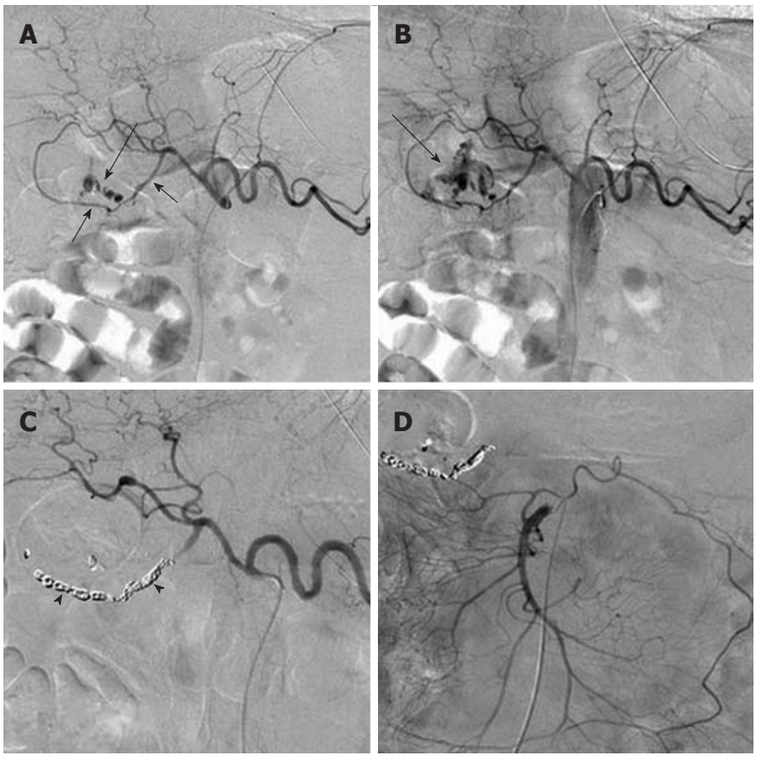

Figure 3 Angiographic diagnosis and transcatheter treatment of duodenal hemorrhage. A: Celiac digital subtraction angiography (DSA) arteriogram obtained in a patient with copious bleeding seen endoscopically in the duodenum shows focal contrast extravasation (black arrow) arising from the gastroduodenal artery (GDA); B: An image slightly later in the arterial phase of the DSA shows increasing extravasation (black arrow); C: The GDA was successfully coil embolized using microcoils (black arrowheads) through a microcatheter; D: An superior mesenteric artery DSA arteriogram was performed after the coil embolization in order to exclude any additional contribution to the duodenal hemorrhage from the pancreaticoduodenal arcade, as the duodenum has a rich collateral blood supply.

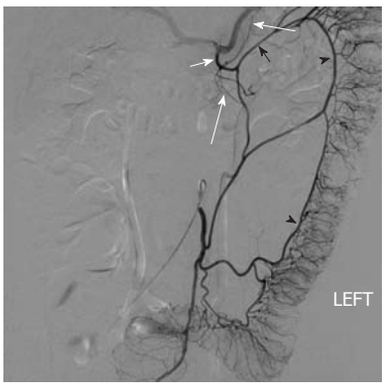

Figure 4 Inferior mesenteric artery digital subtraction angiography arteriogram shows the marginal artery of Drummond (black arrowheads), which courses along the mesenteric border of the colon. There is retrograde filling of the left branch of the middle colic artery (black arrow), which arises from the dorsal pancreatic artery (white arrow) as an anatomic variant. There is also filling of pancreatic branches (long white arrows) along with partial opacification of the splenic and hepatic arteries.

occur in the presence of occlusive disease. Furthermore, congenital variant vascular anatomy must be considered during the angiographic evaluation of gastrointestinal bleeding. For example, the entire middle colic artery may originate from the dorsal pancreatic artery in up to 2% of patients (Figure 4). If this situation exists, a celiac arteriogram may be required when investigating lower gastrointestinal bleeding, in order to fully evaluate the colonic arterial supply, particularly in the presence of negative SMA and IMA arteriograms. If SMA, IMA, celiac, and internal iliac arteriography fail to either localize active hemorrhage or to demonstrate all the mesenteric vascular segments, then variant anatomy must be considered. There are known anomalous vessels that may arise directly from the abdominal aorta, such as an anomalous ileocolic artery or a middle mesenteric artery.

The classic angiographic finding denoting active gastrointestinal bleeding is extravasation of contrast material (Figure 5). Extravasation signifies a breach in the integrity of the arterial wall that permits the angiographic contrast to freely exit from the vessel. Extravasated contrast, therefore, does not have the typical tubular appearance of a vascular structure but instead distributes irregularly and often without a pattern. It may sometimes pool within the gastric rugae or within bowel folds or haustra so that the contrast assumes the appearance of a vein, the "pseudo-vein sign" (Figure 6). This may be differentiated from a true venous structure by the unusual location and appearance, as well as by the persistence beyond the venous phase of the contrast injection. Extravasation must also be differentiated from entities that mimic its appearance, such as a hypervascular bowel mucosa, adrenal gland vascular blush and DSA misregistration artifacts from bowel peristalsis or respiratory motion. There are also angiographic findings other than contrast extravasation that may be seen in certain patho-

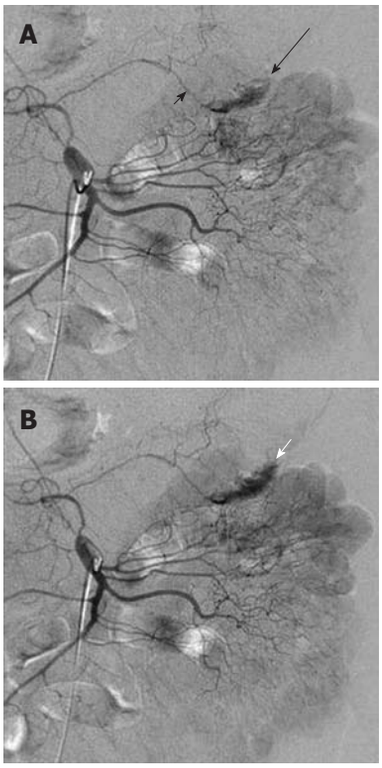

Figure 5 Contrast extravasation demonstrating location of lower gastrointestinal bleeding. A: Superior mesenteric artery arteriogram shows an amorphous contrast collection (black arrow) arising from the left branch (long black arrow) of the middle colic artery; B: Later in the arterial phase the collection has increased and is layering dependently in the colon, assuming the configuration of the haustra. This extravasated contrast medium denotes the site of lower gastrointestinal bleeding.

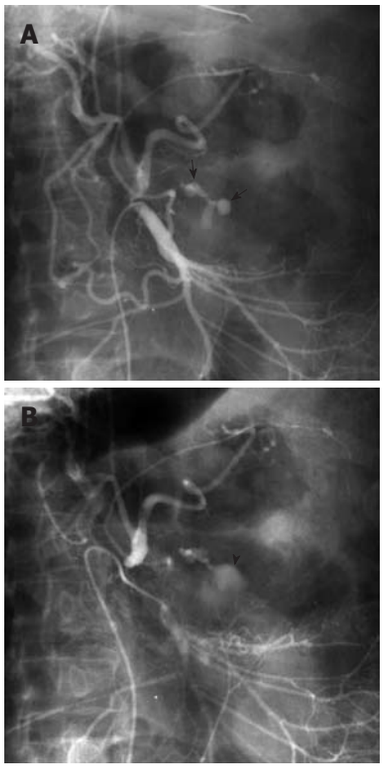

Figure 6 The pseudovein sign in gastrointestinal hemorrhage. A: Superior mesenteric artery arteriogram shows a branching contrast collection (black arrows), overlying the gastric air shadow. The collection has the appearance of a vascular structure such as a vein; B: A later image in the arterial injection shows that the collection (black arrowhead) has a more amorphous appearance, and represents extravasation. The former appearance is an example of the "pseudovein sign".

logic conditions and are suggestive of the cause and/or source of the gastrointestinal bleeding. In peptic ulcer disease, for example, small contrast collections may be seen within an ulcer crater, or may outline the gastric or duodenal mucosa. The angiographic demonstration of a gastric ulcer usually requires subselective catheterization of celiac arterial branches such as the left gastric artery, although the bleeding source may also potentially arise from the right gastric artery, the short gastric artery, or either the left or right gastroepiploic arteries. Additionally, the gastroduodenal artery may supply a bleeding pyloric or duodenal ulcer.

Arterial pseudoaneurysm is another angiographic abnormality that may be identified as a source of gastrointestional hemorrhage. These occur most frequently in patients who have chronic pancreatitis. Hemosuccus pancreaticus refers to bleeding through the pancreatic duct, and may be caused by an arterial pseudoaneurysm that has resulted from chronic exposure of the arterial wall to the inflammatory effects of the pancreatic digestive enzymes[8]. The weakened arterial wall may permit the intermittent bleeding that characterizes hemosuccus pancreaticus, or the pseudoaneurysm may catastrophically rupture and cause acute massive, life-threatening intra-abdominal hemorrhage. Contrast enhanced computed tomographic angiography can be very effective in demonstrating these characteristic pseudoaneurysms (Figure 7) and thus has an increasingly important role in the diagnosis of acute gastrointestinal hemorrhage that is secondary to pancreatitis, while angiographic transcatheter therapy provides the best treatment option in these patients (Figures 8 and 9). Given both the diffuse nature of the inflammatory process seen in pancreatitis, and the pancreatic arterial supply from both the celiac artery and SMA, pseudoaneurysms may be present involving either or both of these arteries or their branches. The angiographic evaluation must thus include both the celiac and SMA and transcatheter treatment may involve multiple sites. Surgical intervention is very difficult in these patients because of the extensive inflammatory process that characterizes this cause of bleeding (Figure 10).

Arterial pseudoaneurysms may also be seen in patients with chronic occlusive disease of the celiac artery, and involve the pancreaticoduodenal arteries (Figure 11). These pseudoaneurysms are rupture-prone and may cause massive acute upper gastrointestinal hemorrhage. Because of the occlusion of the origin of the celiac artery, the angiographic evaluation involves the selective catheterization of the SMA, in order to image the pancreaticoduodenal arcade. Any intervention, such as coil embolization, must typically also be performed *via* an SMA access, although there are cases in which a percutaneous route has been

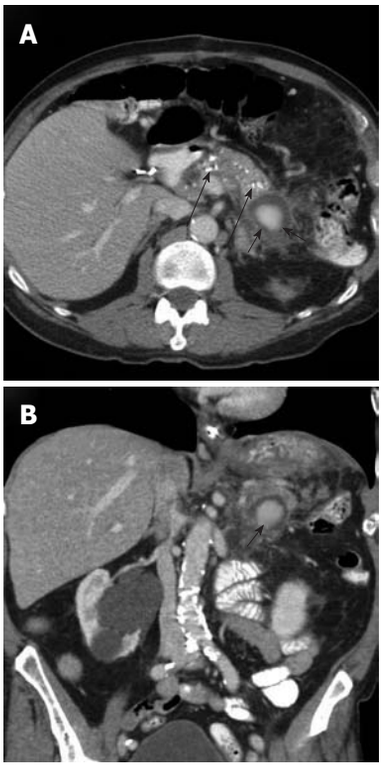

Figure 7 Upper gastrointestinal hemorrhage from pancreatitis related pseudoaneurysm. A: Axial contrast-enhanced computed tomography (CECT) scan, obtained in a patient who presented with hematemesis, shows pancreatic calcifications (long black arrows) indicating chronic pancreatitis and an enhancing mass (black arrows) in the pancreatic tail; B: Coronal CECT shows the rounded enhancing mass (black arrow) with surrounding inflammatory changes. This is suspicious for a pancreatitis-related pseudoaneurysm as the source of the upper gastrointestinal hemorrhage.

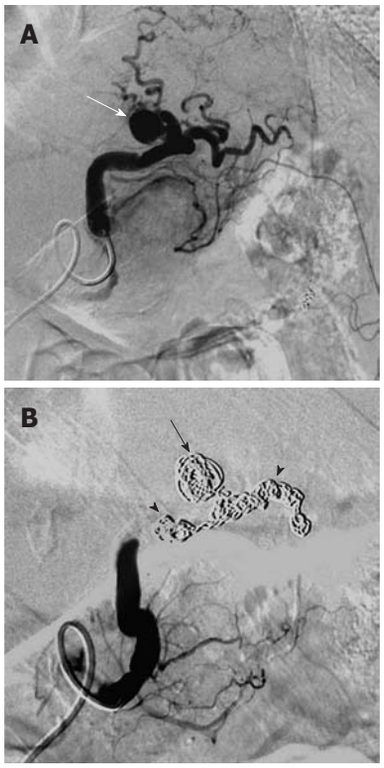

Figure 8 Transcatheter treatment of a pancreatitis related pseudoaneurysm. A: Selective splenic artery digital subtraction angiography arteriogram shows that the pseudoaneurysm (white arrow) arises directly from the splenic artery; B: Coil embolization of the pseudoaneurysm (black arrow) and of the splenic artery (black arrowheads) both proximal and distal to the pseudoaneurysm was successful in controlling the hemorrhage. Arterial pseudoaneurysms associated with pancreatitis have a significant risk of rupture; if this occurs there is an extremely high mortality rate.

used to treat such an abnormality.

Angiodysplasia and arteriovenous malformations (AVMs) of the bowel are characterized by the early and prolonged opacification of a draining vein, accompanied by an abnormal tangle of vessels that may appear as a blush (Figure 12). The simultaneous filling of the feeding artery and the draining vein may give a characteristic "tram-track" sign. Although up to 80% of angiodysplastic lesions are in the right colon, other parts of the gut, particularly the lower small intestine, may be affected. Angiodysplasia is relatively common in older patients, aged 60 to 80 years, and may be an asymptomatic incidentally noted lesion identified at colonoscopy. Because the dilated vessels are superficial, however, they may bleed spontaneously and patients can either present acutely with overt hemorrhage or insidiously with iron deficiency anemia. Once bleeding has begun, recurrent episodic hemorrhage or persistent iron deficiency anemia requiring repeated transfusion is not uncommon. Symptomatic lesions may be effectively treated endoscopically with laser or heat coagulation or with sclerotherapy. These procedures are not risk-free in the thin cecal wall and have been known to cause serosal irritation and post-treatment bleeding. For patients with repeated bleeding from intestinal vascular malformations, pharmacological treatment using estrogens (e.g., 0.05 mg ethinyl estradiol and 1 mg norethisterone) can be effective in reducing transfusion requirements. Although transcatheter arterial embolization of colonic angiodysplasia can be an effective treatment in emergencies[8-11], it carries a significant risk of inducing colonic ischemia[12]. Definitive and curative treatment usually requires surgical resection.

ANGIOGRAPHIC MANAGEMENT OF ACUTE GASTROINTESTINAL HEMORRHAGE

Transcatheter arterial embolization

Transcatheter arterial embolization is an effective means of interrupting blood flow to the bleeding source, while maintaining bowel viability. Although there is a risk of bowel ischemia and/or infarction, the coaxial catheter systems and the variety of available embolic agents that are now used for embolotherapy allow for very selective and precise treatment, and thus have decreased the incidence of these complications. Additionally, the gastrointestinal tract has a rich collateral blood supply, with extensive vascular arcades that permit safe embolization

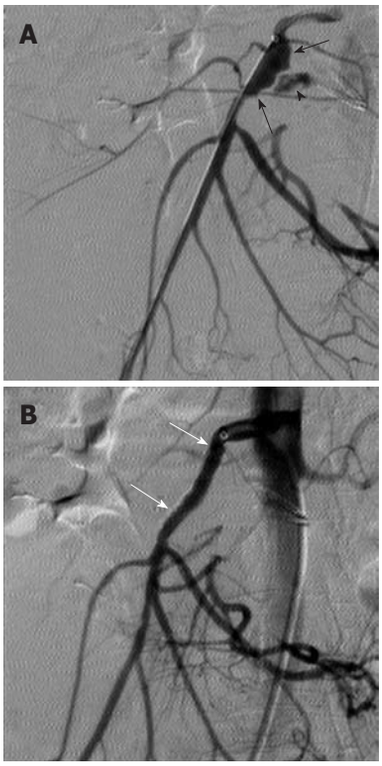

Figure 9 Treatment of ruptured pseudoaneurysm with covered intravascular stent. A: Digital subtraction angiography arteriogram obtained in a patient with upper gastrointestinal bleeding and a history of pancreatitis shows irregular dilatation of the proximal superior mesenteric artery (black arrows) and contrast extravasation (black arrowhead) consistent with pseudoaneurysm rupture; B: The hemorrhage was successfully controlled with covered stent (white arrows) placement.

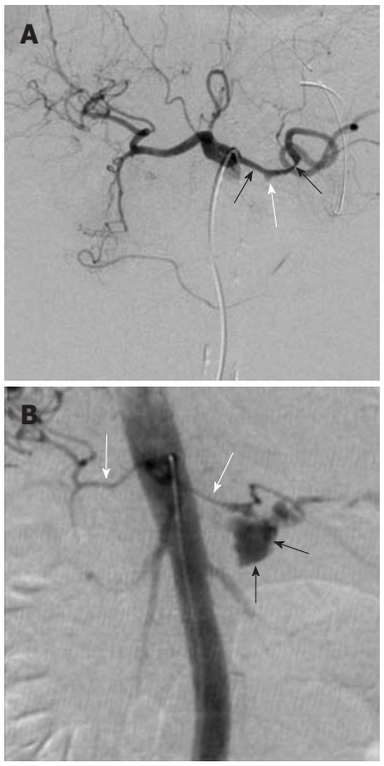

Figure 10 Life-threatening hemorrhage from pancreatitis related pseudoaneurysm. A: Celiac digital subtraction angiography arteriogram obtained in a patient with intermittent upper gastrointestinal bleeding and a history of chronic pancreatitis shows an irregular caliber to the splenic artery (black arrows) and a small pseudoaneurysm (white arrow). No intervention was performed at the time of this examination; B: Two weeks after the celiac arteriogram the patient presented with acute onset of severe abdominal pain and profound hypotension. Repeat angiography showed brisk contrast extravasation (black arrows) from the splenic artery. Note the marked vasoconstriction (white arrows) of the hepatic and splenic arteries due to the life-threatening hemorrhage.

if certain principles are observed. The objective of embolization therapy is to achieve a compromise between selective arterial inflow reduction and maintenance of collateral arterial blood flow. Arterial inflow must be sufficiently decreased to allow for hemostasis, but not to an extent that causes complete devascularization. Achieving a superselective embolization[11] in the presence of an intact coagulation cascade is key to attaining a successful outcome using this form of treatment.

In most of the early literature, embolization was performed proximally in the visceral arteries because microcatheters facilitating superselective embolization were not yet available. The technique that is currently used for transcatheter arterial embolization involves the initial placement of a larger caliber diagnostic catheter (4 or 5 Fr) into the main trunk of the feeding artery, followed by coaxial introduction of a microcatheter. The latter is the crucial step in modern embolization and requires the superselective catheterization of the target artery using a 3-Fr coaxial microcatheter over a 0.018 inch or 0.014 inch guidewire. The guidewire tip must be carefully advanced under fluoroscopic monitoring in a smooth and controlled fashion in order to avoid vasospasm, dissection, or vessel perforation. Vasodilators such as verapamil (100-200 μg) or nitroglycerin (100-300 μg) may be used to treat any vasospasm that may be induced by guidewire and/or microcatheter passage. Using the wire as a guide, the catheter should travel closely behind and eventually engage the target vessel.

The optimal level of embolization varies according to the bleeding site. In general, embolization should not be routinely attempted unless a microcatheter has been advanced close to the bleeding point so that the embolic agent can be deployed as selectively as possible. The risk of infarction is related to both the embolic agent and the proximity of embolization. In the colon, for example, ischemia and infarction may result from embolization of proximal branches supplying a large area of bowel or embolization of multiple distal arteries that do not have sufficient collateral flow. Submucosal collateral blood flow may be preserved only when arteries to a short segment of bowel are embolized, so one should attempt to occlude arteries to as limited a segment of bowel as possible. One must be aware that embolization in the setting of prior gastrointestinal surgery or radiation therapy may impose a greater risk of infarction because of the associated interruption of collaterals.

Various agents may be used for transcatheter embolization. The most commonly used agents include pledgets of absorbable gelatin sponge (Gelfoam®, Pfizer, Inc., NY,

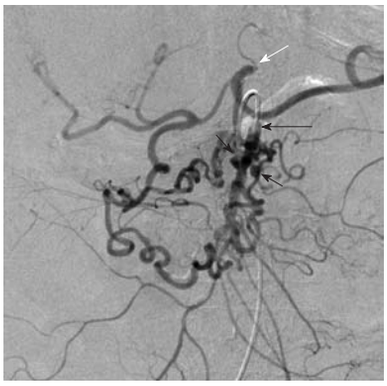

Figure 11 Digital subtraction angiography obtained with the catheter tip (long black arrow) in the superior mesenteric artery shows tortuous and dilated inferior pancreaticoduodenal arteries with at least two small pseudoaneurysms (black arrows). There is retrograde filling of the celiac arterial branches *via* the inferior pancreaticoduodenal arteries arcade, because the celiac origin is occluded (white arrow). The pseudoaneurysms are at risk of rupture.

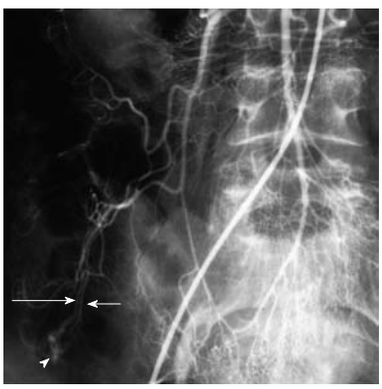

Figure 12 Magnified view from a superior mesenteric artery arteriogram shows an area of abnormal vascularity (white arrowhead) in the cecal area, with an early draining vein (long white arrow) paralleling the arterial inflow (white arrow). This simultaneous filling of the feeding artery and draining vein is known as the "tram-track" sign and is characteristic of angiodysplasia.

United States), particulate agents such as polyvinyl alcohol (e.g., Bead Block™, Biocompatibles International, Farnham, United Kingdom) and other spherical agents (e.g., Embospheres® BioSphere Medical, Inc., Rockland, MA, United States; Embozene™ microspheres CeloNova BioSciences, Inc., Newnan, GA, United States) and microcoils of various sizes and configurations. Microcoils have the advantage of good radiopacity that allows for a precise deployment permitting reduction of the arterial perfusion pressure to the bleeding site while preserving sufficient collateral flow. The wide range of coil sizes allows one to appropriately match the coil to the target vessel diameter. Each microcoil is delivered sequentially, until hemostasis has been achieved. Intra-arterial microcoil placement is analogous to placement of a surgical ligature. The coil physically occludes the vascular lumen and causes a decreased perfusion pressure, while the attached synthetic fibers maximize thrombogenicity.

Gelfoam pledgets and particulate agents may also be used successfully, but are more difficult to control than microcoils. Gelfoam is a temporary agent and often cannot easily be deployed superselectively. A disadvantage of the particulate agents is that small diameters may reach the intramural circulation distal to the collaterals, thereby risking bowel infarction, or may reflux into nontarget arteries.

The liquid embolic agents n-butyl cyanoacrylate, known by the proprietary name Trufill® (Cordis Neurovascular, Miami Lakes, FL, United States), and liquid polyvinyl alcohol copolymer (Onyx®, ev3 Neurovascular, Irvine, CA, United States) have also been used successfully in treating gastrointestinal hemorrhage[13,14]. An advantage of liquid agents is that they may be used effectively in very small caliber vessels. However, the operator must be very familiar with the use of these agents, in order to achieve optimal outcomes and minimize complications.

Technical challenges of transcatheter arterial embolization

Because of the intermittent nature of gastrointestinal hemorrhage, arteriography fails to demonstrate a distinct bleeding site in a considerable number of patients, and thus embolization is not possible. Furthermore, a negative arteriogram fails to guide emergency surgery and delays operative decision-making. In such circumstances, some investigators have advocated provocation of bleeding with vasodilators, anticoagulants, and/or thrombolytics in association with tagged red blood cell scans or angiography[15,16]. This may be appropriate for a patient who has undergone multiple blood transfusions and a prior exhaustive work-up that has failed to localize the occult bleeding site. Additionally there must be no contraindications to the administration of a thrombolytic agent. If provocative angiography is undertaken, one should arrange for surgical backup in the event that uncontrollable bleeding occurs.

Different methods of inducing bleeding and different rates of success have been reported. An optimal protocol has yet to be established and the procedure has also yet to become accepted by clinicians as part of the evaluation of gastrointestinal bleeding. The technique continues to evolve as experience and comfort with the use of thrombolytic agents in the setting of nonlocalized bleeding increases. One reported protocol used a combination of intravenous heparin, intra-arterial tolazoline, and intra-arterial tissue-type plasminogen activator (t-PA) to provoke bleeding. Doses used included 3000-10 000 U heparin, 25-100 mg intra-arterial tolazoline, and 10-50 mg intra-arterial t-PA (mean, 20.3 mg). The investigators also noted that more patients had bleeding provoked after smaller rather than larger doses of t-PA[16]. Tolazoline (Priscoline) was formerly used for a vasodilatory effect, but was withdrawn from the United States market in 2002 by Novartis Pharmaceuticals, the sole manufacturer of this drug. Alternative intra-arterial vasodilators that may be used include verapamil (100-200 μg) and nitroglycerin (100-300 μg), with the former showing the greater vasodilatory effects. In the authors' practice, we have occasionally used a similar transcatheter regimen, in

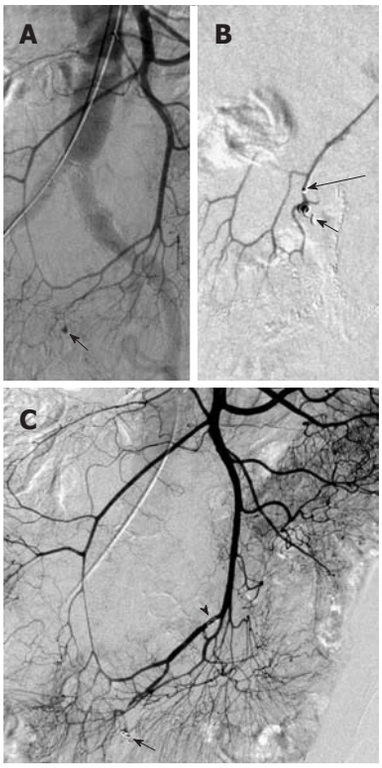

Figure 13 Example of nontarget embolization during treatment of lower gastrointestinal bleeding. A: Superior mesenteric artery arteriogram obtained in a patient with lower gastrointestinal bleeding shows a focal contrast collection (black arrow) arising from a branch of the ileocolic artery; B: A microcatheter was introduced and was subselectively positioned with the tip (long black arrow) immediately proximal to the focal extravasation and a microcoil (black arrow) was placed; C: While attempting to place a second microcoil, the microcatheter tip was displaced proximally resulting in coil placement in a nontarget location (black arrowhead). Repeat digital subtraction angiography shows that the second coil is nonocclusive and the initially placed coil has successfully controlled the bleeding source.

which a vasodilator and a dose of 5000 U intra-arterial heparin and 5-10 mg intra-arterial t-PA were administered, with resultant provocation of bleeding that allowed for subsequent successful transcatheter embolization.

Superselective embolization of the arterial supply to the bleeding source may be technically demanding, particularly in older patients who may have significant atherosclerotic disease. The mesenteric vasculature and the various arterial arcades are often tortuous, and the smaller arteries are prone to vasospasm and care must be taken to avoid dissection.

An arterial bleeding site may receive a dual blood supply as a result of the rich collateral arcades that characterize the mesenteric circulation. One must therefore catheterize and inject both potential sources of perfusion to the lesion and be prepared to embolize two separate vessels if necessary. Although this dual approach will control the hemorrhage, it will also increase the risk of bowel ischemia[17].

While angiodysplasia and AVMs may initially respond to embolization, recurrent hemorrhage is frequent and, as noted, surgical resection of the involved bowel segment is often required. A small bowel AVM is much more easily localized at surgery if an embolization coil has been placed distally in the arterial branch that supplies the lesion, so that it is palpable or visible to the surgeon[18].

Results of transcatheter arterial embolization

The current technique of embolization in the treatment of acute gastrointestinal hemorrhage successfully controls bleeding in about 80%-90% of patients[19-23]. Recurrent hemorrhage is infrequent, with the exception of angiodysplasia, AVMs and inflammatory lesions. Recurrences can usually be angiographically re-evaluated and, if a bleeding source is identified, treated with repeat embolization.

Complications of transcatheter arterial embolization

Transcatheter arterial embolization for the treatment of acute gastrointestinal hemorrhage is safe, with major adverse events occurring in less than 2% of patients. A fraction of patients embolized superselectively will develop minor, asymptomatic and self-limited ischemic changes such as small ulcers that can only be detected incidentally *via* objective follow-up methods such as endoscopy, pathologic surgical specimen or by a radiographic imaging examination. Additionally, superselective microcoil embolization is unlikely to result in delayed infarction. If major bowel ischemia occurs several months to years later, it is more likely to be attributable to a new and acute insult such as thromboembolic disease affecting the mesenteric arterial bed.

Non-target embolization with microcoils is rare, as the coils are introduced only after a microcatheter has been successfully negotiated into the target vessel. One must carefully choose appropriate sized microcoils however, as a coil that is oversized relative to the target vessel may displace the microcatheter from its superselective position. This could lead to deployment of the microcoil in a non-target location (Figure 13). Similarly, undersized coils may fail to adequately occlude the target vessel or may lodge distal to the lesion that is to be treated.

Vasopressin infusion therapy

Vasopressin (Pitressin) is a naturally occurring hormone that causes constriction of both the mesenteric arteries and of the smooth muscle of the bowel wall. The intra-arterial transcatheter infusion of vasopressin proximal to a mesenteric arterial bleeding site will reduce blood flow, thereby lowering the perfusion pressure and permitting clot formation at the lesion (Figure 14). There are several situations in which this form of treatment for gastrointestinal bleeding should not be used. These include bleeding that originates from a large diameter artery such as the gastroduodenal, splenic or proximal SMA or which occurs at a site with a dual blood supply such as the duodenum. It is also contraindicated in patients who have severe coronary artery disease, extreme hypertension, limb ischemia or cardiac arrhythmias. Superselective embolotherapy is now used preferentially over vasopressin infusion for treating gastrointestinal hemorrhage because embolization poses fewer risks and can

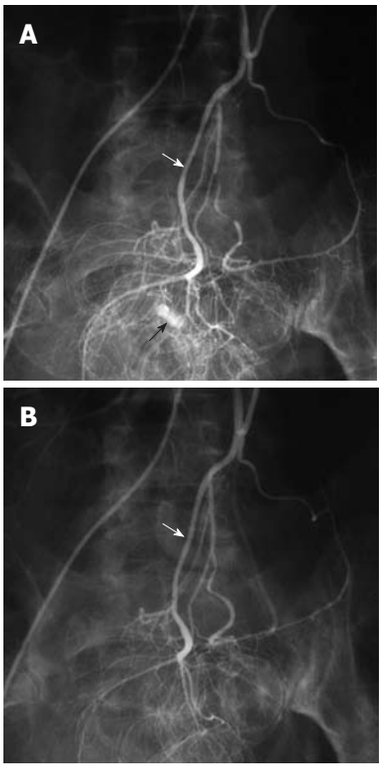

Figure 14 Vasopressin infusion therapy for lower gastrointestinal hemorrhage. A: Inferior mesenteric digital subtraction angiography arteriogram shows contrast extravasation (black arrow) in the rectosigmoid colon, arising from a branch of the superior hemorrhoidal artery (white arrow); B: Following transcatheter arterial vasopressin infusion, there is no longer any evidence of active bleeding from the superior hemorrhoidal (white arrow) arterial distribution.

be completed more rapidly than a vasopressin infusion protocol. Vasopressin may still be useful in certain situations, despite the numerous side effects and the high rebleeding rates. One may consider using vasopressin for treating lesions that are inaccessible to a microcatheter, for diffuse mucosal oozing, and for controlling multiple sites of hemorrhage in high-risk surgical patients.

Technique of vasopressin infusion therapy

Vasopressin is typically infused into the central vessel (e.g., celiac artery, SMA or IMA) that supplies the bleeding site *via* a catheter that has been placed proximally; to avoid bowel ischemia and potential infarction, a distal infusion should not be attempted. Vasopressin (100 U) is mixed in 500 mL of normal saline and the infusion is started at 0.2 U/min for 20 min using an arterial infusion pump set at 60 mL/min. If there is no cessation of bleeding, the dose is increased by 0.1 U/min up to a maximum of 0.4 U/min; each dosage change is followed by a repeat angiogram 20 to 30 min later to assess the effectiveness[24]. If the bleeding stops, the catheter is left in place for a 24-h infusion at the effective dosage, with monitoring in the intensive care unit. After another repeat angiogram shows control of bleeding, the vasopressin infusion is gradually reduced over 24 to 48 h and then vasopressin is replaced with an infusion of normal saline or 5% dextrose in water for 6-12 h.

Results of vasopressin infusion therapy

This form of therapy is particularly effective in controlling diverticular and gastric mucosal hemorrhage, with initial success rates ranging from 60%-90%[24-27]. As previously noted, however, there is a very high rate of rebleeding that may be up to 50%.

Complications of vasopressin infusion therapy

Mild abdominal pain may occur at the initiation of the infusion and should be closely monitored, as persistence and/or worsening can be an indicator of bowel ischemia. If side effects develop during treatment, the vasopressin can be tapered to a lower dose or may need to be discontinued. Additional side effects and potential complications of vasopressin therapy include angina, myocardial infarction, hypertension, volume overload, abdominal cramps, and mesenteric ischemia. Some of the potential adverse effects may be treated or even pretreated. The simultaneous administration of intravenous, sublingual or transdermal nitroglycerin may prevent or reverse the cardiotoxic side effects of vasopressin infusion[28].

CONCLUSION

Technical refinements and advances both in diagnostic angiography and in transcatheter arterial embolization have strengthened these options for the evaluation and management of acute gastrointestinal hemorrhage that is refractory to medical and endoscopic therapy. Highly sensitive noninvasive imaging modalities such as nuclear scintigraphy and contrast-enhanced computed tomography are extremely useful adjuncts to angiography as they often can localize and characterize the bleeding source, confirm active hemorrhage and aid in planning an appropriate transcatheter intervention. Superselective catheterization using a coaxial system that allows for microcoil embolization is an effective and safe alternative to emergency surgery. Other embolic agents such as gelatin sponge, spherical particles and liquids also have a role in transcatheter management of gastrointestinal bleeding as do transcatheter therapies such as covered stent placement and vasopressin infusion.

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TOPIC HIGHLIGHT

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Management of acute nonvariceal upper gastrointestinal bleeding: Current policies and future perspectives

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Abstract

Acute upper gastrointestinal bleeding (UGIB) is a gastroenterological emergency with a mortality of 6%-13%. The vast majority of these bleeds are due to peptic ulcers. Nonsteroidal anti-inflammatory drugs and *Helicobacter pylori* are the main risk factors for peptic ulcer disease. Endoscopy has become the mainstay for diagnosis and treatment of acute UGIB, and is recommended within 24 h of presentation. Proton pump inhibitor (PPI) administration before endoscopy can downstage the bleeding lesion and reduce the need for endoscopic therapy, but has no effect on rebleeding, mortality and need for surgery. Endoscopic therapy should be undertaken for ulcers with high-risk stigmata, to reduce the risk of rebleeding. This can be done with a variety of modalities. High-dose PPI administration after endoscopy can prevent rebleeding and reduce the need for further intervention and mortality, particularly in patients with high-risk stigmata.

INTRODUCTION

Acute upper gastrointestinal bleeding (UGIB) is the most common gastroenterological emergency and has a considerable morbidity and mortality. Management strategies have changed dramatically over recent decades due to the introduction of acid suppressive therapy [histamine-2 receptor antagonists and especially proton pump inhibitors (PPIs)] and endoscopic therapy. This review deals with the current standards and future perspectives in management of acute nonvariceal UGIB.

EPIDEMIOLOGY

The incidence rates of UGIB demonstrate a large geographic variation ranging from 48 to 160 cases per 100 000 population, with consistent reports of higher incidences among men and elderly people[1-5]. Possible

Table 1 Mortality rates in patients with upper gastrointestinal bleeding in various studies

	Czernichow *et al*[5]	Paspatis *et al*[4]	Van Leerdam *et al*[3]	Di Fiore *et al*[7]	Theocharis *et al*[1]	Hearnshaw *et al*[10]
Country	France	Greece	The Netherlands	France	Greece	United Kingdom
Year of publication	2000	2000	2003	2005	2008	2010
No. of patients	2133	353	769	453	353	6750
Mortality rate total (%)	14.3	5.6	13	7.2	6.5	7.4
Varices (%)	22.8	21.4	16	15.2	9	15
Peptic ulcer (%)	13.3	2.6	14	5	4.2	8.7

Table 2 Causes of upper gastrointestinal bleeding according to recent epidemiological studies[1,3-5,7,10]

	%
Peptic ulcer	31-67
Erosive	7-31
Variceal bleeding	4-20
Oesophagitis	3-12
Mallory-Weis	4-8
Neoplasm	2-8
Other	2-8
None	3-19

explanations for the reported geographic variation in incidence are differences in definition of UGIB in various studies, population characteristics, prevalence of ulcerogenic medication, in particular aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs), and *Helicobacter pylori* (*H. pylori*) prevalence. Some but not all time-trend studies have reported a significant decline in incidence of acute UGIB, especially peptic ulcer bleeding, in recent years[1,3,6]. This decline is likely due to a combination of factors, including decreasing prevalence of gastric colonization with *H. pylori*[1], the use of eradication therapy in patients with ulcer disease, and the increased use of PPI therapy, both in general and in patients using aspirin and NSAIDs in particular.

Despite the introduction of therapeutic endoscopy and acid-suppressive therapy, the overall mortality of UGIB has remained stable over recent decades and is still 6%-14% in most studies (Table 1)[1,3-5,7]. The majority of deaths do not directly result from exsanguination, but are related to poorly tolerated blood loss and resultant shock, aspiration, and therapeutic procedures. As such, mortality from UGIB is strongly associated with advanced age and presence of severe comorbidity. The risk of mortality increases with rebleeding, which is thus another major outcome parameter[5]. The incidence of rebleeding in patients with UGIB shows a wide range from 5% to more than 20%, depending on several factors[3,4]. These firstly include the etiology of the bleeding, with rebleeding being more common in patients with variceal bleeding (25%) and uncommon in patients with small mucosal lesions such as Mallory-Weiss lesions. A second factor that determines the frequency of rebleeding is the timing and use of adequate endoscopic therapy. There is strong evidence that the risk of rebleeding is highest in the initial period of admission, and a 24-h time frame for endoscopic therapy is internationally recommended as the optimal window of opportunity[8,9]. Mortality amongst those with recurrent bleeding is considerably higher, therefore, rebleeding must be prevented whenever possible[8].

Peptic ulcer bleeding (PUB) is the most common cause of UGIB, accounting for 31%-67% of all cases, followed by erosive disease, variceal bleeding, esophagitis, malignancies and Mallory-Weis tears (Table 2)[1,3-5,7,10]. In 2%-8% of cases, uncommon causes such as Dieulafoy's lesion, hemobilia, angiodysplasia, vasoenteric fistula, and gastric antral vascular ectasia have been found. In the remainder of this paper, we mainly focus on PUB, yet the approach to and treatment of any patient with nonvariceal UGIB is for the most part comparable. Possible differences will be discussed in the section on endoscopic therapy.

In the subgroup of patients with PUB, bleeding from duodenal ulcers is slightly more frequent than from gastric ulcers[1,4]. NSAID use and *H. pylori* infection are independent risk factors for UGIB, especially PUB[8,11]. The prevalence of *H. pylori* infection in PUB patients varies between 43 and 56%[12-14], and treatment of *H. pylori* significantly reduces the rebleeding rate according to some randomized controlled trials[15,16].

PRE-ENDOSCOPIC MANAGEMENT

Initial resuscitation and risk stratification

Patients with UGIB can present with various symptoms such as hematemesis, hematochezia, melena, or progressive anemia. Immediate evaluation and appropriate resuscitation is of major importance in these patients. Stratification of patients in low- and high-risk categories for rebleeding and mortality can be done using the Blatchford and initial Rockall scores (before endoscopy), or complete Rockall score (after endoscopy) (Table 3)[17,18]. The Blatchford score is more focused on clinical symptoms and laboratory results, whereas the Rockall score considers age as a parameter.

Resuscitation includes intravenous administration of fluids, and supplemental oxygen, correction of severe coagulopathy, and blood transfusion when needed. The threshold for blood transfusion depends on the underlying condition, rate of bleeding, and vital signs of the patient, but is generally set at a hemoglobin level of $\leqslant$ 70 g/L[19]. A recent meta-analysis regarding outcomes following red blood cell transfusion in patients with UGIB, however, suggests that red blood cell transfusion is associated with

Table 3 Comparison of Blatchford and Rockall risk scoring systems

Risk factor	Blatchford score		Initial Rockall score	
	Parameter	Score	Parameter	Score
Age (yr)	-		60-79	1
			⩾ 80	2
Systolic blood pressure (SBP) (mmHg)	100-109	1	< 100	2
	90-99	2		
	< 90	3		
Heart rate (bpm)	> 100	1	> 100 with SBP ⩾ 100	1
Clinical presentation	Melena	1	-	
	Syncope	2		
Comorbidity	Hepatic disease	2	CHF, IHD, major comorbidity	2
	Cardiac failure	2	Renal or liver failure, or disseminated cancer	3
Blood urea, mg/dL (mmol/L)	18.2-22.3 (6.5-7.9)	2	-	
	22.4-27.9 (8-9.9)	3		
	28-69.9 (10-24.9)	4		
	⩾ 70 (⩾ 25)	6		
Hemoglobin, g/dL (mmol/L)	F: 10-11.9 (6.2-7.4) M: 12-12.9 (7.5-8)	1	-	
	M: 10-11.9 (6.2-7.4)	3		
	F/M: < 10 (< 6.2)	6		
			Complete Rockall score	
Endoscopic diagnosis	-		Non-malignant, non-Mallory-Weis diagnosis	1
			Upper GI tract malignancy	2
Evidence of bleeding	-		Blood, adherent clot, active bleeding	2

M: Male; F: Female; CHF: Congestive heart failure; IHD: Ischemic heart disease.

higher mortality and rebleeding rate. The conclusions of this study were limited by the small size of the studies and the large volume of missing data. In addition, the possibility that patients who present with more severe and active bleeding are more rapidly transfused, acted as a potential major confounder in these analyses[20]. This means that prospective studies need to be done with strict predetermined transfusion protocols, and that for now, the risks and benefits of blood transfusion must be carefully weighed individually.

Pre-endoscopic pharmacotherapy

Administration of PPIs before endoscopy has become common practice in patients suspected with PUB. A strongly acidic environment leads to inhibition of platelet aggregation and plasma coagulation as well as to lysis of already formed clots[21]. PPIs quickly neutralize intraluminal gastric acid, which results in stabilization of blood clots. In the longer term, antisecretory therapy also promotes mucosal healing. A recent systematic review has shown that pre-endoscopic PPI administration significantly reduces high-risk stigmata at index endoscopy (37% *vs* 46% respectively, OR: 0.67; 95% CI: 0.54-0.84) and need for endoscopic therapy (9% *vs* 12% respectively, OR: 0.68; 95% CI: 0.50-0.93). However, no effect on clinically important outcome measures such as rebleeding, mortality and need for surgery was seen[22].

Another pharmacotherapeutic approach includes the use of prokinetics before endoscopy, in particular, erythromycin or metoclopramide. A meta-analysis of five studies assessing a total of 316 patients with acute UGIB has found a significant reduction in the need for repeated endoscopy (OR: 0.55; 95% CI: 0.32-0.94) in the prokinetic treatment group compared to the reference group (placebo or no treatment). The groups did not differ in the need for blood products, hospital stay, and need for surgery[23]. Therefore, prokinetics are not routinely recommended, but can be useful in patients who are suspected of having substantial amounts of blood in the stomach[9]. Administration of PPIs and prokinetics should however not delay endoscopy.

ENDOSCOPY

Time to endoscopy

Endoscopy has become a valuable and indispensable tool for diagnosis and treatment of UGIB[24,25]. It allows for identification of the bleeding source and application of treatment in the same session. The optimal timing for endoscopy remains under debate. Emergency endoscopy allows for early hemostasis, but can potentially result in aspiration of blood and oxygen desaturation in insufficiently stabilized patients. In addition, extensive amounts of blood and clots in the stomach can hinder targeted treatment of the bleeding focus, which results in repeated endoscopic procedures. International consensus guidelines recommend early endoscopy within 24 h of presentation, because it significantly reduces the length of hospital stay and improves outcome[19]. Very early endoscopy (< 12 h) has so far not been shown to provide additional benefit in terms of reduction of rebleeding, surgery and mortality, compared with later endoscopy (within 24 h)[26-29]. However, emergency endoscopy should be considered in patients with severe bleeding.

Endoscopic therapy for PUB

The aim of therapeutic endoscopy is to stop any ongoing bleeding and prevent rebleeding. Several techniques, including injection therapy, ablative therapy and mechanical therapy have been studied over recent decades[24,30,31]. Depending on the appearance of the bleeding focus and the related risk for persistent or recurrent bleeding, a suitable technique should be chosen. In PUB, patients with active bleeding ulcers or a nonbleeding visible vessel in an ulcer bed are at highest risk of rebleeding and therefore need prompt endoscopic hemostatic therapy (Figures 1 and 2)[32]. Patients with low-risk stigmata (a clean-based ulcer or a pigmented spot in an ulcer bed) do not require endoscopic therapy.

The role of endoscopic therapy for ulcers with adherent clots has been a topic of debate[19]. The risk of rebleeding depends on underlying lesions, so that clot re-

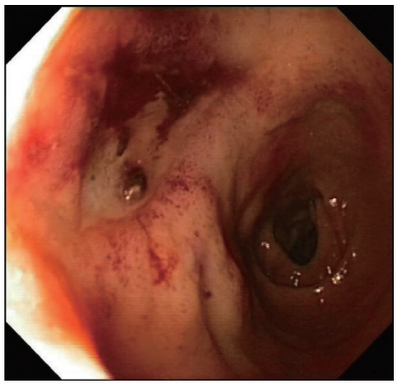

Figure 1 Ulcer with visible vessel.

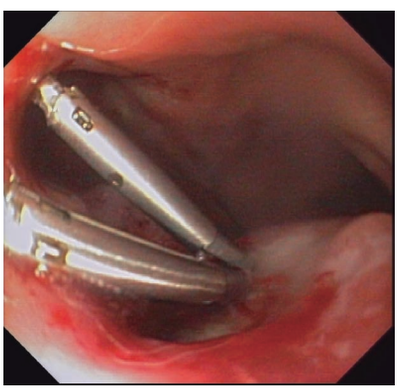

Figure 2 Ulcer with visible vessel after hemoclip placement.

moval should be attempted by vigorous irrigation. Stigmata revealed after clot removal are of high risk in about 70% of cases[33]. In a meta-analysis including 240 patients from six different studies, comparing endoscopic *vs* medical therapy for peptic ulcers with adherent clots, rebleeding was significantly lower in the endoscopic therapy group compared with the control group (8% *vs* 25%, $P = 0.01$)[34]. Another meta-analysis, however, has shown no benefit of endoscopic therapy for bleeding peptic ulcers with adherent clots[35]. These discrepancies could be attributed to inclusion of different studies and heterogeneity in statistical analysis. At present, endoscopic therapy should be considered, although intensive PPI therapy alone might be sufficient in ulcers with adherent clots[19].

Epinephrine injection therapy promotes initial hemostasis by a combination of vasospasm and local tamponade. This effect declines after 20 min, and requires additional treatment with a more durable technique. In several meta-analyses, no superiority of one specific technique was proven; in particular, hemoclip placement, thermocoagulation (e.g., heater probe), and electrocoagulation (e.g., Gold probe, BICAP probe) all seem equivalent alternatives[24,30,31,36]. Patients with recurrent bleeding can usually be managed by endoscopic therapy. However, emergency surgery or angiographic embolization is required on occasion. There have been no randomized trials that have compared surgery and angiographic embolization.

A new promising endoscopic application is the use of a chemical compound which, when sprayed as nanopowder on active bleeding, can lead to immediate hemostasis, with coverage of the bleeding ulcer with a powder layer. In a pilot study of 15 patients with active ulcer bleeding treated with this nanopowder, immediate hemostasis was achieved in 93%, and one patient had recurrent bleeding. No adverse events were reported during the 30-d follow-up[37]. Further studies with this product are ongoing and will elucidate if application is also beneficial for other causes of nonvariceal UGIB.

Endoscopic therapy for other causes of nonvariceal UGIB

Treatment and prevention of (bleeding from) erosions depends upon the cause (e.g., drug-induced, mechanical, or inflammatory). Most cases respond well to PPIs. The offending agent should be discontinued whenever possible and, if present, *H. pylori* should be eradicated. Acute bleeding sometimes needs endoscopic therapy, similar to that for PUB[38].

Hemorrhage due to neoplastic lesions is often difficult to manage because of the diffuse character of the bleeding and vulnerability of the mucosa. Primary endoscopic therapy is recommended, but additional surgical consultation is sometimes necessary. In cases with diffuse tumor bleeding in a palliative setting, radiotherapy is often the treatment of choice.

Most bleeding from Mallory-Weiss tears stops spontaneously. Patients with stigmata of active bleeding, however, might require interventional endoscopy[39]. Endoscopic therapy is the first choice in bleeding Dieulafoy's lesions and is usually performed with clipping or banding of the lesion[40].

The current standard for endoscopic treatment of bleeding angiodysplasia consists of coagulation therapy. Sometimes, pharmacological agents such as estrogen and progesterone, octreotide or thalidomide are given, but their effects remain controversial.

Gastric antral vascular ectasia responds best to endoscopic ablation of the lesion.

POSTENDOSCOPIC MANAGEMENT

Antisecretory therapy

Pharmacotherapy plays a second major role in the treatment of UGIB. PPI therapy is superior over histamine-2 receptor antagonists[19]. PPIs can be administered orally or intravenously depending on the rebleeding risk. In a randomized placebo-controlled trial of 767 multiethnic PUB patients treated with endoscopic therapy because of high-risk stigmata, high-dose intravenous PPI (80 mg esomeprazole bolus, 8 mg/h continuous infusion for 72 h) significantly reduced rebleeding (5.9% *vs* 10.3%, $P = 0.03$) and the need for endoscopic retreatment[41]. Similar results were found by meta-analysis; high-dose intravenous PPI after endoscopic therapy significantly reduced rebleeding [relative risk (RR): 0.40; 95% CI: 0.28-0.59], need for sur-

gery (RR: 0.43; 95% CI: 0.24-0.58) and mortality (RR: 0.41; 95% CI: 0.20-0.84) compared with placebo/no therapy[35]. These data support the guideline recommendation to give high-dose continuous intravenous PPI therapy to patients with PUB with high-risk stigmata.

Additionally, all patients with PUB should be discharged with a prescription for a single-daily-dose oral PPI to reduce the risk of recurrent bleeding. The duration and dose of the PPI depend on the underlying etiology and additional medication use[19].

H. pylori eradication therapy

Testing for *H. pylori* is recommended in all patients with PUB[19]. This should be followed by eradication therapy for those who are *H. pylori*-positive, with subsequent assessment of the effect of this therapy, and renewed treatment in those in whom eradication fails. The efficacy of eradication therapy and maintenance antisecretory therapy for the prevention of rebleeding has been assessed in a meta-analysis of randomized trials. This revealed a significantly lower risk of rebleeding in the *H. pylori* eradication group, that is, 1.6% *vs* 5.6% within a median follow-up of 12 mo. When only patients with successful *H. pylori* eradication were included, the rebleeding rate was even lower (1%)[42]. Therefore, confirmation of eradication is recommended. Diagnostics tests for *H. pylori* have a low negative predictive value in the setting of acute UGIB. This might be due to technical difficulties to collect a sufficient number of representative biopsies, or inaccuracy of the test in a more alkaline environment caused by the blood[43]. Initial negative results on biopsies obtained in the acute setting must therefore be interpreted with caution and repetition of the test during follow-up is recommended[19].

CONCLUSION

The management of UGIB has changed dramatically over recent decades. Endoscopic therapy and pharmacotherapy have become the mainstay in management. Early endoscopy within 24 h of presentation, or earlier in selected cases with signs of ongoing bleeding, improves outcome and reduces length of hospital stay. Endoscopic epinephrine injection in combination with another endoscopic technique reduces the risk for rebleeding and related mortality in patients with high-risk ulcers. Adequate *H. pylori* eradication and PPI therapy after discharge can bring the rebleeding and mortality rates further down.

Ongoing development is expected especially in the area of development of transfusion policies, and new tools for endoscopic hemostasis. Further studies are needed to clarify the optimal approach for patients with adherent clots. These developments should help to reduce the persistent high mortality rate of UGIB, a disease which nowadays in particular occurs in elderly patients with comorbidity and medication use.

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ORIGINAL ARTICLE

Expression of HER2 and bradykinin B_1 receptors in precursor lesions of gallbladder carcinoma

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Abstract

AIM: To determine the expression of HER2 and bradykinin B_1 receptors (B_1R) in the two pathogenic models of gallbladder cancer: the metaplasia-dysplasia-carcinoma and the adenoma-carcinoma pathways.

METHODS: Receptor proteins were visualized by immunohistochemistry on 5-μm sections of paraffin-embedded tissue. Expression of both receptors was studied in biopsy samples from 92 patients (6 males and 86 females; age ranging from 28 to 86 years, mean 56 years). High HER2 expression in specimens was additionally investigated by fluorescence *in situ* hybridization. Cell proliferation in each sample was assessed by using the Ki-67 proliferation marker.

RESULTS: HER2 receptor protein was absent in adenomas and in normal gallbladder epithelium. On the contrary, there was intense staining for HER2 on the basolateral membrane of epithelial cells of intestinal metaplasia (22/24; 91.7%) and carcinoma *in situ* (9/10; 90%), the lesions that displayed a significantly high proliferation index. Protein up-regulation of HER2 in the epithelium with metaplasia or carcinoma *in situ* was not accompanied by *HER2* gene amplification. A similar result was observed in invasive carcinomas (0/12). The B_1R distribution pattern mirrored that of HER2 except that B_1R was additionally observed in the adenomas. The B_1R appeared either as cytoplasmic dots or labeling on the apical cell membrane of the cells composing the epithelia with intestinal metaplasia (24/24; 100%) and carcinoma *in situ* (10/10; 100%) and in the epithelial cells of adenomas. In contrast, both HER2 (4/12; 33%) and B_1R (1/12; 8.3%) showed a low expression in invasive gallbladder carcinomas.

CONCLUSION: The up-regulation of HER2 and B_1R in precursor lesions of gallbladder carcinoma suggests cross-talk between these two receptors that may be of importance in the modulation of cell proliferation in gallbladder carcinogenesis.

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Key words: Bradykinin B_1 receptor; HER2; Gallbladder adenoma; Gallbladder dysplasia; Intestinal metaplasia; Gallbladder cancer

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INTRODUCTION

Gallbladder carcinoma is an aggressive cancer of the gastrointestinal tract with incidence three times greater in the female population of Chile, India and Japan when compared to men[1,2]. The most common type of gallbladder cancer is adenocarcinoma. However, early diagnosis is difficult because most of the cases are detected only at an advanced stage following laparotomy.

Successive bouts of inflammation cause continuous and long lasting damage to the gallbladder epithelium, which thereby transforms into an architecturally abnormal regenerative epithelium very similar to low grade dysplasia. Injury and repair are also related to well known metaplastic changes of the mucosa, such as pyloric and intestinal metaplasia, and to other rare types of metaplasia[3]. Intestinal metaplasia usually develops into dysplasia and is frequently observed adjacent to carcinoma. Pyloric and intestinal metaplasias are associated with gallbladder carcinoma, but intestinal metaplasia show a greater relationship with malignancy[4,5]. Analysis of serial sections indicates the presence of microinvasion foci in the lamina propria next to carcinoma *in situ* and also that dysplasia and carcinoma *in situ* occur close to areas of intact mucosa in nearly all invasive carcinomas[6].

Among the pathogenic models that explain the neoplastic transformation of gallbladder epithelium are the metaplasia-dysplasia-carcinoma and the adenoma-carcinoma pathways[5]. Morphological and molecular studies have shown that these two entities correspond to independent biological events. Therefore, we have used representative gallbladder samples of both putative pathways to investigate the expression of two receptor molecules involved in cell proliferation, namely the HER2 and bradykinin B_1 receptors (B_1R). The HER2 (c-erbB-2) receptor is recognized to be of clinical importance because of its prognostic value in determining progression of some types of breast tumors. Similarly, B_1R has recently been shown to induce the proliferation of estrogen-sensitive breast cancer cells by turning on the transactivation of the epidermal growth factor receptor (EGFR), a signal-transduction pathway involved also in the activation of HER2[7]. As with breast cancer, expression of HER2 in gallbladder carcinoma has been associated with progression of malignancy and linked to poor patient survival[8-11]. Furthermore, constitutive expression of HER2 in transgenic mice causes adenocarcinoma of the gallbladder[12] suggesting a key role for this member of the EGFR family in such neoplasia. Although the expression levels of HER2 have been previously investigated in invasive gallbladder cancer[13], so far no studies appear to have examined its expression in the putative precursor lesions of gallbladder carcinoma.

The B_1R belongs to the family of G protein-coupled rhodopsin-like receptors which, upon stimulation by analogues that lack the Arg^9 from the carboxy terminus of the bradykinin molecule, trigger several second messenger signaling systems that control cell differentiation, proliferation and/or migration[14,15]. The kinin B_1R agonists belong to a family of bioactive peptides produced locally and with paracrine activity, that are formed from precursor molecules by the proteolytic action of enzymes called kallikreins (kininogenases)[14,15]. So far, only a few studies have evaluated the role of B_1R as well as the underlying molecular mechanisms that trigger its activation in cancer cells. Recent reports have suggested that the B_1R is an important player in lung, prostate and breast cancer by regulating tumoral growth, migration and invasion[7,16,17]. In addition, some of the cellular actions of B_1R stimulation are a consequence of EGFR transactivation[7]. However, the status of B_1R in other neoplastic disorders such as gallbladder carcinoma has not been investigated previously. Thus, the primary aim of our study was to perform a comprehensive evaluation of the expression values for HER2 and B_1R in the metaplasia-dysplasia-carcinoma and adenoma-carcinoma pathways.

MATERIALS AND METHODS

Ethics

This study was performed in accordance with the Declaration of Helsinki of the World Medical Association. The designated experiments were approved by the Ethical Committees of Hospital Base Valdivia, Universidad Austral de Chile and the National Fund for Development of Science and Technology in Chile (FONDECYT) that included guidelines for the protection of human subjects.

Patient tissue

The study was performed on 92 routinely resected gallbladders retrieved from the surgical pathology archive of the Servicio de Patologia, Hospital Base Valdivia, Chile. Of the 92 specimens, 6 were from males, and 86 were from females; the patients ranged in age from 28 to 86 years (mean, 56 years). The data on age, sex of patients bearing adenomas, gallbladder adenocarcinomas and their putative precursor lesions are summarized in Table 1. The following categories were recorded: (1) normal mucosa (n = 5), gallbladders that were resected due to lithiasis and elective surgery; (2) pyloric-type adenoma (n = 15); (3) intestinal-type adenoma (n = 6); (4) pyloric metaplasia (n = 20); (5) intestinal metaplasia (n = 24); (6) carcinoma *in situ* (n = 10); and (7) invasive carcinoma (n = 12) (Table 1). Invasive adenocarcinomas examined histologically were classified into well-differentiated with a papillary pattern of growth (8 cases), poorly differentiated (2 cases), mucinous well-differentiated (1 case) and signet ring (1 case) adenocarcinomas.

Table 1 Clinical and pathological data of patients with gallbladder adenomas, adenocarcinoma and its putative precursor lesions

Lesion	Sex	Age (yr, mean ± SD)
Normal mucosa (n = 5)	F (5)	54.6 ± 9.0
Pyloric adenoma (n = 15)	F (13)/M (2)	55.2 ± 3.5
Intestinal adenoma (n = 6)	F (6)	68.5 ± 1.7
Pyloric-type metaplasia (n = 20)	F (20)	51.5 ± 3.2
Intestinal metaplasia (n = 24)	F (24)	50.0 ± 3.4
Carcinoma *in situ* (n = 10)	F (9)/M (1)	65.8 ± 2.5
Invasive carcinoma (n = 12)	F (9)/M (3)	66.0 ± 3.8

According to WHO classification. F: Female; M: Male.

Each gallbladder was fixed in 10% buffered formalin and embedded in paraffin according to conventional protocols. Sections from each block were stained with hematoxylin and eosin for precise histopathological classification before immunostaining. In all cases, two to four blocks were used for immunohistochemical evaluation.

Immunohistochemistry

This method was performed as previously described by Molina *et al*[7]. Briefly, paraffin sections were incubated with primary antibodies overnight at 22 ℃ in a water bath that was used as a moist chamber. The primary antibody sources, dilutions, antigen retrieval and incubation conditions are listed in Table 2. Bound primary antibodies were localized by the biotin/streptavidin immunoperoxidase technique using the LSAB+ kit (Dako, Carpinteria, CA, United States). Peroxidase was developed with diaminobenzidine and hydrogen peroxide. Sections were counterstained with Harris hematoxylin. Negative controls included omission of primary antibody and its replacement by non-immune immunoglobulins of the same species or isotype matched immunoglobulins under identical conditions.

Immunolabeling was scored as positive (presence of staining) or negative (absence of staining) and then expressed as percentage of total number of normal mucosa, metaplasia, carcinoma *in situ* and invasive carcinoma cases examined. Staining was considered positive when over 30% of the gallbladder epithelium was immunostained. Only cell membrane immunoreactivity was considered positive for HER2 staining. Cell proliferation index was determined by recording the immunolabeled Ki-67 antigen in gallbladder carcinoma and its putative precursor lesions. For this purpose, nuclei were counted at x40 magnification in three different fields selected at random. The procedure was repeated twice by two independent observers for each lesion and the counts were averaged. The Ki-67 cell proliferation index was derived by dividing the average Ki-67 count by the average total number of nuclei in one field.

Fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) technique for HER2 was performed on 3-μm thick tissue sections according to the protocol of HER2 FISH PharmDx™ Kit (Dako). Hybridization was performed with a mixture of HER2-Texas Red and cen-17 labeled with fluorescein isothiocyanate. A breast cancer sample classified as HER2 (+3) was used as positive control. For each condition at least two samples were analyzed using a fluorescence Nikon Labophot-2, and at least 50 nuclei were visualized in each sample.

Statistical analysis

Differences in the occurrence of immunolabeling were evaluated using Fischer's exact probability test with the aid of the software JMP Statistical Discovery Software 8.0 (SAS Institute, Cary, NC, United States). Probability values less than 0.05 were considered to be statistically significant.

RESULTS

Histopathological features

Normal, non-metaplastic epithelium was comprised of regular segments of tall columnar cells with basal nuclei and sporadic small apical vacuoles. Pyloric-type adenomas showed their typical morphology that was comprised of tightly packed pyloric or antral-type mucous glands similar to mucous glands of the stomach (Figure 1). Intestinal-type adenomas consisted of papillary structures lined by columnar epithelium with elongated and pseudostratified nuclei (Figure 1). Pyloric-type metaplasia was distinguished by glands lined by columnar cells with vacuolated cytoplasm and flattened nuclei located basally; the glands formed small lobular structures throughout the lamina propria. Epithelium with intestinal metaplasia was composed of tall columnar cells with a brush border and variable proportions of goblet cells. This type of metaplasia was often seen adjacent to invasive adenocarcinoma. Carcinoma *in situ* was characterized by the presence of epithelial cells that showed frequent mitosis and marked alterations in the size and shape of the nuclei such as hypercromasia, overlapping and crowding.

Expression of HER2

No immunolabeled HER2 receptor was visualized in normal gallbladder epithelium and no significant expression of the protein was visualized in adenomas of either pyloric- or intestinal-type (Table 3; Figures 1 and 2). In contrast, an intense immunoreactivity was observed in the epithelium with intestinal metaplasia (22/24) and carcinoma *in situ* (9/10), with decreased staining in invasive carcinomas (4/12) (Table 3; Figure 2) and none in pyloric metaplasia and non-epithelial tissues (not shown). The HER2 immunolabeling was confined to the basolateral cell membrane region of all metaplastic epithelial cells, whereas the luminal membrane was devoid of staining (Figures 2 and 3). At a higher magnification, the boundary between the two segments was clearly delineated by the different immunoreactivity of the cells in the apical

Table 2 Primary antibody sources, dilutions, antigen retrieval and incubation conditions

Antibody	Source	Dilution/incubation	Microwave antigen retrieval
HER2	Novocastra, NCL-CBE-356	1:200/overnight	No
Bradykinin B1R	Santa Cruz, sc-25484	1:300/overnight	90 ℃/7 min, Tris-HCl buffer pH 10
	Merck	1:500/overnight	No
Ki-67	Dako, A0047	1:200/overnight	90 ℃/15 min, Citrate buffer pH 6

Table 3 Expression of immunoreactive HER2, bradykinin B_1 receptor and Ki-67 in gallbladder adenomas and in adenocarcinoma and its putative precursor lesions (%)

Receptor/marker	Normal epithelium	Pyloric adenoma	Intestinal adenoma	Intestinal metaplasia	Carcinoma *in situ*	Invasive carcinoma
HER2	0/5	0/14	1/6 (17)	22/24 (91.7)[a]	9/10 (90)[a]	4/12 (33)
Bradykinin B_1R	0/5	10/15 (67)[a]	6/6 (100)[a]	24/24 (100)[a]	10/10 (100)[a]	1/12 (8.3)
Ki-67[1]	0.05 ± 0.005 (1.1)	0.05 ± 0.01 (5.1)	0.10 ± 0.01 (10.8)	0.36 ± 0.03[b] (36.7)	0.37 ± 0.05[b] (37.5)	0.42 ± 0.03[b] (42)

[1]Proliferation index estimated as immunoreactivity to Ki-67 antigen. [a]$P < 0.05$, [b]$P < 0.01$ *vs* normal epithelium. B_1R: B_1 receptor.

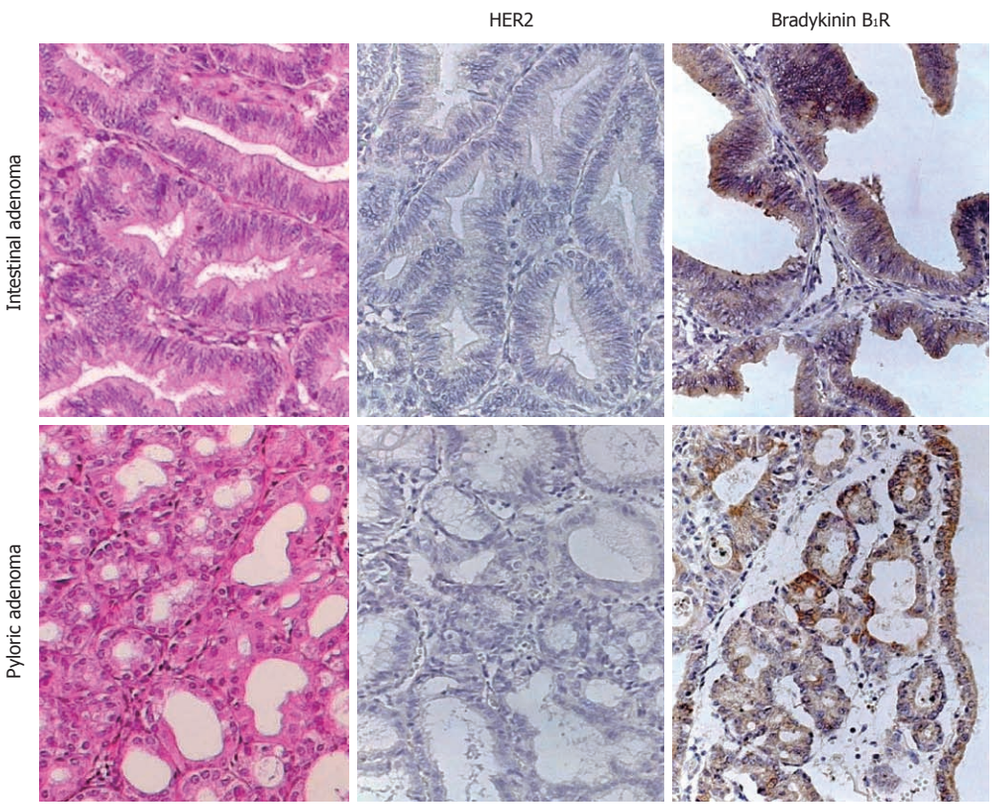

Figure 1 Expression of immunoreactive HER2 and bradykinin B_1 receptor in pyloric- and intestinal-type adenomas. Tissue sections were incubated with each antibody and then the biotin/streptavidin-peroxidase technique was followed. B_1R: B_1 receptor.

membrane when compared to those of the basolateral cell membrane (Figure 3). Loss of polarity in the epithelia with carcinoma *in situ* resulted in a complete membrane staining of some of the neoplastic epithelial cells. With FISH staining, *HER2* gene appeared as two red signals in normal and metaplastic epithelia and two to four signals in carcinoma *in situ* and invasive carcinoma, accompanied by the corresponding green signals pointing out the chromosome 17 centromeres (HER2/CEN-17 < 1.8) that indicated lack of amplification (Figure 3C-F). On the contrary, a clear amplification of *HER2* gene was observed in HER2 (+3) breast cancer samples that were used as positive controls (Figure 3G).

Expression of bradykinin B_1R

The B_1R protein also followed a cell membrane distribution pattern. Immunolabeled B_1R was visualized only in one case of the invasive carcinomas (Table 3). In contrast, samples with intestinal metaplasia and carcinoma *in situ* showed an intense staining for B_1R that appeared as cy-

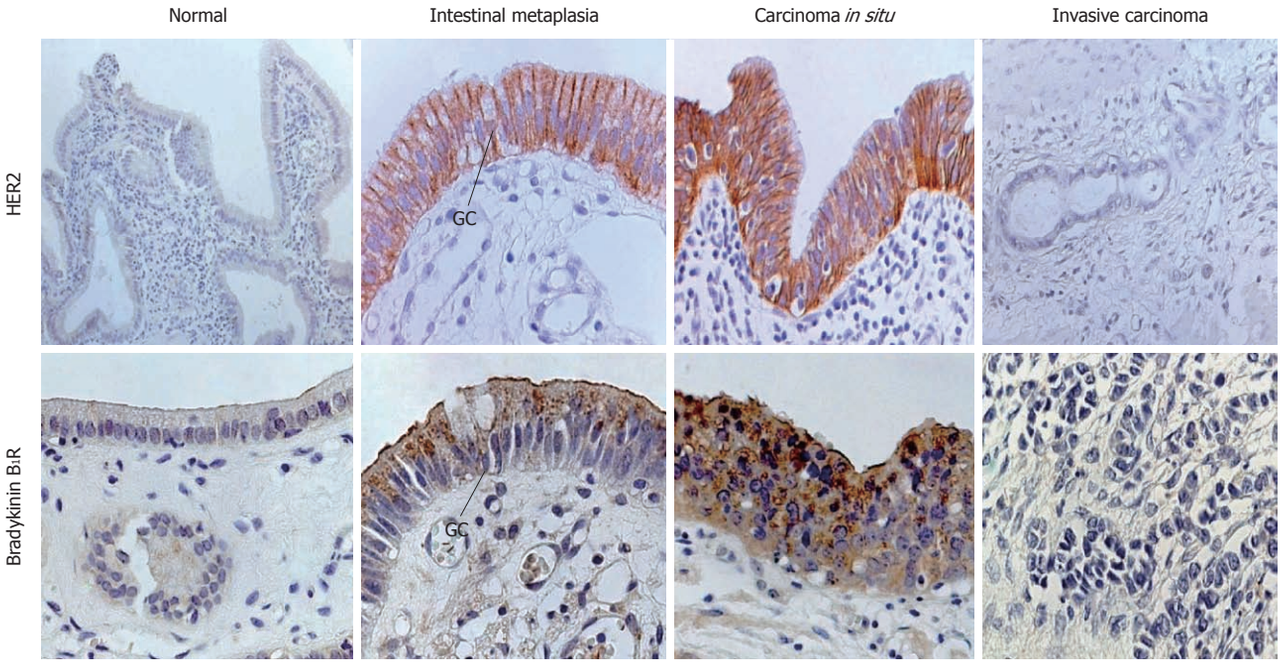

Figure 2 Immunoreactive HER2 and bradykinin B_1 receptor receptors in normal gallbladder, invasive carcinoma and in epithelia with intestinal metaplasia and carcinoma *in situ*. Biotin/streptavidin-peroxidase technique. B_1R: B_1 receptor; GC: Goblet cell.

toplasmic dots or labeling in the apical cell membrane region of metaplastic cells and in the epithelium with carcinoma *in situ* (Figures 2 and 3). Furthermore, B_1R immunolabeling was observed in epithelial cells of pyloric- and intestinal-type adenomas, including the surface epithelium of adenomas (Figure 1 and Table 3).

Ki-67 cell proliferation marker

By using Ki-67, we confirmed that the observed cell proliferation was significantly higher in epithelia with metaplasia, carcinoma *in situ* and in cells of invasive gallbladder carcinomas than in normal epithelium and in epithelial cells of both pyloric- and intestinal-type adenomas (Table 3).

DISCUSSION

Two pathogenic models designated as adenoma-carcinoma and metaplasia-dysplasia-carcinoma have been used to explain neoplastic transformation of the gallbladder epithelium. However, intestinal metaplasia is considered as the major precursor lesion that later progresses into carcinoma *in situ* and invasive adenocarcinoma. The question of whether expression of receptors such as HER2, a tyrosine kinase orphan receptor, and the B_1R, a G protein-coupled receptor, are enhanced in such carcinogenic changes of the gallbladder epithelium formed the focus of the current study[7,15,16,18,19].

Remarkably, the expression of HER2 and bradykinin B_1R followed a similar pattern of distribution in invasive carcinoma and in its putative precursor lesions. Both receptors were absent in the normal epithelium but were strongly expressed in carcinoma *in situ* and the epithelia with intestinal metaplasia. Absence of HER2 staining in normal gallbladder epithelium is in agreement with previous studies performed using normal breast tissue[20]. Two previous reports failed to demonstrate expression of HER2 protein in gallbladder dysplasia[13,21]. The discrepancy between these findings and our results may be explained by the use of different immunostaining procedures (e.g., monoclonal *vs* polyclonal antibodies and sensitivity of the technique), time of fixation, preservation of antigenic sites and number of samples analyzed. In our study, HER2 receptor protein stained intensely along the basolateral plasma membrane of the metaplastic and carcinoma *in situ* cells. A similar pattern of staining has been observed in apocrine metaplasia of the breast where HER2 immunoreactivity appeared restricted to the basolateral plasma membrane of the metaplastic cells[22]. It is well known that tight junctions morphologically divide cell membranes of non-neoplastic polarized epithelial cells into two regions: an apical one which faces the lumen and often has specialized features such as cilia or a brush border of microvilli; and a basolateral region, which covers the rest of the cell[23]. Further, tight junctions prevent proteins and lipids from diffusing between the basolateral and apical regions, so that not only the protein but also the lipid composition of the two membrane regions is different[23]. Therefore, the HER2 immunoreactivity, observed in the basolateral cell membrane domain of metaplastic gallbladder epithelium, is not comparable to that scored by the Hercep Test™ staining protocol, which is based on the immunoreactivity present on the cell membrane of invasive breast cancer cells that are not supported by a basement membrane, i.e., neoplastic non-polarized cells. Despite over-expression

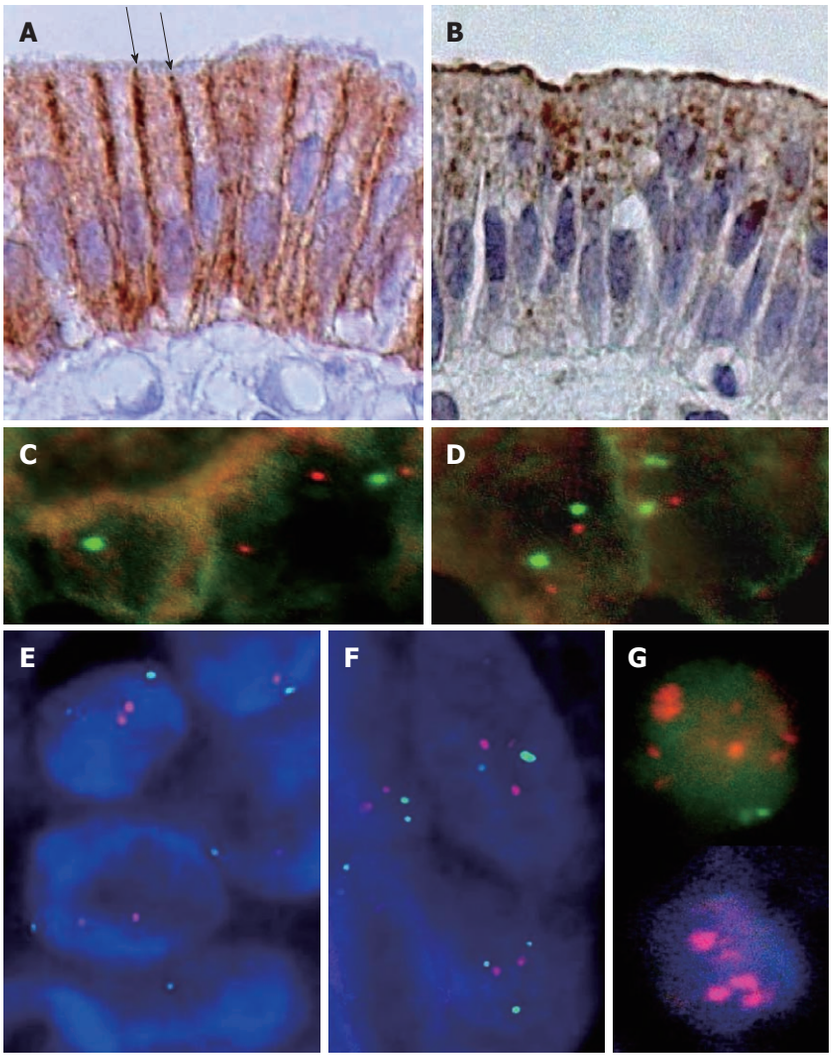

Figure 3 High magnification of gallbladder metaplastic epithelium showing immunoreactivity for HER2 (A) and bradykinin B_1 receptor receptor (B). Arrows show the limit between apical and basolateral cell membrane domains. C-G: Fluorescence *in situ* hybridization for HER2. Tissue sections were hybridized with a mixture of HER2-Texas Red and cen-17 labeled with fluorescein isothiocyanate. C: **Normal gallbladder epithelium; D: Epithelium with intestinal metaplasia; E: Carcinoma** *in situ*; F: **Invasive carcinoma; G: Positive control corresponding to a breast cancer sample classified as HER2 + 3.**

of HER2 receptor protein in metaplastic epithelia and carcinoma *in situ*, our results using FISH revealed that this event was not accompanied by *HER2* gene amplification suggesting that this change may not be relevant for gallbladder cancer. Studies using FISH, but restricted to invasive carcinomas of the gallbladder, have shown amplification of the *HER2* gene only in approximately 10% of the cases investigated[8]. Absence of *HER2* gene amplification in our tissue samples with invasive carcinoma may be due to our low number of cases compared with other published studies[8]. Some authors suggest that *HER2* over-expression is due to gene deregulation rather than gene amplification because in some reports there is no strict correlation between receptor protein expression and gene amplification[24]. The HER2 receptor seems to be a key player since its constitutive expression leads to the development of gallbladder adenocarcinoma in 100% of transgenic mice[12]. Moreover, increased HER2/EGFR (erbB-1) heterodimer formation, hyperphosphorylation of tyrosine residues of both HER2 and EGFR (but not erbB-3 or erbB-4) and activation of the mitogen-activated protein kinases (MAPK) signaling pathway were observed in the gallbladder epithelium of the transgenic mice[12]. Over-expression of the HER2 receptor protein, detected immunohistochemically in nearly all breast specimens showing carcinoma *in situ* of high grade, has been interpreted as an expression of rapid growth since its presence is related to cellular proliferation[25,26]. Our results show that the epithelia with intestinal metaplasia and carcinoma *in situ* display the higher values for Ki-67 cell proliferation marker. The fact that the *HER2* gene shows amplification only in 10% of the invasive gallbladder carcinomas[8] contrasts with the high levels of protein expression observed by us in intestinal metaplasia and carcinoma *in situ,* and suggests that major activity of HER2 may occur in these precursor lesions. Further, the expression of HER2 in other invasive tumors of the gastrointestinal tract such as gastric cancer has revealed

that less than 10% of all invasive tumors showed HER2 expression and that there is no relationship between its expression, patient survival or TNM stage[27].

Members of the EGFR family are activated not only by direct binding of their corresponding ligands but also by transactivation triggered after stimulation of G protein-coupled receptors such as the B_1R[15,19]. We have recently reported B_1R binding sites in breast carcinoma and showed that B_1R stimulation induces the proliferation of MCF-7 and ZR-75 breast cancer cells, an effect that depends on the transactivation of the EGFR and the subsequent activation of the MAPK signaling pathway[7]. Moreover, stimulation of the B_1R increases the release of metalloproteases-2 and -9 from breast cancer cells[28]. Both metalloproteases are considered key enzymes for tumor invasion and metastasis, because they have the capacity to degrade type IV collagen, the major protein component of basement membranes. Here, we have described, for the first time, the expression of B_1R in precursor lesions of gallbladder carcinomas (i.e., intestinal metaplasia and carcinoma *in situ*), conditions in which its expression is maximal and associated with high proliferation rates of the epithelial cells.

The kinin B_1R agonists are short-lived peptides that exert most of their actions in a paracrine fashion for which the presence of kallikreins, kinin precursors and expression of the appropriate receptor is required[14]. In a previous report, we showed that both tissue kallikrein and the kinin precursors are present in the human gallbladder suggesting that kinin formation is feasible in this tissue[29]. Tissue kallikrein (*KLK1*/hK1, kininogenase) is expressed by gallbladder epithelial cells whereas the kininogens, substrates of hK1, diffuse from submucosal blood vessels to fill the interstitial space between epithelial cells[29]. A further source of tissue kallikrein is the neutrophil that infiltrates the inflamed gallbladder during acute episodes of cholecystitis and thereby releases hK1 in the vicinity of epithelial cells[29]. Additionally, the natural B_1R agonist induces migration of glioma cells *via* up-regulation of cyclooxygenase-2 expression[30]; whether the B_1R produces a similar effect in gallbladder cancer cells remains to be investigated using gallbladder cancer cell lines.

The identical pattern of expression observed for B_1 and HER2 receptors in precursor lesions of gallbladder carcinoma suggests a cooperative relationship, orientated towards promoting functional cell signaling that may result in an increased proliferation of the gallbladder epithelium.

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COMMENTS

Background

Gallbladder carcinoma is an aggressive malignancy of the gastrointestinal tract, and its early diagnosis is difficult, because most of the cases are detected at an advanced stage at laparotomy. It is well known that the HER2 receptor is involved in the carcinogenesis of many malignancies. Further, recent reports show that the bradykinin B_1 receptor (B_1R) stimulates the proliferation of breast cancer cells. It is not known whether HER2 and B_1R are overexpressed in precursor lesions of gallbladder carcinoma and whether they serve as biomarkers for early neoplastic transformation of gallbladder epithelium.

Research frontiers

Most studies related to HER2 and gallbladder cancer have been focused on advanced stages of the disease, but not in their precursor lesions. So far, there have been no investigations on the expression levels of B_1R in this neoplasm. Because HER2 has a low expression in invasive gallbladder cancer, it has been proposed by some authors that it is not relevant to gallbladder carcinoma.

Innovations and breakthroughs

The novel finding of high expression of HER2 and B_1R in precursor lesions of gallbladder carcinoma suggests that they enhance cell proliferation and play a significant role in gallbladder carcinogenesis.

Applications

Because of the high HER2 and B_1R expression data in precursor lesions of gallbladder carcinoma, and the therapeutic efficacy of anti-HER2 treatments in breast tumors, future studies should focus on HER2 and B_1R as biomarkers for the detection of early neoplastic transformations in the gallbladder epithelium.

Terminology

The HER2 is a tyrosine kinase receptor, member of the epidermal growth factor receptor family. Growth factors bind to receptors and initiate cell proliferation, migration, invasion, resistance to apoptosis and angiogenesis. B_1R belongs to the family of G protein-coupled receptors, which upon stimulation by analogues trigger cell proliferation and secretion of metalloproteases in cancer cells: proteases that assist cancer cells to invade normal human tissue. The results clearly indicate that both HER2 and the B_1R play a significant role in transformation of epithelial cells through the metaplasia-dysplasia-carcinoma pathway.

Peer review

This article has very well explored the HER2 and B1R in gallbladder carcinoma. While HER2 and B1R are scarcely expressed in invasive gallbladder carcinoma, their expression is upregulated in precursor lesions of this neoplasm which may help in early diagnosis and also provide some pathways of carcinogenesis. This study is worthy.

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ORIGINAL ARTICLE

Proteomic analysis of gastric cancer and immunoblot validation of potential biomarkers

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Abstract

AIM: To search for and validate differentially expressed proteins in patients with gastric adenocarcinoma.

METHODS: We used two-dimensional gel electrophoresis and mass spectrometry to search for differentially expressed proteins in patients with gastric adenocarcinoma. A set of proteins was validated with immunoblotting.

RESULTS: We identified 30 different proteins involved in various biological processes: metabolism, development, death, response to stress, cell cycle, cell communication, transport, and cell motility. Eight proteins were chosen for further validation by immunoblotting. Our results show that gastrokine-1, 39S ribosomal protein L12 (mitochondrial precursor), plasma cell-induced resident endoplasmic reticulum protein, and glutathione S-transferase mu 3 were significantly underexpressed in gastric adenocarcinoma relative to adjacent non-tumor tissue samples. On the other hand, septin-2, ubiquitin-conjugating enzyme E2 N, and transaldolase were significantly overexpressed. Translationally controlled tumor protein was shown to be differentially expressed only in patients with cancer of the gastric cardia/esophageal border.

CONCLUSION: This work presents a set of possible diagnostic biomarkers, validated for the first time. It might contribute to the efforts of understanding gastric cancer carcinogenesis.

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Key words: Two-dimensional gel electrophoresis; Mass spectrometry; Gastric adenocarcinoma; Proteome; Biomarkers

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INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer worldwide[1]. However, incidence rates have steadily declined; this is largely determined by trends in the fundus and distal stomach[1]. Gastric cardia cancer, on the other hand, is on the increase, and is usually of the diffuse histological type[2]. However, despite declining incidence rates, GC is still the second leading cause of cancer death and thus remains a major health problem[1]. Radical surgery still offers the only chance of a cure for GC that invades the muscular layer, but only half of patients qualify for this at the time of their diagnosis[2]. The majority of patients are diagnosed at an advanced stage, where a systemic spread of the tumor cells has to be anticipated. The 5-year survival rate is very low and despite consistent improvements the prognosis remains poor[3]. On the other hand, the 5-year survival rate exceeds 90% for patients in an early stage of the disease[4].

A definitive diagnosis of GC requires a gastroscopic or surgical biopsy[5], which means there is a need for a non-invasive test, e.g., the detection of serum/plasma biomarkers. The most widely investigated serum markers include carcinoembryonic antigen, carbohydrate antigen (CA) 19.9, CA 72.4, cytokeratins and the β subunit of human chorionic gonadotropin. However, their low sensitivity and specificity precludes their use in screening and early diagnosis. Therefore, a search for more appropriate biomarkers is necessary.

One possible source of potential new biomarkers is the proteome. As opposed to static genomic data, the proteome is necessary for understanding the dynamic processes in cells[6]. It is also more complex, e.g., due to alternative splicing and post-translational modification, and the protein levels often do not correlate with the mRNA[7]. Proteomics is a rapidly developing field and is now applied to all areas of the life sciences[8]. Gel-based approaches, for example, belong to the most frequently used assays in protein separation and analysis[9]. Two-dimensional gel electrophoresis (2-DE) is still the method of choice for complex protein samples, although, due to its limitations with hydrophobic and large proteins, alternative or complementary methods based on mass spectrometry (MS) are gaining popularity.

2-DE, dating back to the 1970s[10,11], in conjunction with MS, has allowed the identification of differentially expressed proteins in various diseases, including GC[12-17]. However, the process of translating basic proteomic discoveries to the clinic is very time consuming and expensive, and despite all the efforts put into biomarker research, very few diagnostic protein biomarkers have been approved by the United States Food and Drug Administration[8]. The lack of specific and sensitive biomarkers thus creates a persistent need for the expedited development of biomarkers and their use in improving the diagnosis and treatment of cancer. Furthermore, the validity of potential biomarkers, as found by proteome analysis, needs to be investigated and incorporated as the second step of the research, e.g., by immunohistochemical and/or western blot analyses, in order to fulfill the criterion of a systematic investigation of the protein biomarkers in GC.

In this study, we used 2-DE and MS to search for differentially expressed proteins in patients with gastric adenocarcinoma: a total of 30 different protein alterations were found. Gastrokine-1 precursor (GKN1), 39S ribosomal protein L12 (mitochondrial precursor) (MRPL12), plasma cell-induced resident endoplasmic reticulum protein (PACAP), glutathione S-transferase mu 3 (GSTM3), septin-2 (SEPT2), ubiquitin-conjugating enzyme E2 N (UBE2N), transaldolase (TALDO1), and translationally controlled tumor protein (TPT1) were further validated with the immunoblot analysis, making them a set of possible biomarkers for gastric adenocarcinoma.

MATERIALS AND METHODS

Subjects and tissues

A total of 32 pairs of gastric adenocarcinoma tissues and adjacent control tissue from 32 patients were obtained from the tissue bank of the Institute of Oncology Ljubljana after the National Medical Ethics Committee's approval. They were stored at -70 ℃ until further use. The patients' and tumors' parameters are listed in Table 1.

Sample preparation

The tissues were ground with a mortar and pestle in the presence of liquid nitrogen and then lysed with 7 mol/L urea, 2 mol/L thiourea, 40 g/L 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 20 mmol/L dithiothreitol (DTT) and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, United States). For every 10 mg of tissue, 50 μL lysis buffer was added. After sonication on ice, three times for 10 s, the samples were incubated for 1 h on ice with occasional vortexing and then centrifuged for 1 h at 20 000 × *g* at 4 ℃. The supernatant was collected and the protein concentration was determined according to a commercial Bradford reagent (Thermo Fisher Scientific, Waltham, MA, United States) with bovine serum albumin as the standard.

2-DE

2-DE was conducted on 12 pairs of tissues and repeated in triplicate. For the isoelectric focusing, 100 μg protein was diluted in 450 μL rehydration solution (7 mol/L urea, 2 mol/L thiourea, 20 g/L CHAPS, 20 mmol/L DTT, 0.5% immobilized pH gradient (IPG) buffer, pH 4-7), loaded by in-gel rehydration onto IPG strips with an acidic pH range of 4-7 (GE Healthcare, Stockholm, Sweden), and focused in an Ettan IPGphor Ⅱ isoelectric focusing system (GE Healthcare) to a total of 63.5 kVh. Next, the IPG strips were equilibrated for 15 min in 6 mol/L urea, 30% glycerol, 20 g/L sodium dodecyl sulfate (SDS), 50 mmol/L Tris-HCl, pH 8.8, with 10 g/L DTT, and for another 15 min in the same solution with DTT replaced by 25 g/L iodoacetamide. The strips were then transferred to 12% polyacrylamide gels and the

Table 1 Patient and tumor characteristics

Characteristics	*n* (%)
Sex	
Male	19 (59.4)
Female	13 (40.6)
Lauren's classification	
Intestinal	20 (62.5)
Diffuse	11 (34.4)
Mixed	1 (3.1)
Location	
Corpus	8 (25)
Antrum	7 (21.9)
Cardia/gastroesophageal border	10 (31.2)
Several parts	7 (21.9)
Grade	
Well-differentiated	2 (6.2)
Moderately differentiated	12 (37.5)
Poorly differentiated	12 (37.5)
Undifferentiated	6 (18.8)
pT	
pT1b	1 (3.1)
pT2a	5 (15.6)
pT2b	15 (46.9)
pT3	11 (34.4)
pN	
pN0	8 (25)
pN1	16 (50)
pN2	6 (18.8)
pN3	2 (6.2)

second dimension was carried out in an Ettan DALTsix electrophoresis unit (GE Healthcare) under constant power in a Laemmli buffer system until the bromophenol blue reached the end of the gel.

Silver staining

The gels were silver stained according to Mortz *et al*[18]. Briefly, they were fixed overnight in 50% methanol, 12% acetic acid, and 0.05% formalin, washed for 2 × 20 min with 35% ethanol and sensitized for 2 min with 0.02% sodium thiosulfate. Next, they were washed for 3 × 5 min with water and stained for 20 min in 0.2% silver nitrate and 0.076% formalin. Then they were washed again for 2 × 1 min with water and developed with 6% sodium carbonate, 0.05% formalin and 0.0004% sodium thiosulfate. The reaction was stopped with 50% methanol and 12% acetic acid and the gels were left in 1% acetic acid until the scanning.

Image acquisition and analysis

The gels were scanned with an ImageScanner Ⅱ (GE Healthcare) at 300 dpi and analyzed with an ImageMaster 2D Platinum v7 (GE Healthcare). The spots were detected, matched and quantified by relative volume. The normalization of the spot values (relative spot volumes) was based on the total spot volume. Next, the data were analyzed with Excel. The spots of interest were determined using a pair-by-pair comparison. The spots were considered to be differentially expressed if they matched the following criteria: at least a twofold change in the relative spot volume, the occurrence of this change in at least three patients, and statistical significance.

In-gel digestion

The enzymatic digestion of the excised spots was performed using a Progest robot (Genomic Solutions, Holliston, MA, United States). Briefly, the protein spots were excised from the gel, placed into 96-well plates and washed with 50 μL acetronitrile (CH_3CN) for 3 min and then with 50 μL 25 mmol/L ammonium bicarbonate (NH_4HCO_3) for 3 min. This washing step was repeated three times to eliminate SDS, Tris and glycine. After the washing procedure, the excised spots were treated with 100 μL 10 mmol/L DTT for the reduction step and the reaction was left to proceed at 57 ℃ for 1 h. After DTT removal, 100 μL 55 mmol/L iodoacetamide was added for the cysteine carbamidomethylation and the reaction was left to proceed at room temperature (RT) for 1 h. After removal of the supernatant, the washing procedure was repeated three times and the gel slices were dried with a SpeedVac (5 min). Based on the gel-slice volume, 5-10 μL 12 ng/μL Porcine Trypsin (Promega, Madison, W, United States) was added. The enzyme was freshly diluted in 25 mmol/L NH_4HCO_3 and the digestion was performed overnight at RT. Trypsin was the protease of choice for the MS protein identification because of its reliability and its substrate specificity, yielding peptides with C-terminal basic residues (Arg and Lys), which facilitated positive ionization and subsequent MS detection. Finally, 10 μL of 39% H_2O/60% CH_3CN/1% HCOOH was added and the samples were left for 3 h at RT to favor the extraction of peptides from the gel.

MS and database search

Trypsin-generated peptide mixtures were analyzed by matrix-assisted laser desorption ionization coupled with a time-of-flight analyzer (MALDI-TOF) (Voyager, Applied Biosystems, Carlsbad, CA, United States). Samples that provided an ambiguous identification with MALDI-TOF were analyzed by tandem MS (MS/MS) using nanoliquid-chromatography electrospray ionization coupled with the quadrupole and time-of-flight analyzer (nanoLC-ESI-Q-TOF) (nanoAcquity, Q-TOF Premier; Waters, Milford, MA, United States).

For MALDI-TOF MS, the peptide extracts (0.5 μL) were mixed with an equal volume of 2,5-dihydroxybenzoic acid (10 mg/mL; Sigma-Aldrich) dissolved in 20% CH_3CN. Trypsin-digested ovalbumin was used for external calibration. Crystals were obtained using the dried-droplet method on a 100-spots metallic plate and approximately 500 MALDI mass spectra were averaged per spot to optimize the signal-to-noise ratio. The laser fluency was adjusted to the threshold to achieve the best resolution and mass accuracy. The MS measurements were carried out at a maximum accelerating potential of 20 kV, in the positive reflectron mode. The acquisition range was set to m/z 800-3500 with a low-mass gate at m/z 700. For m/z values of about 1500, the mean mass resolution was 15 000.

Proteins were identified by peptide mass fingerprinting with two search programs: Mascot and ProFound. The following search parameters were applied: SWISS-PROT and NCBI were used as the protein-sequence databases; a mass tolerance of 50 ppm and one missed cleavage were allowed; the alkylation of cysteine by carbamidomethylation was considered complete; while the oxidation of methionine was considered as a possible modification.

For nanoLC-ESI-Q-TOF MS/MS analysis, peptide mixtures were SpeedVac-treated for 10 min to eliminate CH_3CN, then 25 mmol/L NH_4HCO_3 was added before injection into the nanoAcquity/Q-TOF system equipped with a trapping column (Symmetry C18, 180 μm × 20 mm, 5 μm particle size) and an analytical column (BEH130 C18, 75 μm × 100 mm, 1.7-μm particle size) (Waters). The aqueous solvent (buffer A) was 0.1% formic acid and the organic phase (buffer B) was acetonitrile with 0.1% formic acid. A 2%-40% B gradient was set for 25 min. The MS parameters were as follows: positive ion mode; capillary voltage, 3 kV; cone voltage, 40 V; ion-source block temperature, 80 ℃; and collision energy ramping from 15 to 40 eV. For the exact mass measurements, the glufibrinopeptide reference (m/z = 785.8426) was continuously supplied during the nanoLC-MS/MS experiments using the lockspray device. The peptide mass measurements were corrected by the PLGS software (ProteinLynx Global Server; Waters) during data processing. Peak lists were generated by PLGS and the processed data were submitted to Mascot searching using the following parameters: data bank, NCBI; peptide tolerance, 15 ppm; fragment tolerance, 0.1 Da; number of missed cleavages, one; variable modifications, oxidation; and fixed modifications, carbamido methylation.

Immunoblot analysis

To validate the differential expression from the 2-DE gels, an immunoblot analysis was performed on an expanded number of samples, on 27 pairs. For TPT1, additional four pairs of cardia/gastroesophageal border adenocarcinoma were used. A total of 30 μg protein per sample was loaded on 12% or any kD gels (Bio-Rad, Hercules, CA, United States), transferred onto PDVF membranes (Millipore, Billerica, MA, United States), and blocked in 50 g/L skimmed milk overnight at 4 ℃. Primary antibodies were used in the following dilutions: anti-GKN1 at 0.5 μg/mL (WH0056287M1; Sigma–Aldrich), anti-MRPL12 at 1 μg/mL (WH0006182M1; Sigma-Aldrich), anti-PACAP at 1:1000 (ab96308; Abcam, Cambridge, United Kingdom), anti-GSTM3 at 0.75 μg/mL (ab74749; Abcam), anti-SEPT2 at 1 μg/mL (ab88657; Abcam), anti-UBE2N at 0.5 μg/mL (ab25885; Abcam), anti-TALDO1 at 1:1000 (ab67467; Abcam), and anti-TPT1 at 1 μg/mL (WH0007178M1; Sigma-Aldrich). Horseradish-peroxidase-conjugated secondary antibodies were used in the following dilutions: goat anti-mouse at 1:5000 (115-035-062; Jackson ImmunoResearch, Newmarket, Suffolk, United Kingdom) and goat anti-rabbit at 1:10 000 (111-035-003; Jackson ImmunoResearch). The proteins were detected chemiluminescently with an LAS-4000 CCD camera (Fujifilm, Tokyo, Japan) at 10-s intervals. The blots were then quantified with Multi Gauge software (Fujifilm) and the intensity values were exported to Excel. Differential expression was determined after normalization to Ponceau S-stained membranes for loading and transfer differences and statistical significance was assessed.

Statistical analysis

To assess the statistical significance of differential protein expression (tumor *vs* non-tumor) in 2-DE as well as in immunoblotting, Wilcoxon signed-rank test was used. The test was double-sided and values with $P < 0.05$ and a 95% CI were considered to be statistically significant. To assess the correlation of the differential protein expression from immunoblotting with the histopathological parameters, repeated-measures analysis of variance was used. The values with $P < 0.05$ and a 95% CI were considered to be statistically significant. Bonferroni post-test was used to narrow down where the differences were significant. All analyses were performed using Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, WA, United States) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, United States).

RESULTS

Subject parameters

Altogether, we used 32 pairs of human gastric adenocarcinoma and adjacent normal tissue samples from 32 patients. The mean age of the patients was 68.3 ± 10.5 (range 40-84) years. The subject group contained predominantly males (59.4%). According to Lauren's classification, the majority of tumors were intestinal (62.5%) and the largest part was located in the cardia/gastroesophageal border (31.2%). The tumors were mainly moderately (37.5%) and poorly (37.5%) differentiated. According to the TNM classification, most were pT2b (46.9%) and pN1 (50%). The data on pM were only available for some patients and were thus not included in any further analysis. For more details, refer to Table 1.

2-DE and MS

To determine the differentially expressed proteins in the gastric adenocarcinoma patients, we first performed 2-DE on 12 pairs of human gastric tissue samples. For good resolution we used large (24 cm) IPG strips with an acidic pH gradient (4-7). We performed the experiments in triplicate, which produced well-resolved spots and reproducible 2-DE patterns. On average, 1197 ± 150 spots per gel were detected after silver staining. Figure 1 shows a typical image of a tumor- and a non-tumor-sample-derived gel. In the lower part of the figure, one can see differentially expressed spots from the tumor/non-tumor pairs, obtained from a pair-by-pair comparison.

After the proteolytic digestion of 32 excised spots,

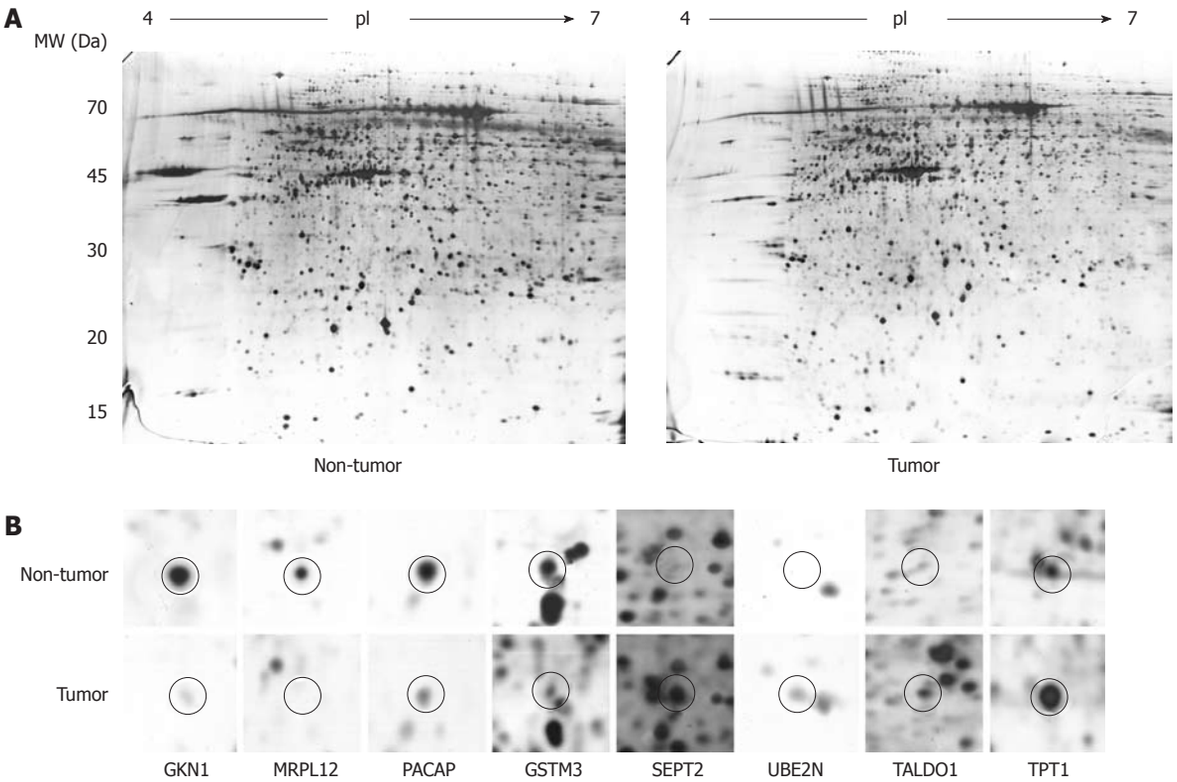

Figure 1 Results of two-dimensional gel electrophoresis from gastric adenocarcinoma patients. A: Examples of silver-stained two-dimensional gel electrophoresis gels for non-tumor and tumor tissue; B: Examples of differentially expressed spots (circled) for the non-tumor and tumor tissue that were chosen for immunoblot validation. MW: Molecular weight; GKN1: Gastrokine-1 precursor; MRPL12: 39S ribosomal protein L12 (mitochondrial precursor); PACAP: Plasma cell-induced resident endoplasmic reticulum protein; GSTM3: Glutathione S-transferase mu 3; SEPT2: Septin-2; UBE2N: Ubiquitin-conjugating enzyme E2 N; TALDO1: Transaldolase; TPT1: Translationally controlled tumor protein.

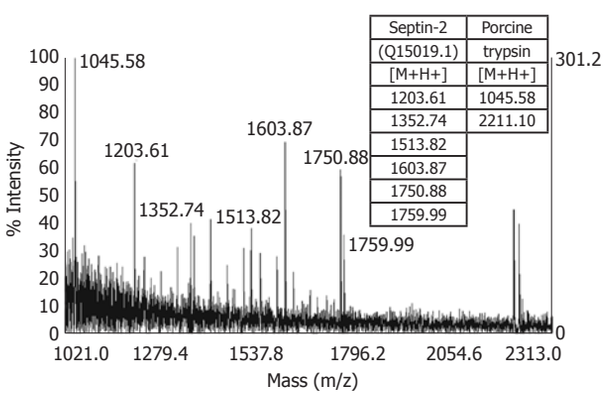

Figure 2 An example of mass spectrometry identification results for septin-2.

we identified 30 different proteins by MALDI-TOF MS or nanoLC-ESI-Q-TOF MS/MS. Eleven proteins were found to be underexpressed, while 19 were found to be overexpressed in the gastric adenocarcinoma relative to adjacent non-tumor tissue samples. These results are summarized in Table 2 and an example of the MS identification is shown in Figure 2.

The identified differentially expressed proteins could be classified into eight groups according to the biological process in which they are involved (Figure 3), by information from the GO Classification for *Homo sapiens* from European Molecular Biology Laboratory - European Bioinformatics Institute (http://www.ebi.ac.uk/integr8/GOAnalysisPage.do?orgProteomeID=25). These groups were: metabolism (*HPGD, ECHS1, MRPL12, ACADS, NDUFV2, PBLD, PACAP, ATP5H, GSTM3, PGK1, ACTB, HSPA1, CBX3, UBE2N, TALDO1, MTPN, ENO1, NNMT, EIF2S1, NME1*), development (*HPGD, NDUFV2, GSTM3, SEPT2, SERPINB5, ACTB, NGFR, MTPN, NME1*), death (*PACAP, PKM2, NGFR, CTSD, ANXA4, TPT1, ANXA5, NME1*), response to stress (*ACTB, HSPA1, UBE2N, ENO1, EIF2S1, ANXA5*), cell cycle (*GKN1, HPGD, SEPT2, NGFR, HSPA1, NME1*), cell communication (*GSTM3, ACTB, NGFR, ANXA4, ANXA5*), transport (*ATP5H, TTR, SEPT2, TPT1*), and cell motility (*SERPINB5, ACTB*).

Immunoblot analysis

In order to validate the results from the 2-DE and to investigate the possibility of the identified proteins becoming relevant biomarkers, we performed an immunoblot analysis on an expanded group of samples: 11 that were used for 2-DE as well as an additional 16 (n = 27). The selection of proteins subjected to the analysis was based on their putative relevance. GKN1 was the most abundantly underexpressed protein and was already validated at the protein level[19], so it was chosen as a general control in our 2-DE and immunoblot experiments. MRPL12 has, to the best of our knowledge, not yet been found

Table 2 Results of mass spectrometry identification

Protein name	Gene name	Swiss-Prot ID	pI/MW	Frequency[1]	Fold[2]	*P* value	Coverage[3]	No. of pep.[4]
Down-regulated								
Gastrokine-1 precursor	*GKN1*	Q9NS71	5.3/21.0	75% (9/12)	15.17	0.000	16%	6
15-hidroxyprostaglandin dehydrogenase isoform 1	*HPGD*	P15428	5.6/25.2	66% (8/12)	3.85	0.016	23%	5
Enoyl CoA hydratase, mitochondrial precursor	*ECHS1*	P30084	5.8/27.9	66% (8/12)	3.38	0.001	49%	15
39S ribosomal protein L12, mitochondrial precursor	*MRPL12*	P52815	5.1/20.6	58.3% (7/12)	10.95	0.016	16%	4
Acyl-CoA dehydrogenase	*ACADS*	P16219	6.1/39.9	58.3% (7/12)	4.94	0.005	12%	5
NADH dehydrogenase (ubiquinone) flavoprotein 2, mitochondrial precursor	*NDUFV2*	P19404	5.7/24.2	50% (6/12)	2.26	0.027	58%	16
Phenazine biosynthesis-like domain-containing protein isoform a	*PBLD*	P30039	6.1/31.8	50% (6/12)	3.60	0.007	25%	5
Plasma cell-induced resident endoplasmic reticulum protein	*PACAP*	Q8WU39	5.2/22.0	41.6% (5/12)	4.41	0.042	39%	5
ATP synthase subunit d, mitochondrial	*ATP5H*	O75947	5.2/20.5	41.6% (5/12)	2.40	0.012	26%	5
Glutathione S-transferase mu 3	*GSTM3*	P21266	5.3/26.3	33.3% (4/12)	3.35	0.042	29%	4
Transthyretin	*TTR*	P02766	5.4/16.4	25% (3/12)	2.74	0.027	53%	7
Up-regulated								
Septin-2	*SEPT2*	Q15019	6.0/41.0	75% (9/12)	4.58	0.009	34%	7
Maspin	*SERPINB5*	P36952	5.6/40.2	58.3% (7/12)	4.99	0.007	14%	5
Phosphoglycerate kinase 1	*PGK1*	P00558	6.0/40.1	58.3% (7/12)	6.46	0.016	22%	6
Pyruvate kinase, muscle	*PKM2*	P14619	6.0/38.8	58.3% (7/12)	5.25	0.021	20%	8
Actin, cytoplasmic 1	*ACTB*	P60709	4.8/32.2	58.3% (7/12)	7.77	0.005	44%	12
Tumor necrosis factor receptor superfamily member 16 precursor	*NGFR*	P08138	5.2/35.5	50% (6/12)	3.94	0.002	17%	5
Heat shock 70 kDa protein 1A/1B	*HSPA1*	P08107	5.1/36.9	50% (6/12)	4.77	0.009	14%	8
Heterochromatin-like protein 1	*CBX3*	Q13185	5.2/22.0	50% (6/12)	4.12	0.027	19%	3
Ubiquitin-conjugating enzyme E2 N	*UBE2N*	P61088	5.7/16.4	41.6% (5/12)	4.94	0.001	40%	5
Transaldolase	*TALDO1*	P37837	5.9/38.5	41.6% (5/12)	3.12	0.003	18%	5
Cathepsin D precursor	*CTSD*	P07339	5.3/28.4	41.6% (5/12)	3.49	0.034	27%	11
Annexin A4	*ANXA4*	P09525	5.6/31.3	41.6% (5/12)	13.31	0.027	54%	26
Myotrophin	*MTPN*	P58546	5.1/13.5	41.6% (5/12)	2.45	0.000	22%	4
Enolase 1 variant	*ENO1*	P06733	5.5/42.6	41.6% (5/12)	3.95	0.034	19%	7
Nicotinamide N-methyltransferase	*NNMT*	P40261	5.4/28.5	41.6% (5/12)	4.24	0.000	9%	3
Eukaryotic translation initiation factor 2 subunit 1	*EIF2S1*	P05198	4.9/33.5	41.6% (5/12)	6.61	0.042	24%	8
Translationally-controlled tumor protein	*TPT1*	P13693	4.8/20.7	25% (3/12)	2.50	0.002	18%	4
Annexin A5	*ANXA5*	P08758	5.0/32.4	25% (3/12)	3.24	0.005	70%	25
Nucleoside diphosphate kinase A isoform a	*NME1*	P15531	5.7/19.0	25% (3/12)	3.11	0.016	33%	5

[1]Frequency shows the number of patients where the protein was differentially expressed in two-dimensional gel electrophoresis analysis; [2]Fold stands for the average fold change in the relative spot volume between non-tumor and tumor (downregulated) or tumor and non-tumor (upregulated) tissue; [3]Coverage stands for the sequence coverage in mass spectrometry (MS) analysis; [4]No. of pep. stands for the number of matched peptides in MS analysis. MW: Molecular weight.

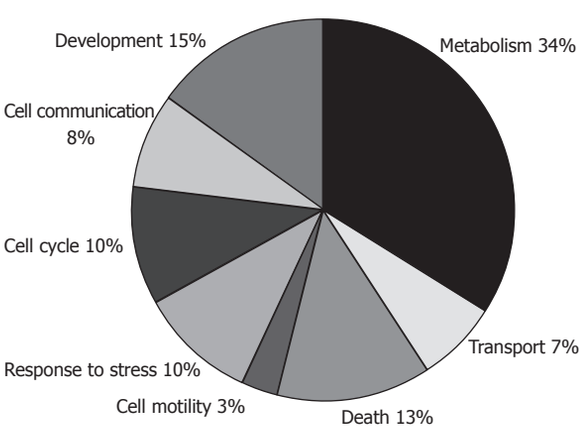

Figure 3 Distribution of differentially expressed proteins according to the biological processes in which they are involved.

to be associated with gastric cancer, while SEPT2 (as the most abundantly overexpressed), UBE2N, TALDO1, TPT1, PACAP, and GSTM3 were already found to be associated with gastric adenocarcinoma, but were not previously validated at the protein level.

Our results (Figure 4) show that GKN1 ($P = 0.0007$), MRPL12 ($P = 0.0033$), PACAP ($P = 0.0015$), and GSTM3 ($P = 0.0002$) were significantly underexpressed in gastric adenocarcinoma. SEPT2 ($P = 0.0001$), UBE2N ($P = 0.0017$), and TALDO1 ($P = 0.0006$), on the other hand, were significantly overexpressed. The immunoblot results confirmed our results from the 2-DE analysis. TPT1, on the other hand, did not appear as generally differentially expressed in the tumor samples compared to the non-tumor samples. However, we also checked for a correlation of the differential expression with the histopathological parameters. We observed (Figure 5) significant differences in the MRPL12 expression and tumor location ($P = 0.017$), as well as in TPT1 expression and tumor location ($P = 0.011$). TALDO1 expression was observed to correlate with pN status ($P = 0.036$). A higher rate of MRPL12 overexpression was found in the antrum ($P < 0.001$) and a higher rate of TPT1 overexpression

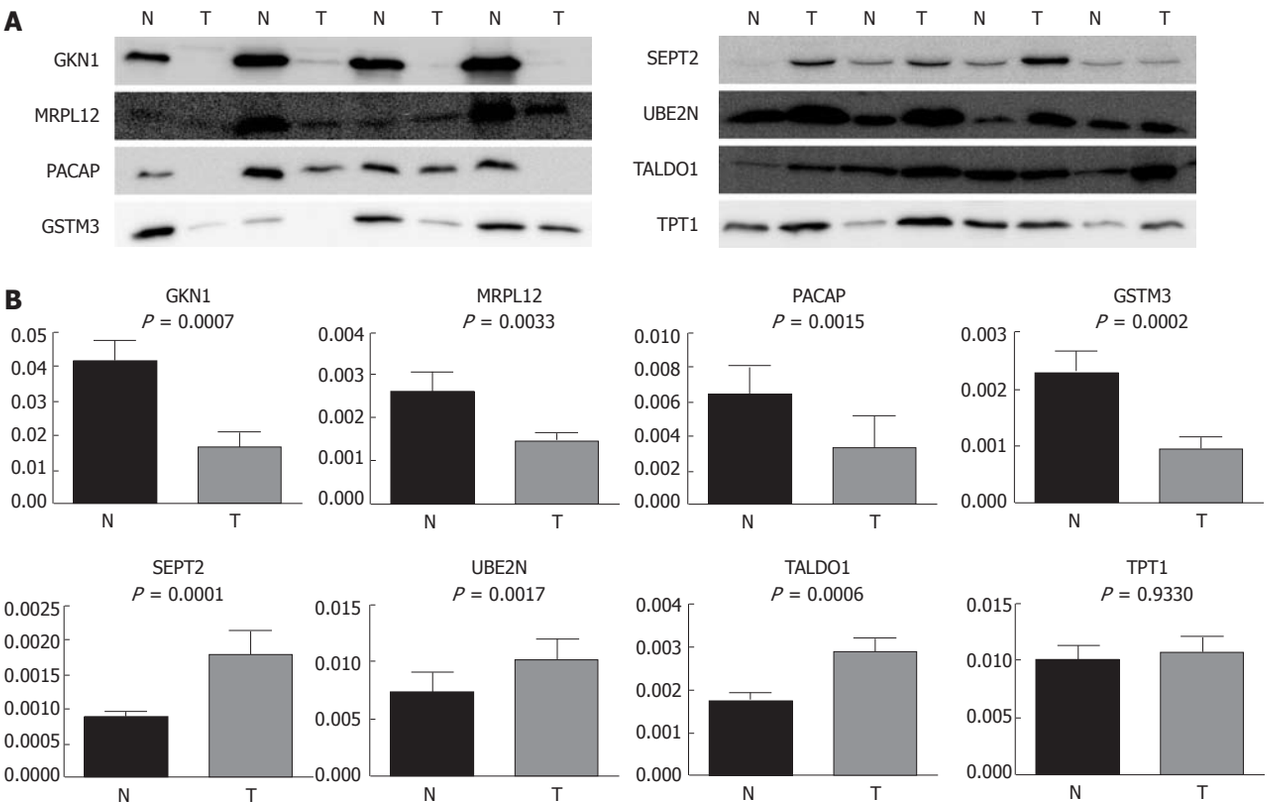

Figure 4 Examples of immunoblotting results on four pairs of non-tumor and tumor tissue for eight differentially expressed proteins (A) and densitometric results from immunoblotting for the same eight proteins (B). The x-axis represents non-tumor (N) and tumor (T) tissues and the y-axis represents relative band density. The *P* values are from the Wilcoxon signed-rank test. GKN1: Gastrokine-1 precursor; MRPL12: 39S ribosomal protein L12 (mitochondrial precursor); PACAP: Plasma cell-induced resident endoplasmic reticulum protein; GSTM3: Glutathione S-transferase mu 3; SEPT2: Septin-2; UBE2N: Ubiquitin-conjugating enzyme E2 N; TALDO1: Transaldolase; TPT1: Translationally controlled tumor protein.

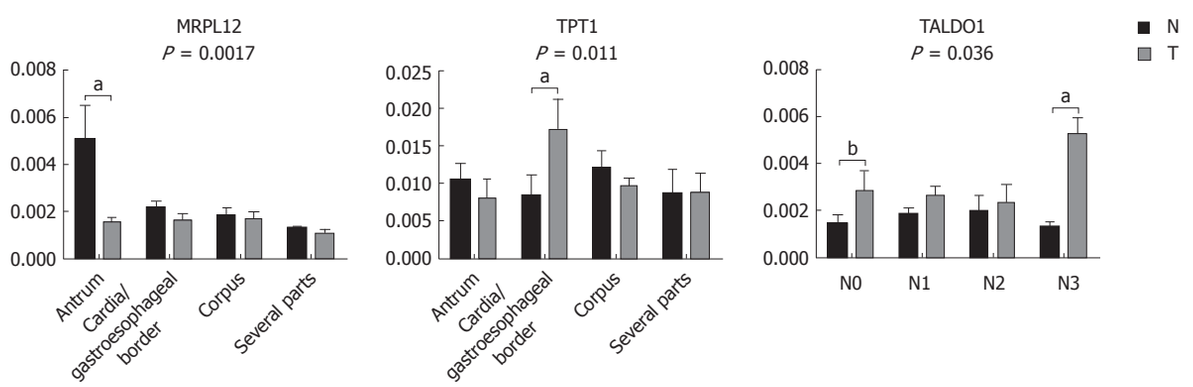

Figure 5 Densitometric results from immunoblotting for three proteins describing potential associations of differential protein expression and clinical parameters. The x-axis represents different clinical parameters and the y-axis represents relative band density. The *P* values are from the repeated measures analysis of variance. [a]*P* < 0.01 and [b]*P* < 0.05 are from the Bonferroni post-test. MRPL12: 39S ribosomal protein L12 (mitochondrial precursor); TPT1: Translationally controlled tumor protein; TALDO1: Transaldolase; N: Non-tumor; T: Tumor.

was found in the cardia/gastroesophageal border ($P < 0.01$). A higher rate of TALDO1 overexpression was found in pN0 ($P < 0.05$) and pN3 ($P < 0.01$) tumors.

Due to the interesting results of TPT1 and cardia/gastroesophageal border correlation, we used additional tissue samples from four patients with cardia/gastroesophageal border adenocarcinoma to confirm it. The trend remained the same: TPT1 was upregulated. When combining tissues from all 10 patients with cardia/gastroesophageal border adenocarcinoma (six from before and four additional), Wilcoxon signed-rank test confirmed the differential expression (Figure 6).

DISCUSSION

General observations

Using a gel-based proteomic approach, we aimed to find and validate the differentially expressed proteins in a set

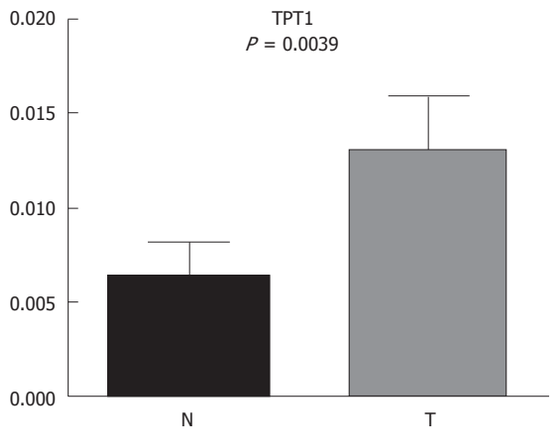

Figure 6 Densitometric results from immunoblotting of 10 pairs of non-tumor and tumor tissue from cardia/gastroesophageal border adenocarcinoma patients. The x-axis represents non-tumor (N) and tumor (T) tissues and the y-axis represents relative band density. The *P* value is from the Wilcoxon signed-rank test. TPT1: Translationally controlled tumor protein.

of gastric adenocarcinoma patients. A total of 30 different proteins were identified in the study. They belonged to different biological processes, including metabolism, development, death, response to stress, cell cycle, cell communication, transport, and cell motility. The largest group, metabolism, contained 20 proteins with at least some role in cell metabolism.

A set of these proteins has already been found in similarly conducted experiments for GC. Among those downregulated in the tumor, enoyl CoA hydratase, mitochondrial precursor[20,21], acyl-CoA dehydrogenase[22], NADH dehydrogenase (ubiquinone) flavoprotein 2, mitochondrial precursor[22], phenazine biosynthesis-like domain-containing protein isoform a[22], and ATP synthase subunit d, mitochondrial[23] have been reported with the same alterations as in our study. In contrast to our observation, ATP synthase subunit d was reported to be upregulated in another study[22]. Among the proteins upregulated in the tumor, phosphoglycerate kinase 1[19], pyruvate kinase isozymes M1/M2[24,25], heat shock 70 kDa protein 1A/1B[24,25], cathepsin D precursor[22,24,26], annexin A4[26], alpha-enolase[24,25], nicotinamide N-methyltransferase[20,27,28], annexin A5[20,29], and actin, cytoplasmic 1 in rat GC metastases[30] have been found elsewhere with the same trend as in our study. However, other studies have reported that pyruvate kinase[22], alpha-enolase[26], and actin[26,31] have the opposite expression patterns compared to our results. This discrepancy could perhaps result from the heterogeneity that is often present in studies of human tumor tissue. In this paper, we present and discuss eight putative biomarkers for gastric carcinogensis that were identified in our study, among which seven were validated for the first time by means of immunoblotting.

Specific protein alterations

GKN1 has already been identified as a downregulated gene in GC[32]. It is suggested to maintain gastric mucosal integrity and mediate repair after injury[33]. The protection of the mucosal barrier is thought to be due to the ability of GKN1 to alter the distribution of specific tight-junction proteins and to stabilize perijunctional actin[34]. It was later demonstrated to bind with F-actin in smooth muscle cells, suggesting a role in cell–cell adhesion and the assembly of actin stress fibers[35]. Recently, GKN1 has been shown to be a modulator of apoptotic signals[36]. It has been confirmed as a secreted protein and as being present in native and metaplastic gastric epithelium, but absent from the gastric carcinoma and the precursor lesion of intestinal metaplasia, making it a possible tumor suppressor in gastric carcinogenesis[37].

GKN1 expression is downregulated in *Helicobacter-pylori*-positive patients[38]. In another study, a loss of GKN1 occurred, especially in the diffuse-type tumor, but was associated with a significantly worse outcome in the intestinal type[39]. It has been found to be downregulated in GC, using 2-DE[20,23,25,27], and these results have been validated at the protein and mRNA levels[19]. Consistently, we found that GKN1 was underexpressed in tumor tissue, using 2-DE, and we also validated the results by immunoblotting. Our result is in agreement with other reports and, like other research groups, we were also unable to find any correlation with histopathological parameters at the protein level. The overlapping of both steps of the biomarker identification with a number of other studies in this case contributed to the confidence in the approach for the analysis of additional biomarkers found in our proteomic analysis.

MRPL12 is the first cloned and characterized mammalian mitochondrial ribosomal protein encoded by the nucleus[40]. It accumulates in cells at the mRNA and protein levels upon growth-factor stimulation. The enhanced expression later contributes to transcriptional activation[41]. *MRPL12* mRNA levels have been detected in different organs, being especially high in the colon and skeletal muscle[41]. Besides being a component of the mitochondrial ribosome (its dimers bind the large ribosomal unit), MRPL12 binds to mitochondrial RNA polymerase and enhances transcription *in vitro*[42]. It has been speculated that it may either directly couple transcription and translation by binding simultaneously to polymerase and ribosomes, or alone bind to polymerase and activate its transcriptional activity in some way[43]. However, Litonin *et al*[44] have observed no such stimulation, so further experiments are necessary to clarify the possible role of this protein in transcription.

MRPL12 is differentially expressed, and it has previously been observed by 2-DE to be overexpressed in prostate cancer[45] and in hepatitis B virus (HBV)-associated hepatocellular carcinoma[46]. This is not in accordance with our results because we showed that MRPL12 was downregulated in gastric adenocarcinoma. We also observed it correlation with location: higher rates of underexpression were found in the antrum. In the context of cancer, the knockdown of MRPL12 decreases mitochondrial activity, increases glycolysis and accelerates

tumor growth[47]. We speculate that it may also be the case in gastric adenocarcinoma.

PACAP was found as an expressed sequence tag from a microarray analysis where it exhibited downregulation in intestinal-type GC[48]. The protein was found in the endoplasmic reticulum of lymphocytes, where it exhibited upregulation in the course of B-cell differentiation[49,50]. It was found to assist in the oxidative folding of Ig domains; however, both research groups were uncertain as to whether it acted as an oxidoreductase or as a chaperone. In a very recent report[51], PACAP was described as helping to diversify peripheral B-cell functions by regulating Ca^{2+} stores, antibody secretion and integrin activation.

A patent application[52] has disclosed the use of PACAP as a universal marker of different types of cancer, GC included. However, they claim that increased concentrations of the protein and/or its fragments are associated with cancer, whereas we discovered just the opposite for GC. The same was reported by Huang *et al*[31] in a 2-DE experiment (although this was without validation), by Hasegawa *et al*[48] and Katoh *et al*[53]. However, in the latter two cases, this was at the non-protein level. We validated our results by immunoblotting. Due to its downregulation in the tumor, we strongly support the previous speculation that PACAP might be a candidate tumor-suppressor gene[53].

GSTM3 is a glutathione S-transferase (GST) that belongs to the mu class[54]. It is rather unusual; it is not only about 70% identical in its protein sequence to the other mu-class transferases, but it is also considerably shorter and transcribed in the reverse orientation[55].

Several polymorphisms have been found in GSTs, which can alter the susceptibility to carcinogens and toxins and influence the toxicity and efficacy of drug treatment[54]. They have been studied in relation to several cancers, GC included. For example, Martinez and colleagues have found no association with the *GSTM3* genotype and GC risk[56], whereas Tatemichi and co-workers have described a possible association between *GSTM3* polymorphisms and Ig titer levels in serum against *H. pylori*[57].

GSTM3 is found in several normal tissues, including the stomach[58]. A comparison of the differential GSTM3 expression in cancers is made rather difficult by the fact that many studies have focused on the whole GST family or class, but not on individual isoforms. For instance, antral GST enzyme activity has been found to be significantly lower in the stomach of *H. pylori*-infected patients[59], but the contributions of separate isoforms has not been studied. For GSTM3 specifically, the gene is highly expressed in a subgroup of patients with head and neck squamous cell carcinoma[60]. The protein is upregulated in neuroblastoma[61] and in polycystic ovary syndrome[62]. We found that GSTM3 was downregulated in gastric adenocarcinoma. Our results are in agreement with a study reporting downregulation in the seminomatous germ cell tumor, where the changes were also reflected at the transcriptional level[63]. This reduced expression could be indicative of the decreased detoxification capacity of tumor cells[59].

Septins belong to a family of conserved GTP-binding proteins[64]. They have been implicated in many cellular processes. SEPT2 is thought to be involved in cytokinesis, as well as chromosome congression and segregation[65,66]. Its fibers appear to contact actin bundles and focal adhesion complexes physically, thereby linking it to a functional interaction with actin-based cytoskeletal systems in interphase cells[65]. It has also been found in the microtubule spindle during metaphase and is proposed to form a mitotic scaffold for different effectors to coordinate cytokinesis with chromosome congression and segregation[66]. Several other binding partners and functions have been proposed for SEPT2, such as the DNA damage response, the regulation of the efficiency of vesicular transport, and FCγR-mediated phagocytosis[64]. Recently, it has been reported that SEPT2 is part of a diffusion barrier between the primary cilia and the cell and it is essential for retaining receptor-signaling pathways in primary cilia[67]. Also, in response to physiological and pathological stimuli, SEPT2 redistributes and its interaction with actin increases, which allows for the dynamic modulation of the airway epithelial barrier function[68]. Despite all the progress, the exact molecular mechanisms, cellular, and physiological functions of septins are still poorly understood and interactome studies could help[69].

Septins have been linked to diseases such as neurodegeneration and cancer[70]. It is proposed that altered SEPT2 expression can lead to disordered chromosomal dynamics and underlie the development of the aneuploidy common to cancers[66]. SEPT2, among others, has been found to be a fusion partner of the mixed lineage leukemia (*MLL*) gene in therapy-related acute myeloid leukemia[71]. Such fusion is associated with downregulation of *SEPT2* and *MLL* in myeloid neoplasia[72]. It has also been shown to be downregulated in glioblastoma[73]. On the other hand, it has been identified as upregulated in hepatoma carcinoma cells, where its phosphorylation on Ser218 by casein kinase 2 has been determined as crucial for hepatoma carcinoma cell proliferation[74]. In a 2-DE experiment, SEPT2 was, in agreement with our results, determined to be upregulated in GC[22]; however, no validation was carried out. The same expression pattern was found in renal cell carcinoma[75] and in late-stage human colon cancer tissue[76], and it is abundantly expressed in several brain tumors and brain-tumor cell lines[77]. Taken as a whole, these results suggest its possible role as an oncogene.

Ubiquitination is a post-translational modification carried out in several steps[78], one of them being conjugation of an activated ubiquitin to an ubiquitin-conjugating enzyme (E2) *via* a highly conserved catalytic cysteine residue. By directly influencing the type of lysine used to label the substrates, they influence the fate of the substrates. One of the E2s is UBE2N, which acts as part of a complex that enables the formation of the non-canonical Lys63-mediated polyubiquitin chains[79]. As opposed to Lys48-mediated ones, these do not target proteasome degradation but mediate other processes. Among other functions, it has been shown that UBE2N in a complex with Mms2 functions *via* Lys63-mediated polyubiquitination in DNA repair, whereas in a complex with Uev1A, it functions in activating nuclear factor-κB signaling[78,80]. Both of the partner proteins, however, are dispensable for the RNF8-dependent propagation of DNA damage signals *via* ubiq-

uitination[81], although there are some questions as to the importance of this activity[82].

UBE2N is differentially expressed between different types of leukemia and lymphoma cell lines[83]. It has significantly lower transcriptional expression levels in non-small-cell lung cancer and is correlated with pN and the stage of the disease[84]. In a breast-cancer metastatic model using iTRAQ technology, UBE2N was downregulated when comparing cells with the most metastatic potential and non-metastatic cells[85]. On the other hand, it was observed in a 2-DE experiment to be overexpressed in HBV-associated liver cancer[46], which is consistent with our results. It has already been shown to be differentially expressed in GC[86]; however, it has not been validated whether it is up- or downregulated. UBE2N-dependent Lys63-mediated polyubiquitination regulates processes that often enhance cell survival in response to certain forms of stress[87], therefore, our result supports its implication in the regulation of similar processes in gastric cancerogenesis.

TALDO1 is an almost ubiquitous cofactor-less enzyme of the pentose phosphate pathway[88]. Its activity is tissue specific and, in the brain, it is selectively expressed in oligodendrocytes, thus connecting it to different autoimmune diseases, such as multiple sclerosis[89]. Its expression is developmentally controlled[88].

TALDO1 is the rate-limiting enzyme of the non-oxidative part of the pentose phosphate pathway[88] that catalyzes the reversible transfer of a three-carbon unit between various sugar phosphates (from ketose to aldose sugar phosphates). It has a role in regulating the balance between the two branches of the pentose phosphate pathway and its overall output, as measured by NADPH and glutathione production, and thus influences the sensitivity to cell-death signals[90].

When comparing tumor and normal TALDO1, its activity is increased in neoplastic liver[91]. Its gene is highly expressed in a subgroup of patients with squamous cell carcinoma of the head and neck[60]. Furthermore, it is upregulated in late-stage human colon-cancer tissue[76] and in the sera of colorectal cancer patients[92]. In metastatic, compared to non-metastatic GC cell lines[93] and in GC tissue[86], TALDO1 was overexpressed, as shown by 2-DE. However, in both studies, again, no validation was performed. All these studies are consistent with our results, which were also validated by immunoblotting. We also found that TALDO1 correlated with pN status at stages pN0 and pN3. A higher TALDO1 expression in the tumor tissue could reflect an increased metabolism of glucose for the synthesis of nucleic acids in malignant cells[92].

TPT1 is a ubiquitously expressed and highly conserved protein. It is associated with various cellular processes, such as cell-cycle progression, release of histamine and various interleukins, apoptosis, malignant transformation, and tumor reversion[94,95]. Very recently, it was also discovered as a glucose-regulated protein, important for the survival of pancreatic beta cells[96].

It has been implicated in cancer, although it is not tumor-specific. It is upregulated in various tumor tissue cell lines when compared to normal tissue cell lines[97], in breast[98] and colon cancer[99]. As for the gastric tissue, TPT1 has been reported as cDNA present in libraries only from normal gastric tissues[100]. In our study, TPT1 was not validated as generally differentially expressed in the whole group of samples. Instead, its expression was location-correlated; TPT1 was upregulated in gastric adenocarcinoma from the cardia/gastroesophageal border. In contrast to the general worldwide decline of GC rates, an increasing incidence of gastric cardia cancer has been observed in several countries[1]. This suggests that it is a distinct clinical entity[26]. Therefore, it is possible that TPT1 is implemented only in gastric cardia/gastroesophageal border carcinogenesis.

Comparison of tumor and adjacent, non-tumor gastric tissues by means of proteome analysis, including differential 2-DE coupled to MS analysis, revealed 30 protein alterations. Some of the differentially expressed proteins had already been observed in GC in previous studies, which supports the reliability of our analysis. Several other proteins were found with the same trend of differential expression in other types of cancer, which could suggest that they are commonly involved in carcinogenesis. The high mortality rate from GC is due to delayed detection and surgical resection at advanced stages of the disease. A breakthrough in the early diagnosis of GC has not occurred yet and there are currently very few markers that are clinically in use; however, advances in proteomic research are facilitating the identification of novel diagnostic, prognostic, or therapeutic biomarkers. It is apparent that a collection of protein biomarkers will be necessary for reliable cancer detection and monitoring, as single biomarkers often have an inadequate predictive value[101]. There is, therefore, a need for the expedited development of new, validated biomarkers to be added to the list of clinically relevant tumor-associated proteins in the proteome databases of gastric tissue and cell lines. To the best of our knowledge, we are the first to observe aberrant expression of MRPL12 in gastric adenocarcinoma, and, in addition, aberrant expression of PACAP, GSTM3, SEPT2, UBE2N, TALDO1 and TPT1 for the gastric cardia/esophageal border were validated in gastric adenocarcinoma, also for the first time. Future experiments are planned to use these biomarkers in the design of a combinatory microarray and to translate the obtained results to blood samples, so the proteins would ultimately be useful as biomarkers for early detection.

ACKNOWLEDGMENTS

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COMMENTS

Background

Gastric cancer (GC) is the fourth most common cancer worldwide. Despite declining incidence rates, it is still the second leading cause of cancer death and thus remains a major health problem. The majority of patients are diagnosed at an advanced stage when the 5-year survival rate is very low.

Research frontiers

Current diagnosis is invasive, whereas blood biomarkers lack sensitivity and

specificity. This study investigated proteome changes in gastric cancerous *vs* non-cancerous tissue in the hope of discovering new biomarker candidates. It reports the validation of seven aberrantly expressed proteins, one of them being reported as a novel candidate biomarker.

Innovations and breakthroughs

This is believed to be the first study that reports aberrant expression of 39S ribosomal protein L12 in gastric adenocarcinoma, and for the first time, validates aberrant expression of plasma cell-induced resident endoplasmic reticulum protein, glutathione S-transferase mu 3, septin-2, ubiquitin-conjugating enzyme E2 N, transaldolase, and translationally controlled tumor protein for the cardia/gastroesophageal border at the protein level.

Applications

Future work will be focused on using the validated biomarkers in the design of a diagnostic protein microarray and on translating the research to blood samples, so that the proteins would ultimately be useful as biomarkers for early detection. By showing their differential expression, this work might contribute to the efforts to understand GC carcinogenesis, as well as present a set of possible diagnostic biomarkers for gastric adenocarcinoma.

Peer review

Proteomic analysis was done in the present study. And several potential biomarkers were selected as an important protein for gastric cancer carcinogenesis. These fields are very important for further developments of the clinical treatment in patients with various malignancies including gastric cancer.

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BRIEF ARTICLE

High-dose infliximab for treatment of pediatric ulcerative colitis: A survey of clinical practice

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Abstract

AIM: To assess attitudes and trends regarding the use of high-dose infliximab among pediatric gastroenterologists for treatment of pediatric ulcerative colitis (UC).

METHODS: A 19-item survey was distributed to subscribers of the pediatric gastroenterology (PEDSGI) listserv. Responses were submitted anonymously and results compiled in a secure website.

RESULTS: A total of 113 subscribers (88% based in the United States) responded (101 pediatric gastroenterology attendings and 12 pediatric gastroenterology fellows). There were 46% in academic medical institutions and 39% in hospital-based practices. The majority (91%) were treating >10 patients with UC; 13% were treating >100 patients with UC; 91% had prescribed infliximab (IFX) 5 mg/kg for UC; 72% had prescribed IFX 10 mg/kg for UC. Using a 5-point Likert scale, factors that influenced the decision not to increase IFX dosing in patients with UC included: "improvement on initial dose of IFX" (mean: 3.88) and "decision to move to colectomy" (3.69). Lowest mean Likert scores were: "lack of guidelines or literature regarding increased IFX dosing" (1.96) and "insurance authorization or other insurance issues" (2.34). "Insurance authorization or other insurance issues" was identified by 39% as at least somewhat of a factor (Likert score ⩾ 3) in their decision not to increase the IFX dose. IFX 10 mg/kg was more commonly used for the treatment of pediatric UC among responders based in the United States (75/100) compared to non-United States responders (6/13, P = 0.047). Induction of remission was reported by 78% of all responders and 81% reported maintenance of remission with IFX 10 mg/kg. One responder reported one death with IFX 10 mg/kg.

CONCLUSION: IFX 10 mg/kg is more commonly used in the United States to treat pediatric UC. Efficacy and safety data are required to avoid insurance barriers for its use.

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Key words: Immunosuppression; Inflammatory bowel disease; Ulcerative colitis; Children; Pharmacology

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INTRODUCTION

In the last decade, infliximab has become an alternative treatment for moderate to severe ulcerative colitis (UC). The landmark ACT1 and ACT2 trials in adults showed that infliximab (IFX) successfully induced remission in patients with corticosteroid sensitive and corticosteroid resistant UC[1]. More recently, the Pediatric Inflammatory Bowel Disease Collaborative Research Group showed that children with corticosteroid-dependent or corticosteroid-refractory UC treated with IFX have a significant decrease in corticosteroid dependency and avoidance of colectomy in 72% after 1 year and 61% after 2 years of treatment[2].

The Food and Drug Administration (FDA) has approved the use of IFX for treatment of moderate to severe UC for adult patients at a dose of 5 mg/kg[3]. However, in patients who lose responsiveness, the dose of IFX is commonly increased up to 10 mg/kg. The ACT1 and ACT2 trials demonstrated a response at this higher dose[1]. No studies have documented a benefit at this higher dose in pediatric patients.

Given the increased use of IFX for treatment of UC and the ongoing issues regarding efficacy, safety and insurance approval, we conducted a survey of pediatric gastroenterologists to collect information regarding dosing practices of IFX for the treatment of UC in the pediatric population. The results of this survey provide a glimpse into the attitudes of pediatric gastroenterologists regarding the use of high-dose IFX for pediatric UC.

MATERIALS AND METHODS

An anonymous survey was created using the web-based survey software SurveyMonkey.com® (Portland, OR). The survey was distributed to all United States based and international pediatric gastroenterologists participating in the pediatric gastroenterology (PEDSGI) listserv with the approval of the listserv administrator. This survey included an electronic consent form and was free of identifiers. In addition, the survey forms were secured using secure sockets layer encryption to maintain the Health Insurance Portability and Accountability Act compliance. An "Opt Out" link was available at any point during the survey. This survey was approved by the University of California, San Francisco Committee on Human Research.

Demographic information was obtained by asking responders to provide data regarding their specialty (pediatric gastroenterology attending, pediatric gastroenterology trainee, or other specialty), practice type (academic, private, hospital based or other), practice size (< 10, 10-50, 51-100, 101-200 or > 200 patients) and location (Western United States, Midwest United States, Northeast United States, South United States, Europe, Middle East, Asia or "Other").

To determine the extent of IFX use among pediatric gastroenterologists, responders were asked, "Have you ever prescribed IFX at 5 mg/kg?" Those pediatric gastroenterologists that responded positively were asked, "What were the diagnoses for those patients receiving IFX 5 mg/kg (Crohn's, UC, inflammatory bowel disease-unspecified (IBD-U) or other)?" Responders were then asked, "Have you ever prescribed IFX at 10 mg/kg?" Those that responded positively were again asked, "What were the diagnoses for those patients receiving IFX 10 mg/kg (Crohn's, UC, IBD-U or other)?" Responders who listed UC as a diagnosis were subsequently asked to specify all indications for which they prescribed IFX 10 mg/kg (steroid refractory UC, mild-to-moderate UC for induction or maintenance of remission, moderate-to-severe UC for induction or maintenance of remission, severe UC for induction or maintenance of remission).

Difficulty in achieving dose escalation was evaluated by asking those with experience in prescribing IFX 5 mg/kg: "… was an increase in IFX dosing considered at any point?" Responders who answered positively were subsequently asked: "…was an increase in IFX dose achieved?"

To further evaluate barriers to IFX dose escalation, a 5-point Likert scale was created whereby responders were asked to rank (scale of 1-5) factors that may have influenced their decision not to increase IFX to 10 mg/kg. The factors listed included: (1) patient improvement on the initial dose of IFX; (2) patient improvement on alternate medical therapy; (3) decision to move to colectomy; (4) lack of guidelines or literature regarding dose escalation; and (5) insurance authorization or other insurance issues. Responders were also asked to list other reasons that may have influenced their decision not to increase IFX dosing.

To evaluate the variation in administration of concomitant immunosuppressive medications, responders were asked to specify all medications instituted prior to IFX 10 mg/kg. In a separate question, responders were asked to specify all medications instituted concomitant with IFX 10 mg/kg for the treatment of UC. Answer choices included: corticosteroids, aminosalicylates, azathioprine (AZA), 6-mercaptopurine (6-MP), and methotrexate. Responders were also asked to specify any other medications that were not listed.

Methods for administration of IFX 10 mg/kg such as dosing interval and continuous or episodic treatment were not surveyed.

Responders were also asked to select any adverse events their patients experienced as a result of IFX 10 mg/kg. Answer choices included: worsening UC, headache, arthralgia, profound anemia, infection, oncologic process, neurologic event, antibodies against IFX or other autoimmune antibodies and infusion reactions. Responders were asked to specify any other adverse event that was not listed.

Finally, responders were asked to list all outcomes their patients experienced as a result of IFX 10 mg/kg (clinical remission, maintenance of remission, colectomy, continued or worsening colitis, death). Again, responders were asked to specify any other unlisted outcome.

Response frequencies were tabulated and expressed as percentages of total responses. Differences among groups with regard to frequency and Likert scale variables were assessed for significance using a Wilcoxon

Table 1 Indication for initiation of infliximab 10 mg/kg

Answer options	Percentage (*n*)
Steroid refractory UC	70.5 (55)
Severe UC for maintenance of remission	64.1 (50)
Moderate-to-severe UC for maintenance of remission	62.8 (49)
Severe UC for induction of remission	55.1 (43)
Moderate-to-severe UC for induction of remission	38.5 (30)
Mild-to-moderate UC for maintenance of remission	9.0 (7)
Mild-to-moderate UC for induction of remission	2.6 (2)
Other	6.4 (5)

78 respondents. UC: Ulcerative colitis.

Table 2 Therapies introduced prior to infliximab 10 mg/kg

Answer options	Percentage (*n*)
Corticosteroids	96.2 (75)
Infliximab 5 mg/kg	92.3 (72)
Aminosalicylates	89.7 (70)
Azathioprine[1]	76.9 (60)
6-mercaptopurine[1]	70.5 (55)
Methotrexate	25.6 (20)
Other	14.1 (11)
None	1.3 (1)

[1]Reported use by physicians included either azathioprine or 6-mercaptopurine in different patients. 78 respondents.

Rank Sum test for comparing two groups, or a Kruskal-Wallis test for more than two groups as the data were not normally distributed. Differences between categorical variables were tested using χ^2 and Fisher exact tests. Differences were considered significant for $P \leqslant 0.05$.

RESULTS

Demographics

Our survey yielded 113 responders (5.7%) out of a total of 1993 subscribers to the PEDGI listserv. Of those responders, 101 were pediatric gastroenterology attendings and 12 were trainees in pediatric gastroenterology programs. Most responders identified themselves as based in an academic medical institution (46.0%) or a hospital-based practice (38.9%). A minority of respondents identified themselves as working in a private practice (12.4%). Most responders also identified themselves as practicing within the United States (88.5%). Ninety-one percent of responders reported practices that treat at least 10 patients with UC, 23.0% reported 50 to 100 patients, and 13.3% reported over 100 patients with UC.

Initiation of IFX

Ninety-one percent (103/113) of respondents had prescribed IFX 5 mg/kg for treatment of UC, while 71.7% (81/113) had prescribed IFX 10 mg/kg for treatment of UC. The indications for prescribing IFX 10 mg/kg are listed in Table 1. A large majority of respondents selected steroid refractory UC as an indication for IFX 10 mg/kg (70.5%). In addition, a majority of respondents listed severe UC for induction and maintenance of remission as an indication for prescribing IFX 10 mg/kg (55.1% and 64.1%, respectively). A large number of respondents had also used IFX 10 mg/kg for treatment of moderate-to-severe UC, both for induction and maintenance of remission (38.5% and 62.8%, respectively).

The overall use of IFX 10 mg/kg to treat UC did not differ significantly when comparing responders in academic and non-academic practices. However, more responders working in non-academic practices had prescribed IFX 10 mg/kg for maintenance of remission for severe UC (29/39) compared with those in academic practices (19/39, $P = 0.034$).

Responders based in the United States were more likely to use IFX 10 mg/kg to treat UC (75/100) compared with non-United States responders (6/13, $P = 0.047$). However, the indication for initiation of IFX 10 mg/kg for UC did not differ between these two groups.

Increasing the IFX dose

Pediatric gastroenterologists will often increase IFX dosing in patients that respond poorly to IFX 5 mg/kg. Respondents who had prescribed IFX 5 mg/kg for the treatment of UC were subsequently asked, "Have you ever considered an increase in IFX dosing above 5 mg/kg?" Overwhelmingly, 87.4% (90/103) of those using IFX had at least considered an increase in IFX dosing.

Using a 5-point Likert scale, respondents were asked to rank factors that may have influenced their decision not to increase IFX dosing in patients with UC (Figure 1). Among the 82 respondents, "improvement on initial dose of IFX" and "decision to move to colectomy" had the highest mean Likert scores (3.88 and 3.69, respectively). "Lack of guidelines or literature regarding increased IFX dosing" and "insurance authorization or other insurance issues" had the lowest mean Likert scores (1.96 and 2.34, respectively). Of note, however, 39.0% (32/82) identified "insurance authorization or other insurance issues" as at least somewhat of a factor (Likert score $\geqslant 3$) in their decision not to increase the IFX dose in patients with UC.

Respondents were asked to list all medical therapies that may have been instituted prior to IFX 10 mg/kg (Table 2). Many of the respondents selected all the alternative medical therapies listed, and only one reported prescribing IFX 10 mg/kg without attempting any prior medical therapy. "Other" medications that were specified included tacrolimus, probiotics, metronidazole and other antibiotics.

Respondents were also asked what therapies they had instituted concurrently with IFX 10 mg/kg (Table 3). Most used corticosteroids with IFX 10 mg/kg. In addition, aminosalicylates were commonly administered with IFX 10 mg/kg. Conversely, fewer prescribed AZA or 6-MP along with IFX 10 mg/kg. A small minority administered IFX 10 mg/kg alone. Methotrexate was also noted to be used concurrently with IFX 10 mg/kg.

No significant differences in practice patterns with regards to medications initiated prior to and concurrent with IFX 10 mg/kg were observed between those in

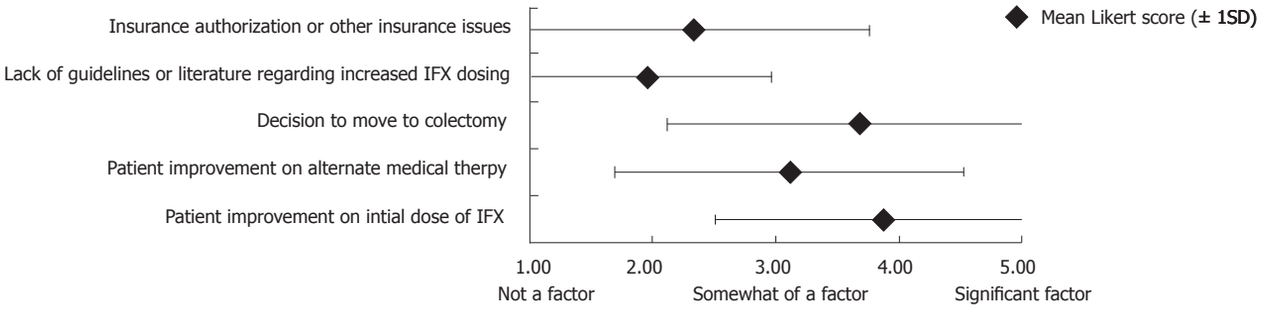

Figure 1 Factors that influenced pediatric gastroenterologists to not increase infliximab dosing depicted as a 5-point Likert scale. IFX: Infliximab.

Table 3 Therapies instituted with infliximab 10 mg/kg

Answer options	Percentage (*n*)
Corticosteroids	70.5 (55)
Aminosalicylates	61.5 (48)
Azathioprine[1]	39.7 (31)
6-mercaptopurine[1]	34.6 (27)
Antibiotics	44.9 (35)
None	11.5 (9)
Methotrexate	6.7 (5)
Other	1.3 (1)

[1]Reported use by physicians included either azathioprine or 6-mercaptopurine for different patients. 78 respondents.

Table 4 Outcomes reported using infliximab 10 mg/kg

Answer options	Percentage (*n*)
Maintenance of remission	80.8 (63)
Clinical remission	78.2 (61)
Colectomy	60.3 (47)
Continued or worsening colitis	51.3 (40)
Other	2.6 (2)
Mortality	1.3 (1)

78 respondents.

academic and non-academic practices.

A larger number of United States based responders had prescribed 6-MP prior to instituting IFX 10 mg/kg to treat UC (54/75, 72.0%) compared with non-United States based responders (1/6, 16.7%, $P = 0.012$). In addition, more United States based responders had prescribed 5-aminosalicylates concurrently with IFX 10 mg/kg (47/75, 62.7%) compared with non-United States responders (1/6, 16.7%, $P = 0.039$). Similarly, antibiotics was more commonly prescribed concurrently with IFX 10 mg/kg among United States responders (35/75, 46.7%) compared with non-United States responders (0/6, $P = 0.034$).

Outcomes with high-dose IFX

Table 4 lists the outcomes reported by respondents as a result of using IFX 10 mg/kg. A majority of pediatric gastroenterologists surveyed reported either clinical remission (61/78, 78.2%) or maintenance of remission with this dose (63/78, 80.8%). Over half of the surveyed pediatric gastroenterologists who used 10 mg/kg IFX had patients who continued to suffer worsening colitis (40/78, 51.3%) or who moved to colectomy (47/78, 60.3%).

Table 5 Side effects and adverse reactions

Answer options	Percentage (*n*)
None	64.1 (50)
Antibodies to infliximab	17.9 (14)
Infusion reaction or delayed hypersensitivity reaction	16.7 (13)
Worsening ulcerative colitis	14.1 (11)
Infection	12.8 (10)
Headache	9.0 (7)
Arthralgia	9.0 (7)
Development of autoimmune antibodies (e.g., antinuclear antibodies, anti-dsDNA antibodies)	5.1 (4)
Other	2.6 (2)
Profound anemia	1.3 (1)
Oncologic process	0 (0)
Neurologic event (e.g., neuritis, neuropathy)	0 (0)

78 respondents.

Most pediatric gastroenterologists (50/78, 64.1%) reported no adverse effects of the higher dose (Table 5). The most common adverse events reported by prescribers were antibodies to IFX (14/78, 17.9%) and infusion reactions (13/78, 16.7%); 14.1% (11/78) respondents reported discontinuing IFX 10 mg/kg as a result of these adverse reactions. No oncologic or major neurologic events were reported as a consequence of administering IFX 10 mg/kg. However, a single respondent reported one death while using IFX 10 mg/kg.

DISCUSSION

This survey was designed to understand the attitudes and driving forces of pediatric gastroenterologists regarding the use of high-dose IFX for the treatment of UC. In our sample, a majority of the pediatric gastroenterologists surveyed had experience treating pediatric UC with IFX 10 mg/kg.

One of the strengths of this study is that the respondents were evenly split between academic institutions and non-academic practices, capturing the major practice settings of pediatric gastroenterologists.

One of the weaknesses of this study is the low response rate, which may represent a non-response bias. However, it should be noted that a large number of the 1993 PEDSGI listserv subscribers are not pediatric gastroenterologists. Many of the listserv subscribers are surgeons, pathologists, nurse practitioners and other healthcare professionals. Although the exact number of pediatric gastroenterologists that subscribe to the listserv is unknown, the response rate is likely much higher among this particular group. According to the American Board of Pediatrics, at the time this survey was conducted, 974 board certified pediatric gastroenterologists were registered in the United States[4]. Based on these numbers, our survey results represent the opinions of 9.3% (91/974) of the registered pediatric gastroenterologists practicing within the United States.

In addition, the results of this survey study are subject to recall bias. Respondents who have experienced more extreme outcomes with IFX or difficulty with insurance approval for higher doses of IFX are more likely to respond in an exaggerated manner.

While the results of this uncontrolled study are subject to various biases, they may also be indicative of the trends regarding the use of high-dose IFX among pediatric gastroenterologists.

IFX is effective for both induction and maintenance of remission for steroid-refractory and steroid-dependent UC[1,2,5]. Predictably, most of the pediatric gastroenterologists surveyed indicated steroid refractoriness as an indication for prescribing IFX 10 mg/kg. In addition, nearly all pediatric gastroenterologists surveyed indicated that both corticosteroids and IFX 5 mg/kg had been employed prior to increasing the IFX dose to 10 mg/kg. IFX 10 mg/kg may also be a corticosteroid-sparing agent as reflected in the response that fewer pediatric gastroenterologists reported using corticosteroids after the 10 mg/kg dose was instituted.

While nearly three-quarters of pediatric gastroenterologists surveyed have prescribed 6-MP or AZA therapy prior to instituting IFX, only about one-third of pediatric gastroenterologists surveyed have administered 6-MP or AZA concurrently with IFX 10 mg/kg. This practice may be in flux, as recent reports suggest improved disease responsiveness and lower risk of antibodies to IFX with concomitant immunomodulators, despite concerns of hepatosplenic T-cell lymphoma[6-10]. In our survey, no oncologic or serious neurologic adverse outcomes were reported. Future studies should further clarify the risk *vs* benefits of concomitant immunomodulator therapy.

Our results also suggest that pediatric gastroenterologists in the United States are more likely to prescribe IFX 10 mg/kg to treat UC compared with those working outside the United States. The only difference found for responders in academic compared with non-academic settings was an increased use of high-dose IFX for maintenance of remission for severe UC among non-academic pediatric gastroenterologists.

These differences highlight the lack of universal guidelines and need for further studies regarding the optimal use of IFX 10 mg/kg for the treatment of UC.

The most common reported side effects were antibodies to IFX and infusion reactions. Infections were also reported by 12.8% of responders. One respondent reported death as an adverse outcome. The events that lead to this death are not known. It is thought, however, that this fatality was not a result of oncologic, neurologic or infectious complications as the same respondent did not report experiencing any of these adverse effects while using IFX 10 mg/kg.

Most pediatric gastroenterologists surveyed reported improved outcome as a result of prescribing IFX 10 mg/kg, documenting a benefit of the higher dose in pediatric UC. However, over half of the respondents reported worsening disease and the need for colectomy, possibly due to loss of responsiveness, or in some cases a lack of any response to the increased dose.

IFX is approved by the FDA for the treatment of moderate to severe UC at a dose of 5 mg/kg[3]. Insurance companies have often rejected appeals for reimbursement for increased doses of IFX due to lack of published data showing efficacy and safety for pediatric patients with UC. Despite this, however, our survey suggests that insurance approval or other insurance issues did not uniformly prevent pediatric gastroenterologists from prescribing IFX 10 mg/kg.

Insurance approval for higher doses of IFX is achieved *via* an exception process or appeal process. An exception process involves an initial request for a health plan to consider coverage for a prescribed medication therapy or to reevaluate the possible benefits. An appeal is a request for an insurance carrier to reconsider a coverage decision that was initially denied[11]. The exception and appeal processes require letters of support from the prescribing physician. The time-consuming processes to initiate higher doses of IFX provide some patients with potentially lifesaving therapy. On the other hand, this litigious process is often unsuccessful and may delay alternative intervention for some patients.

Modification of UC treatment often becomes necessary in patients who lose responsiveness to an initial dose or standard maintenance regimen of IFX. Modification may consist of an increase in dose, decrease in dosing interval (not queried here), or addition of alternative medical or surgical therapy[12]. Treatment strategies with IFX should optimize drug pharmacokinetics to maximize dose response and minimize the development of antibodies to IFX. This may delay the need to increase IFX dose and reduce the risk of complications. Among pediatric gastroenterologists who prescribe IFX, IFX 10 mg/kg has a recognized role and perceived benefit in the treatment of some pediatric UC patients. Future prospective controlled clinical trials using high-dose IFX and other anti-tumor necrosis factor-α antibody agents, and testing of specific dose increases may provide more

standardized guidelines for the treatment of pediatric patients with UC.

COMMENTS

Background

In the last decade, infliximab (IFX) has become an alternative treatment of moderate to severe ulcerative colitis (UC) in both the adult and pediatric population. Based on current Food and Drug Administration recommendations the use of IFX for treatment of moderate to severe UC in adults is started at a dose of 5 mg/kg. However, in patients who lose responsiveness, the dose of IFX is commonly increased up to 10 mg/kg.

Research frontiers

No studies have documented benefit at this higher dose in pediatric patients. The efficacy, safety and insurance approval for high-dose IFX have also been of growing concern in the medical community. The authors conducted a survey of pediatric gastroenterologists to collect information regarding the use of IFX for the treatment of UC in the pediatric population.

Innovations and breakthroughs

Recently, the Pediatric Inflammatory Bowel Disease Collaborative Research Group (PIBDCRG) showed that children with corticosteroid-dependent or corticosteroid-refractory UC treated with IFX have a significant decrease in corticosteroid dependency and improved rates of colectomy. In the PIBDCRG study, dose escalation and decreased dose interval was at the discretion of prescribing physicians. The survey study conducted here is the first to report the extensive use of IFX 10 mg/kg by pediatric gastroenterologists for treatment of UC in the pediatric population.

Applications

This study offers a glimpse in the attitudes and practice patterns of pediatric gastroenterologists regarding the use of high-dose IFX for the treatment of pediatric UC. The results highlight the recognized role of IFX 10 mg/kg for the treatment of UC in the pediatric population and the need for more standardized guidelines regarding dose escalation.

Terminology

IFX is a biologic therapy used to treat autoimmune diseases such as UC. IFX is a monoclonal immunoglobulin G antibody that is genetically engineered to target tumor necrosis factor-alpha (TNF-α). By selectively targeting specific players in the inflammatory cascade such as TNF-α, biologic agents may spare the need for treatment with systemic corticosteroids and thereby reduce the number of side effects associated with such treatment.

Peer review

The authors examined the practice patterns of pediatric gastroenterologists regarding the use IFX for the treatment of UC. They found that a majority of those pediatric gastroenterologists surveyed have experience using IFX 10 mg/kg. In addition, significantly more pediatric gastroenterologists based in the United States have used IFX 10 mg/kg compared to those not based in the United States. Over one-third of pediatric gastroenterologists surveyed indicated insurance issues as a barrier to increasing IFX dosing.

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BRIEF ARTICLE

Polymorphisms of the *TLR2* and *TLR4* genes are associated with risk of gastric cancer in a Brazilian population

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Abstract

AIM: To investigate toll-like receptor 2 (*TLR2*) -196 to -174 del, and *TLR4* (+896A/G rs4986790 and +1196C/T rs4986791) polymorphisms at risk of chronic gastritis and gastric cancer in a Brazilian population and association of gastric lesions with risk factors such as smoking, alcohol intake and *Helicobacter pylori* infection.

METHODS: In this case-control study, polymorphism at *TLR2* -196 to -174 del was investigated by using the allele-specific polymerase chain reaction (PCR) method, while the PCR-restriction fragment length polymorphism technique was carried out to identify the *TLR4* (rs4986790 and rs4986791) genotypes in 607 Brazilian individuals (208 with chronic gastritis-CG, 174 with gastric cancer-GC and 225 controls -C).

RESULTS: The single nucleotide polymorphisms *TLR4*+1196C/T was not associated with risk of chronic gastritis or gastric cancer and the homozygous genotypes *TLR4*+896GG and *TLR4*+1196TT were absent in the studied population. However, the frequency of *TLR2* -196 to -174 ins/del + del/del and *TLR4*+896AG genotypes was significantly higher ($P < 0.01$ and $P = 0.01$, respectively) in the cancer group (33.4% and 11.5%, respectively) than in the control group (16.9% and 4.5%, respectively). It was also observed that the G-C haplotype of the *TLR4*+896A/G+1196C/T ($P = 0.02$) and the combination of variant alleles of the *TLR2*/*TLR4*+896G ($P = 0.02$) are associated with susceptibility to gastric cancer. In addition, the multiple logistic regression showed that male gender [odds ratio (OR) = 2.70; 95% CI: 1.66-4.41; $P < 0.01$], alcohol intake (OR = 2.93; 95% CI: 1.76-4.87; $P < 0.01$), *TLR2* -196 to -174 del (OR = 2.64; 95% CI: 1.56-4.44; $P < 0.01$) and *TLR4*+896G (OR = 3.19; 95% CI: 1.34-7.61; $P < 0.01$) polymorphisms were associated with a higher susceptibility to developing this neoplasm.

CONCLUSION: Our data indicate that *TLR2* -196 to -174 del and *TLR4*+896G may increase the risk of gastric cancer in a Brazilian population.

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Key words: Polymorphisms; Toll-like receptor 2; Toll-like receptor 4; Gastric cancer; Gastritis

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INTRODUCTION

Gastric cancer is one of the most serious health problems in many countries, including Brazil, which ranks third in incidence and mortality, and with an estimated incidence in 2010 of about 21 500 new cases, with an incidence of 14.25 per 100 000 males and 7.70 per 100 000 females[1]. The *Helicobacter polori* (*H. pylori)* is the major etiological risk factor for this malignancy, which progresses through a multi-step process, developing from gastritis, to gastric atrophy, intestinal metaplasia, dysplasia, and finally to carcinoma[2]. It is widely accepted that chronic *H. pylori* infection induces a gastric atrophy and hypochlorhydria, which are precursors of all pathophysiological changes of gastric carcinogenesis[3]. However, colonization with *H. pylori* can lead to various outcomes. Nearly all *H. pylori* positive subjects have chronic gastritis, and only 1%-2% development of stomach cancer among infected[4]. Hence, other factors are likely to be involved in gastric tumorigenesis such as host genetic factors, as well as the diversity of *H. pylori* virulence genes.

Host genetic factors, as polymorphisms in inflammatory and immune response genes, are mainly related to the recognition of the bacteria by the immune system and the variation in the level of cytokine response[5]. Among host factors, several inflammatory proteins including cytokines, growth factors, and chemokines have been known to control immune response against *H. pylori* infection[6,7]. Therefore, many studies have focused on the analyses of polymorphisms in genes associated with the inflammatory response in the gastric mucosa and risk for malignancy[8-11]. Other mediators also have polymorphic variants that modulate the innate immune response pattern, as the toll-like receptors (TLRs), which provide first line of host defense against harmful pathogens[12].

Among the TLRs, it has been reported that the TLR2, lipoproteins bacterial receptor and the TLR4, the lipopolysaccharide (LPS) receptor, are involved in the response to infection by *H. pylori* on gastric epithelial cells[3,13-15]. Both the TLR2 and the TLR4 promote transcription of genes involved in immune activation including nuclear factor kappa B (*NF-κB)* and also mintage-activated protein (*MAP*) kinase pathways[16]. *TLR4* is up regulated in gastric epithelial cell lines infected with *H. pylori* and in macrophages and expression of TLR4 protein has been demonstrated in chronic active gastritis, in precancerous lesions, and also in gastric tumor cells. TLR2 activates NF-κB in epithelial cells, in response to *H. pylori* infection, causing the expression of interleukin (IL)-8, macrophage inflammatory protein-3α and growth-regulated oncogene alpha[7]. Thus, it is conceivable that functionally relevant polymorphisms in *TLR* genes can alter the host immune response to pathogens as infection induced by *H. pylori*.

Single nucleotide polymorphisms (SNPs) in *TLR2* have been associated with susceptibility to various infectious and inflammatory diseases such as leprosy[17], increased risk of Gram-negative sepsis[18], asthma[19], recurrent bacterial infections[20] and sporadic colorectal cancer susceptibility[21]. The specific polymorphism *TLR2* -196 to -174 del/del genotype has been reported to show decreased transcriptional activity of the *TLR2* gene[22]. Such fact aroused the interest for this polymorphism, and previous studies in the Japanese population demonstrated its association with increased susceptibility to non-cardia gastric cancer[23] and intestinal metaplasia[24].

Likewise, the *TLR4* presents some polymorphisms implicated in increased susceptibility to various diseases such as atherosclerosis[25], asthma[3], malaria[26], and also infection with the *H. pylori* associated with gastric cancer and its precursors[27]. Two SNPs in *TLR4*+896A/G (rs4986790) and +1196C/T (rs4986791) have received special attention in some studies, although the results are still controversial[6,28,29].

Therefore, the aim of this study was to evaluate the influence of the 22-bp nucleotide deletion -196 to –174 del, in the promoter region of the gene *TLR2* and +896A/G and +1196C/T polymorphisms (Asp299Gly and Thr399Ile) respectively in *TLR4* gene on the risk of chronic gastritis and gastric cancer in a Brazilian population and whether there is an association of gastric lesions with risk factors such as smoking, alcohol intake and *H. pylori* infection.

MATERIALS AND METHODS

Subjects

This was a case-control study on chronic gastritis and gastric cancer, in which a total of 607 DNA samples from peripheral blood leukocytes were genotyped. The case groups comprised 208 individuals (102 men and 106 women) with a histopathologically confirmed diagnosis of chronic gastritis - CG (Sidney System)[30], with a mean age of 52.8 + 14.5 years (range 19 to 84 years), and 174 individuals (134 men and 40 women) with a histopathologically confirmed diagnosis of gastric cancer - GC (Lauren's classification)[31], with a mean age of 62.2 ± 12.2 years (range 28 to 93 years). All subjects were recruited from the Hospital de Base in São José do Rio Preto, SP, and from the Pio XII Foundation in Barretos, SP, Brazil. *H. pylori* infection was histologically established either by the Giemsa staining technique or by the urease test, performed at the Pathology Services of the Hospital de Base and the Pio XII Foundation. Results of *H. pylori* infection were obtained for the available cases. The cancer-free control group (C) with no previous history of gastric disease was composed of 225 healthy individuals (112 men and 113 women), mainly blood donors, with a mean age of 56.5 + 18.1 years (range 20 to 93 years). Epidemiological data on the study population were collected using a standard interviewer-administered questionnaire, with questions about current and past occupation, smoking habits, alcohol intake and family history of cancer. All the individuals were ethnically classified on their visual appearance as Caucasians in the three groups evaluated. The few cases of African descent were excluded from the study (about 10%).

Table 1 Primer sequences, restriction enzymes and fragment sizes for toll-like receptor 2 and toll-like receptor 4 gene polymorphisms and interleukin-1β gene

Genes	Primers	Enzyme T°/time	Fragment (bp)	Ref.
TLR2 *del* -196 to -174	F: 5′-CACGGAGGCAGCGAGAAA-3′ R: 5′-CTGGGCCGTGCAAAGAAG-3′	-	286 ins/ins: 286 ins/del: 286, 264 del/del: 264	[24]
TLR4+896A/G rs4986790	F: 5′-AGCATACTTAGACTACCACCTCGATG 3′ R: 5′-GTTGCCATCCGAAATTATAAGAAAAG 3′	*Bst*XI 37 ℃, 1 h	131 A/A: 131 A/G: 131, 108 G/G: 108	[34]
TLR4+1196C/T rs4986791	F: 5′-GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA-3′ R: 5′-ACCTGAAGACTGGAGAGTGAGTTAAATGCT-3′	*Hin*fI 37 ℃, 1 h	407 C/C: 407 C/T: 407, 378 T/T: 378	[33]
IL1-β	F: CATGTGACCTGCTCGTCAGT R: CCCTAGGGATTGAGTCCACA	*Hin*fI 37 ℃, 1 h	370 195, 175	[47]
TLR2	F: 5′-CACGGAGGCAGCGAGAAA-3′ R: 5′-CTGGGCCGTGCAAAGAAG-3′	*Bst*XI 37 ℃, 1 h	286 188, 98	[24]

TLR: Toll-like receptor; IL: Interleukin; T°: Temperature.

The National Research Ethics Committee approved this work, and written informed consent was obtained from all individuals.

Genotyping

About 5 mL of whole blood were collected from all study participants in sterile EDTA-coated vacutainers. DNA was extracted according to a previous report[32], and stored at -20 ℃ until use for genotyping.

Polymorphism at *TLR2* -196 to -174 del was investigated using the allele-specific polymerase chain reaction (PCR) method[24], and the PCR-restriction fragment length polymorphism (RFLP) technique was carried out in order to identify the *TLR4* (rs4986790 and rs4986791) genotype[33,34] in cases and control groups. In brief, the procedure was carried in a total reaction volume of 25 μL, containing 2.5 μL 10 × PCR buffer, 2 μL deoxyribonucleotide triphosphatess (1.25 μmol/L), 0.5 μL $MgCl_2$ (25 mmol/L), 1.25 μL of each primer (25 mmol/L, Sigma-Aldrich, United States), 15.3 μL dH_2O, 2 μL DNA (100 ng/μL), and 0.2 μL Taq DNA polymerase (5 U/μL, Invitrogen, United States). PCR for *TLR2* -196 to -174 *del* was as follows: initial denaturation step at 95 ℃ for 5 min, amplification was carried out by 35 cycles at 95 ℃ for 30 s, at 60 ℃ for 40 s, and at 72 ℃ for 40 s, followed by a final elongation cycle at 72 ℃ for 7 min (Table 1). PCR for both *TLR4* polymorphisms were as follows: after an initial denaturation step at 94 ℃ for 3 min, amplification was carried out by 30 cycles at 94 ℃ for 30 s, at 62 ℃ for 30 s, and at 72 ℃ for 30 s, followed by a final elongation cycle at 72 ℃ for 7 min. Then, 10 μL of *TLR4* +896 A/G and *TLR4* +1196 C/T polymorphism PCR products were digested with 0.5 μL (5 U/μL) of the *Bst*XI and *Hin*fI specific enzymes, respectively in a 10 μL volume including 2.5 μL 10 × buffer 1 (New England Biolabs, United States) and 7.0 μL dH_2O (Table 1). The products were then electrophoresed on a 3% agarose 1000 (Invitrogen, United States) gel, to allow detection by ethidium bromide staining.

In order to confirm the veracity of the results, one confirmed polymorphic case was used as positive control for every RFLP procedure, to attest the good functioning of the restriction enzyme. For the *TLR4*+896A/G (rs4986790) and +1196C/T (rs4986791) polymorphisms, we also used other fragments of the *TLR2* and *IL-1β* gene that was known to have the enzyme recognition site to verify the correct functioning of the enzymes *Bst*XI and *Hin*fI (Table 1), considering we did not detect any polymorphic homozigous subjects.

Statistical analysis

Fisher's exact test was used to compare the groups regarding genotype and allele frequencies, and the chi-square test for determining Hardy-Weinberg equilibrium. Multiple logistic regression models were used to determine the effects of the variables in gastric cancer and chronic gastritis. The models included age (reference: < 61 and 53 years old - median of the groups), gender (reference: female), smoking habits (reference: nonsmokers), drinking habits (reference: nondrinkers), and *H. pylori* infection (reference: *H. pylori*-negative). The results are shown as odds ratio (OR), showing 95% CI. ORs were calculated using a dominant model due to low frequency of polymorphic homozygous (i.e., combining heterozygous and homozygous for the minor allele *vs* homozygous for the major allele) for all SNPs. The haplotype frequencies of *TLR4* were inferred by Haploview program (4.0 version). Statistical analyses were performed using the GraphPad Instat, and SPSS (11.5 version) computer software programs. A probability level (*P*) of less that 0.05 was adopted as a significance criterion.

RESULTS

The samples of the 607 subjects were genotyped for the *TLR2* -196 to -174 *del* and *TLR4* (+896A/G rs4986790

Table 2 Genotype and allele frequencies of toll-like receptor 2 (-196 to -174 *del*) and toll-like receptor 4 (rs4986790 and rs4986791) polymorphisms in gastric cancer, chronic gastritis and control groups

Genotypes/alleles	GC *n* = 174 (%)	C *n* = 225(%)	CG *n* = 208 (%)
TLR2 -196 to -174			
ins/ins	116 (66.6)	189 (84.0)	160 (76.9)
del/ins	50 (28.7)	34 (15.1)	41 (19.7)
del/del	8 (4.7)	2 (0.9)	7 (3.4)
OR (95% CI)	2.62 (1.63-4.22)		1.57 (0.97-2.54)
P value	< 0.01		0.06
Alleles			
ins	81.0	91.5	87.0
del	19.0	8.5	13.0
OR (95% CI)	2.53 (1.65-3.88)		1.65 (1.06-2.55)
P value	< 0.01		0.02
TLR4 +896A/G (rs4986790)			
AA	154 (88.5)	215 (95.5)	187 (89.9)
AG	20 (11.5)	10 (4.5)	21 (10.1)
GG	0	0	0
OR (95% CI)	2.79 (1.27-6.13)		2.41 (1.10-5.25)
P value	0.01		0.02
Alleles			
A	94.0	97.0	95.0
G	6.0	3.0	5.0
OR (95% CI)	2.68 (1.23-5.81)		2.33 (1.08-5.02)
P value	0.01		0.02
TLR4 +1196C/T (rs4986791)			
CC	165 (94.8)	219 (97.3)	202 (97.1)
CT	9 (5.2)	6 (2.7)	6 (2.9)
TT	0	0	0
OR (95% CI)	1.99 (0.69-5.70)		1.08 (0.34-3.41)
P value	0.28		1.00
Alleles			
C	98.0	99.0	98.0
T	2.0	1.0	2.0
OR (95% CI)	1.96 (0.69-5.57)		1.08 (0.34-3.38)
P value	0.29		1.00

OR: Odds ratio; GC: Gastric cancer; CG: Chronic gastritis; C: Control.

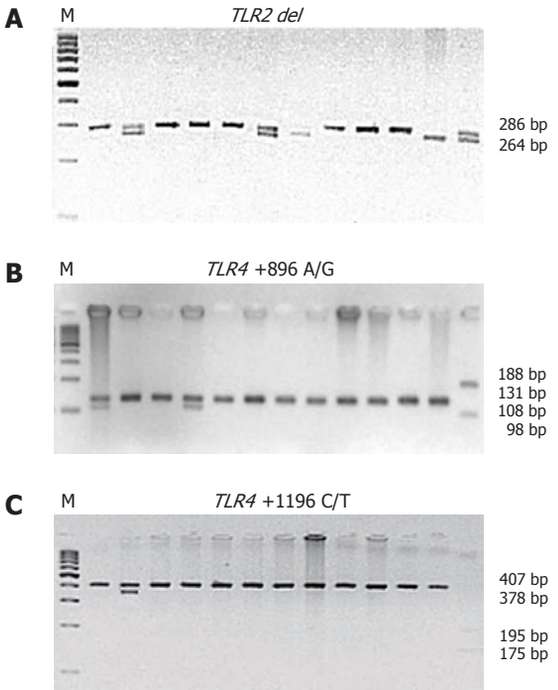

Figure 1 Eletrophoretic pattern of fragments generated by polymerase chain reaction-allele specific and polymerase chain reaction-restriction fragment length polymorphism for the polymorphisms. A: Toll-like receptor (*TLR*)2 -196 to -174 del: ins/ins = 286 bp; ins/del = 286 + 264 bp; del/del = 264 bp; B: *TLR4* + 896A/G: A/A = 131 bp; A/G = 131 + 108 bp and positive control of enzyme *BstXI*, *TLR2* gene fragment of 286 bp with the enzyme cutting site: 188 + 98 bp (last lane); C: *TLR4* + 1196 C/T: C/C = 407 bp; C/T: 407 + 378 bp and positive control of enzyme *Hinf*I, interleukin-1β gene fragment of 370 bp with the enzyme cutting site: 195 + 175 bp (last lane). M: Molecular weight marker of 100 bp.

Table 3 Toll-like receptor 4 haplotype frequency distribution between gastric cancer, chronic gastritis and control groups

Haplotypes *TLR4* +896/+1196	GC (%)	C (%)	χ^2	*P* value	CG (%)	C (%)	χ^2	*P* value
A-C	91.4	95.7	6.802	< 0.01	94.0	95.7	1.361	0.24
G-C	63.0	31.0	5.247	0.02	46.0	31.0	1.598	0.20
A-T	21.0	11.0	1.461	0.22	NF	NF	NF	NF

Haplotype G-T not found. NF: Not found; GC: Gastric cancer; CG: Chronic gastritis; C: Control.

and +1196C/T rs4986791). The genotype and allele frequencies for these polymorphisms are presented in Table 2. The genotype and allele frequency distribution of the three polymorphisms complied with Hardy-Weinberg equilibrium in both cases and control groups (data not shown). The banding patterns of these SNPs are represented in Figure 1.

For *TLR2* -196 to -174 del, the genotype (ins/del and del/del) and allele (del) frequencies were increased statistically ($P < 0.01$) in gastric cancer group (33.4% and 19% respectively) than in control group (16.0% and 8.5% respectively). In addition, among the groups of chronic gastritis (13.0%) and control (8.5%), the allele frequencies (del) was statistically significant ($P = 0.02$).

Similarly, for *TLR4*+896A/G (rs4986790), the genotypes (A/G) and allele (G) frequencies were increased statistically in gastric cancer group (11.5% and 6.0%, respectively; $P = 0.01$) and chronic gastritis (10.1% and 5.0%, respectively; $P = 0.02$) than in control group (4.5% and 3%, respectively). This results due to the higher frequency of polymorphic allele (*TLR4* +896G) in the gastric cancer and chronic gastritis groups.

In contrast, for *TLR4*+1196C/T (rs4986791), no significant difference was found between gastric cancer and control group (Table 2). Homozygous genotypes *TLR4*+896GG and *TLR4*+1196TT were absent in the studied population. We also compared the genotype and allele frequencies between gastric cancer and gastritis groups and no significant difference was found for the polymorphisms studied (data not shown).

The *TLR4* haplotype analysis (Table 3) demonstrated higher frequency of both wild alleles (haplotype A-C) in control subjects compared with gastric cancer (95.7% and 91.4%, respectively; $P < 0.01$). However, the opposite was observed for frequency of variant haplotype

Table 4 Combined effect of toll-like receptor 2 (-196 to -174 *del*) and toll-like receptor 4 (rs4986790 and rs4986791) polymorphisms on risk of gastric cancer and chronic gastritis

Risk genotype	Groups								
	GC (*n* = 174)	C (*n* = 225)	OR (95% CI) *P* value	CG (*n* = 208)	C (*n* = 225)	OR (95% CI) *P* value	GC (*n* =174)	CG (*n* = 225)	OR (95% CI) *P* value
Neither	113	187	1.00 (reference)	160	187	1.00 (reference)	113	160	1.00 (reference)
TLR2 ins/del or del/del/*TLR4*+896 AG	10	4	4.13 (1.26-13.50) 0.02	11	4	3.21 (1.00-10.29) 0.06	10	11	1.28 (0.52-3.13) 0.64
TLR2 ins/del or del/del/*TLR4*+1196 CT	4	1	6.61 (0.73-59.99) 0.07	1	1	1.16 (0.07-18.84) 1.00	4	1	5.66 (0.62-51.37) 0.16
TLR4+896 AG/+1196 CT	1	1	1.65 (0.10-26.73) 1.00	3	1	3.50 (0.36-34.05) 0.34	1	3	0.47 (0.04-4.59) 0.64

C: Control group; OR: Odds ratio; GC: Gastric cancer; CG: Chronic gastritis; TLR: Toll-like receptor.

Table 5 Distribution of risk factors, genotypes of toll-like receptor 2 (-196 to -174 *del*) and toll-like receptor 4 (rs4986790 and rs4986791), and odds ratios for gastric cancer, chronic gastritis and control groups

Variables	F (GC/C) %	OR (95% CI)	*P* value	F (CG/C) %	OR (95% CI)	*P* value
Gender						
Female	23/49.7	Reference	< 0.01	51.0/50.3	Reference	0.96
Male	77/50.3	2.70 (1.66-4.41)		49.0/49.7	0.99 (0.66 -1.48)	
Age (yr)						
< 61	40.9/56.0	Reference	0.30	52.8/43.5	(< 53 yr) Reference	0.03
⩾ 61	59.1/44.0	1.26 (0.81-1.98)		47.2/56.5	(⩾ 53 yr) 0.64 (0.43-0.95)	
Smoking						
Nonsmokers	30.5/34.3	Reference	0.13	44.8/34.3	Reference	0.01
Smokers	69.5/65.7	0.67(0.39-1.13)		55.2/65.7	0.57 (0.37-0.87)	
Alcohol						
Nondrinkers	46.6/74.3	Reference	< 0.01	68.2/74.3	Reference	0.06
Drinkers	53.4/25.7	2.93 (1.76-4.87)		31.8/25.7	1.54 (0.97-2.44)	
TLR2						
ins/ins	66.6/84.0	Reference	< 0.01	76.9/84.0	Reference	0.18
ins/del	33.4/16.0	2.64 (1.56-4.44)		23.1/16.0	1.39 (0.84 -2.28)	
del/del						
TLR4 +896A/G (rs4986790)						
AA	88.5/95.5	Reference	< 0.01	89.9/95.5	Reference	0.04
AG	11.5/4.5	3.19 (1.34-7.61)		10.1/4.5	2.29 (1.02-5.13)	
TLR4 +1196C/T (rs4986791)						
CC	94.8/97.3	Reference	0.63	97.1/97.3	Reference	0.91
CT	5.2/2.7	1.33 (0.41-4.33)		2.9/2.7	0.93 (0.28-3.05)	

F: Frequency of individual (%); OR: Odds ratio; GC: Gastric cancer; CG: Chronic gastritis; C: Control; TLR: Toll-like receptor.

G-C, which was higher in the gastric cancer group compared with control group (63.0% and 31.0%, respectively; $P = 0.02$).

In another statistical analysis, which evaluated the combined effect between the three polymorphisms (*TLR2* -196 to -174 del, *TLR4*+896A/G and +1196C/T), the combination of variant alleles of the polymorphisms *TLR2* ins/del and del/del with *TLR4* +896 AG showed a higher risk of gastric cancer compared to healthy individuals (OR = 4.13; 95% CI: 1.26-13.50; $P = 0.02$). The other combinations of variant alleles did not show any significant difference (Table 4). The combination of the three variant alleles was found in only one individual of the group of chronic gastritis (data not shown).

The potential associations between the distributions of *TLR2* -196 to -174 del and *TLR4* (+896A/G and +1196C/T) genotypes adjusting for risk factors for gastric cancer and chronic gastritis in comparison of control group are presented in Table 5.

In the gastric cancer group, the multiple logistic regression shows that male gender (OR = 2.7; 95% CI: 1.66-4.41; $P < 0.01$), alcohol intake (OR = 2.93; 95% CI: 1.76-4.87; $P < 0.01$), *TLR2* -196 to -174 ins/del+del/del (OR = 2.64; 95% CI: 1.56-4.44; $P < 0.01$) and *TLR4* +896AG (OR = 3.19; 95% CI: 1.34-7.61; $P < 0.01$) were associated with a higher susceptibility to developing this neoplasm. The comparison between gastritis and control group showed that only *TLR4*+896AG polymorphism was associated with risk of chronic gastritis (OR = 2.29; 95% CI: 1.02-5.13; $P = 0.04$), while age above 53 years (OR = 0.64; 95% CI: 0.43-0.95; $P = 0.03$) and smoking (OR = 0.57; 95% CI: 0.37-0.87; $P = 0.01$) were negatively associated with the development of gastritis. In another multiple logistic regression analysis considering also the individuals tested for *H. pylori* infection (95 with gastric cancer and 177 with gastritis), there was no statistically

significant association (OR = 0.73, 95% CI: 0.29-1.78; P = 0.49, data not shown in Table 5). There was also no association of three polymorphisms with *H. pylori* when evaluated negative and positive individuals within a group of gastric cancer and gastritis (data not shown).

DISCUSSION

TLRs participate in *H. pylori* bacterium recognition in gastric mucosa, and SNPs in TLRs are associated with impaired immune response, inducing a potent inflammatory response. Therefore, it is relevant to carry out studies on host genetic factors that can be associated with susceptibility of gastric diseases. Hence, we investigated whether *TLR2* -196 to –174 del and *TLR4* (+896A/G rs4986790 and +1196C/T rs4986791) polymorphisms affect the risk of developing gastric cancer and chronic gastritis in a Brazilian population. Our results have demonstrated for the first time in this population, an association of *TLR2* -196 to -174 del and of *TLR4*+896 G polymorphisms with susceptibility to gastric cancer. The polymorphism *TLR4*+1196 T was not associated with risk to the gastric lesions evaluated, and the homozygous genotypes *TLR4*+896GG and *TLR4*+1196TT were absent in the studied population.

Some studies that investigated the association of *TLR2* -196 to -174 del polymorphisms at risk of developing diseases related to an inflammatory process have shown conflicting results. For instance, del allele or del/del genotype of *TLR2* -196 to-174 polymorphism was significantly associated with cervical cancer susceptibility[35] and risk of non-cardia gastric cancer in a Japanese population, but not for gastritis, gastric ulcer and duodenal ulcer[23], while *TLR2* -196 to -174 ins allele was associated with more severe intestinal metaplasia in older patients[24]. However, Wang *et al*[36] failed to show association of *TLR2* -196 to -174 del/del and ins/del carriers with ulcerative colitis.

The -196 to -174 del polymorphism in *TLR2* gene located on chromosome 4, causes a 22-bp nucleotide deletion that alters the promoter activity of gene. The *TLR2* del/del genotype is reported to show decreased transcriptional activity this gene[22]. In our study, we observed significantly higher frequencies of genotypes *TLR2* ins/del and del/del in the gastric cancer group compared to the healthy individuals, emphasizing its role in the gastric carcinogenesis.

The *TLR4* gene is mapped on chromosome 9 and consists of three exons. In exon 3, two non-synonymous SNPs *TLR4*+896A/G and +1196C/T allows the substitution of amino acids Asp299Gly and Thr399Ile, respectively. In the analysis by Haploview, the frequencies of *TLR4* G-C (299Gly-399Thr) and G allele were higher in patients with gastric cancer indicating an association of this haplotype with increased risk of gastric cancer to its carriers. The substitution of Asp299Gly amino acids disrupt the normal structure of the extracellular region of the TLR4 and may cause decreased ligand recognition or protein interaction, and decreased responsiveness to lipopolysaccharide, disrupting transport of *TLR4* to the cell membrane[13,37]. This change leads to an exaggerated inflammatory response with severe tissue destruction, likely due to a failure in stimulating regulatory cells and production of IL-10 cytokine[38]. Arbour *et al*[13] were the first to report that individuals having either the Asp-299Gly and/or Thr399Ile polymorphisms had a blunted response towards inhaled LPS. Thus, during the cascade of progression of gastric carcinogenesis, the subjects with this polymorphism can have an increased risk of severe inflammation followed by development of hypochlorhydria and gastric atrophy, which are regarded as important precursor alterations of gastric cancer[12].

Both SNPs in *TLR4* are presented in about 10% of Caucasian and African populations and are reported to have a positive correlation with susceptibility to infectious diseases, whereas studies in Asian populations have shown the absence of these polymorphisms[26,39]. However, in our study with a Southeastern Brazilian population, we found that both SNPs *TLR4* (+896A/G and +1196C/T) were present in heterozygous in about 4.5% to 11.5% and 2.7% to 5.2% respectively in the gastric cancer and control groups. Other studies in the Brazilian population found similar frequencies of heterozygous *TLR4*+896A/G polymorphism in Chagas disease (5.6%), ulcerative colitis (7.1%) and Crohn's disease (7%)[40,41]. But, to the best of our knowledge, there are not studies on *TLR2* and *TLR4* polymorphisms in gastric cancer of Brazilian population.

With regards to *TLR4*+896A/G and +1196C/T polymorphisms, Garza-Gonzalez *et al*[29] showed no association with the risk of gastric cancer in the Mexican population, while Trejo de la O *et al*[7] observed that both SNPs in *TLR4* had an association with duodenal ulcer and gastric cancer also in Mexican patients. Yet, other studies have demonstrated association with only one of these polymorphisms and risk of gastric cancer and precancerous lesions, either *TLR4*+1196C/T (Thr399Ile)[6,42] or *TLR4*+896A/G (Asp299Gly) polymorphisms[3] are associated with susceptibility to gastric carcinogenesis. In addition, in this study in Caucasian population[3], homozygous polymorphic *TLR4*+896 GG was not found, corroborating our results.

Concerning *H. pylori* infection, we evaluated the association between *TLR2* and *TLR4* polymorphisms in the case groups with the available information in their medical records (95 with gastric cancer and 177 with gastritis) and no association was found. The reduced number of samples available for statistical analysis may have harmed these results. Rad *et al*[8] studied the role of various TLRs (2/4/7 and 9) in response to *H. pylori* using mice mutants lacking these receptors. The results demonstrated the importance of the TLR2 in response to this bacterium, unlike the TLR4. TLR4-receptor lacking mice had little or no change in response to *H. pylori* compared with controls. Although the role that the effect of polymorphism in *TLR2* in the activity of this receptor in

gastric cells is not fully understood, this deletion is likely to alter their activity. Since *TLR2* has an important role in immune response against the *H. pylori* bacterium, the change of its function becomes relevant in carcinogenesis of the stomach[8].

Besides the influence of the *H. pylori*, other risk factors as gender, age, smoking and alcohol intake were analyzed. There are statistically significant results for male gender and alcohol intake in the gastric cancer group compared with the control group. According to the National Cancer Institute the highest incidence of gastric cancer occurs in men around age 70 years, and about 65% of patients diagnosed with this type of cancer were over 50 years[1]. Another risk factor well established in the literature in relation to gastric carcinogenesis is the excessive consumption of alcohol[43-45]. Really, ethanol oxidation generates acetaldehyde, which presents carcinogenic effects, since it interferes with many DNA synthesis and repair sites, leading to tumor development[46].

In conclusion, our findings indicate a significant role of both *TLR2* -196 to -174 del and *TLR4*+896G (Asp299Gly) polymorphic variant with susceptibility to gastric cancer in the Southeastern Brazilian population evaluated, whereas no association was observed for *TLR4* +1196T polymorphism. Thus, it is feasible to highlight that host genetic factors as the interaction of polymorphisms in genes of toll-like receptors can play an important role in gastric carcinogenesis.

COMMENTS

Background

Gastric cancer (GC) has high rate of incidence and mortality in Brazilian population. Thus is important to establish host genetic factors, as polymorphisms in genes related with inflammatory and immune response associated with higher risk of development of this neoplasia. In this study, authors have shown, for the first time association of toll-like receptor (*TLR)2* -196 to -174 *del* and *TLR4*+896AG (Asp299Gly) polymorphisms with gastric cancer in a sample of Brazilian population.

Research frontiers

Epidemiological studies on association of polymorphisms with susceptibility to disease as cancer frequently present conflicting results, possibly due to different factors as ethnicity, sample number, population sub-sampling, which can contribute to this discrepancy. Thus, are relevant studies in different populations that help clarify these aspects.

Innovations and breakthroughs

In this study it was possible to make the combined analysis of three polymorphisms (*TLR2* -196 to -174 *del*, *TLR4*+896A/G and +1196C/T) and to show that the combination of single nucleotide polymorphisms *TLR2 ins/del and del/del* with *TLR4* +896 AG led to a higher risk of gastric cancer.

Applications

Considering the high incidence of gastric cancer in the Brazilian population, the data show that carriers of polymorphisms in genes involved with immune response as *TLR2 ins/del* and *del/del* and *TLR4* +896 AG, together with other genetic and environmental factors constitute a risk group to gastric carcinogenesis.

Peer review

This is a cross sectional study on the role of the TLR2 and TLR4 polymorphisms in GC in a Brazilian population. The main point that needs to be further clarified is the ethnic composition of the population. An important observation is that the TLR4 variants are at similar frequencies on the African and Caucasian populations.

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BRIEF ARTICLE

Adenoma incidence decreases under the effect of polypectomy

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Abstract

AIM: To investigate whether, under the influence of polypectomy, the incidence of adenoma decreases with age.

METHODS: Consecutive patients with colonic adenomas identified at index colonoscopy were retrospectively selected if they had undergone three or more complete colonoscopies, at least 24 mo apart. Patients who had any first-degree relative with colorectal cancer were excluded. Data regarding number of adenomas at each colonoscopy, their location, size and histological classification were recorded. The monthly incidence density of adenomas after the index examination was estimated for the study population, by using the person-years method. Baseline adenomas were excluded from incidence calculations but their characteristics were correlated with recurrence at follow-up, using the χ^2 test.

RESULTS: One hundred and fifty-six patients were included (109 male, mean age at index colonoscopy 56.8 ± 10.3 years), with follow-up that ranged from 48 to 232 mo. No significant correlations were observed between the number, the presence of villous component, or the size of adenomas at index colonoscopy and the presence of adenomas at subsequent colonoscopies (P = 0.49, 0.12 and 0.78, respectively). The incidence of colonic adenomas was observed to decay from 1.4% person-months at the beginning of the study to values close to 0%, at 12 years after index colonoscopy.

CONCLUSION: Our results suggest the sporadic formation of adenomas occurs within a discrete period and that, when these adenomas are removed, all neoplasia-prone clones may be extinguished.

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Key words: Colorectal cancer; Colorectal adenoma; Incidence; Age; Polypectomy

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INTRODUCTION

It has long been believed that initiation of sporadic colorectal cancer (CRC) increases over time, due to toxicity or loss of fidelity of DNA replication. Therefore, it is believed that the risk of adenoma formation is a function of age[1], which justifies lifelong colonoscopy surveillance. However, current evidence has started to challenge these perceptions.

The data that have suggested that incidence of adenoma increases with age were mostly collected from autopsy studies[2,3]. Although relevant, these studies are prone to bias because the age-specific incidence rates do not adequately represent the time trend for newly formed lesions after removal of index lesions. Moreover, the rather important information of family aggregation of CRC was lacking in all of them.

More recent studies, based on surveillance colonoscopies[4-6], have been mainly limited by short follow-up (3-5 years), which may lead to the inclusion, as new adenomas, of polyps that were missed at a previous examination. These time intervals are also likely to be too short to predict the lifelong dynamics of adenoma formation.

The notion that the formation of colon adenomatous polyps peaks at a certain age and then rapidly declines was first presented in 1975 by Henry *et al*[7]. He stated that: "some undefined stimulus to continuous polyp formation persists for up to 4 years in about one-third of patients who develop a colonic polyp. Thereafter, either the stimulus to neoplasia is no longer present, or the colonic mucosa adapts so that polyp formation does not persist." We propose that this undefined stimulus corresponds to the presence of a limited number of mutated clones that are scattered in the colonic epithelium and evolve through cumulative critical gene events selected over several decades. This sequence of events ends at a specific time window, in which most adenomas become endoscopically detectable and after which its incidence declines.

A study in Germany[8] has shown that individuals with negative findings at colonoscopy had a reduced risk of CRC for at least 20 years. Furthermore, when the examination was performed at 55-64 years of age and older, the risk of CRC was even lower. Additionally, according to the latest Guidelines for Colonoscopy Surveillance after Polypectomy issued by the American Gastroenterological Association (AGA)[9], age is not considered to be a reliable predictor of subsequent advanced adenomas.

Taking these data into account and considering the possibility of only a limited time window for sporadic adenoma expression, we aimed to study the temporal trend for adenoma formation, in a standard-risk population under colonoscopic surveillance. According to our hypothesis, which stated that a discrete number of mutated clones were scattered in the colon, we expected the adenoma incidence to decrease under the effect of polypectomy.

MATERIALS AND METHODS

Patients and procedures

Consecutive patients from three Portuguese hospitals (a tertiary oncology center, a tertiary general hospital and a regional hospital) with colonic adenomas who were identified at an index colonoscopy were retrospectively reviewed. These patients were included if they had undergone three or more complete colonoscopies that were at least 24 mo apart. The index colonoscopies were performed between 1978 and 2000, for screening or diagnostic purposes. The subsequent colonoscopies were performed according to the assistant physicians' choice, based on surveillance guidelines that changed during the time of the study (data were collected until 2007). Only colonoscopies reaching the cecum, with adequate bowel preparation and complete removal of all of the identified polyps were considered for inclusion in the present study.

The patient files were reviewed, and the patients were excluded if they had a previous history of CRC or adenomas, inflammatory bowel disease, hereditary non-polyposis CRC, familial adenomatous polyposis syndrome, a family history of CRC in any first-degree relative, or CRC at the index colonoscopy.

The location and size of all of the polyps had been recorded and the specimens had been sent for pathological evaluation and were classified according to the criteria of the World Health Organization[10]. All of the colonoscopies were performed by certified gastroenterologists, and sedation using intravenous midazolam (with or without pethidine association) or intravenous propofol (performed by an anesthesiologist) was administered on a case-to-case basis. The bowel preparation methods varied among the centers and over time, but were based on oral solutions that contained polyethylene glycol or senna.

The endoscopic and pathological reports were reviewed by the authors, for the results of the index colonoscopy, and of each colonoscopy reported thereafter. The left colon was defined as the splenic flexure and the segment distal to it.

The baseline adenoma characteristics were recorded and correlated with recurrence of adenomas at follow-up. However, these adenomas were specifically excluded from incidence ratio evaluation, because the time frame for their formation was unknown.

Data analysis

The data regarding number of adenomas observed at each colonoscopy, along with their location, size and

Table 1 Outcomes of first four colonoscopies

Outcomes	Mean	SD
Index colonoscopy: 156 patients studied		
Adenomas	1.68	1.01
Tubular adenomas	1.49	1.13
Tubulo-villous/villous adenomas	0.14/0.05	0.35/0.22
Size of the larger adenoma (mm)	15.51	10.36
Adenomas in the left colon/remaining colon	1.34/0.35	0.93/0.69
Second colonoscopy: 156 patients studied (41.6 ± 21.0 mo since the first)		
Adenomas	0.54	0.86
Tubular adenomas	0.51	0.85
Tubulo-villous/villous adenomas	0.02/0.01	0.14/0.11
Size of the larger adenoma (mm)	8.02	7.39
Adenomas in the left colon/remaining colon	0.36/0.17	0.67/0.49
Third colonoscopy: 156 patients studied (83.7 ± 27.7 mo since the first)		
Adenomas	0.47	0.92
Tubular adenomas	0.46	0.92
Tubulo-villous/villous adenomas	0.02/0.0	0.18/0.0
Size of the larger adenoma (mm)	6.19	4.22
Adenomas in the left colon/remaining colon	0.21/0.26	0.47/0.71
Fourth colonoscopy: 44 patients studied (116.9 ± 34.1 mo since the first)		
Adenomas	0.32	0.74
Tubular adenomas	0.32	0.74
Tubulo-villous/villous adenomas	0.0/0.0	0.0/0.0
Size of the larger adenoma (mm)	5.78	1.92
Adenomas in the left colon/remaining colon	0.16/0.16	0.43/0.48

histological classification were recorded. The analysis of adenoma incidence over time was based on the person-years method. We assumed that the incidence rate of adenomas was constant between two consecutive colonoscopies in each individual and, in that time interval, the monthly incidence of adenomas in each patient was estimated by dividing the number of adenomas found at colonoscopy by the number of months that had elapsed since the previous examination. The incidence of adenomas in a given month after the index colonoscopy was determined for the entire sample by summing the incidence for that month across all of the patients that had been observed up to that time. The monthly incidence density of adenomas was then obtained by dividing the estimated incidence in that month by the population that was still at risk.

The statistical analysis was performed using Excel XP (Microsoft Inc) and Stata 10.0 (Stata Corporation, College Station, TX, United States). The baseline adenoma characteristics were correlated with recurrence at follow-up using the χ^2 test.

Ethics

The present study was a retrospective observational study, in which no experimental intervention was used, and all of the data were kept anonymous. Therefore according to the local regulations, no approval by the Ethics Committee was necessary. All of the patients signed an informed consent document before each endoscopic examination.

RESULTS

The present study included a total of 156 patients (109 male and 47 female). The mean age at the time of the index colonoscopy was 56.8 ± 10.3 years. No procedural complications were recorded. All 156 patients underwent three colonoscopies and 44 of them underwent a fourth examination. The outcomes of these colonoscopies are summarized in Table 1. The index colonoscopy was performed for screening in 31 patients and for diagnostic purposes in the remaining patients (and the symptoms were considered to be unrelated to the adenomas in the majority of cases).

Table 2 Correlation between baseline adenoma characteristics and presence of adenomas in the second colonoscopy

Index colonoscopy	Second colonoscopy		χ^2 test
	0 adenomas (*n*)	⩾ 1 adenoma (*n*)	
> 1 adenoma	39	28	$P = 0.24$
⩾ 3 adenomas	14	10	$P = 0.57$
⩾ 1 TV adenoma	8	14	$P = 0.004$
⩾ 1 villous adenoma	5	3	$P = 0.95$
⩾ 1 TV or V adenoma	13	17	$P = 0.01$
⩾ 1 adenoma > 1 cm	67	42	$P = 0.43$

TV: Tubulo-villous; V: Villous.

The total number of adenomas and the numbers of each adenoma subtype (according to the histology or location) declined over the course of the four colonoscopies. Additionally, 12 patients underwent a fifth examination (162.3 ± 32.7 mo after the first), and two of these had a sixth examination (211.5 ± 20.5 mo after the first). No adenomas were found in any of these last examinations.

Of the initial 156 patients, who underwent three colonoscopies, 107 presently have scheduled colonoscopies, in agreement with the latest guidelines for surveillance after polypectomy; 27 patients have been released from follow-up due to advanced age or significant comorbidity; two patients have died of unrelated causes; and 20 have been lost from follow-up.

There was no significant correlation between the number of adenomas at the index colonoscopy and the presence or absence of adenomas at the second or all subsequent colonoscopies ($P = 0.68$ for the second colonoscopy, $P = 0.49$ for all subsequent colonoscopies). The presence of three or more adenomas at the index colonoscopy did not correlate with the presence of adenomas at the subsequent colonoscopies ($P = 0.57$ for the second, $P = 0.21$ for all subsequent colonoscopies), nor did the presence of adenomas > 1 cm at the index colonoscopy ($P = 0.43$ for the second colonoscopy, $P = 0.78$ for all subsequent colonoscopies). The presence of adenomas with a villous component at the index colonoscopy correlated with the presence of adenomas at the second colonoscopy ($P = 0.01$), but there was no significant correlation when all of the subsequent colonoscopies were considered together ($P = 0.12$) (Tables 2 and 3).

The presence of adenomas of the left colon at the index colonoscopy did not predict recurrence in the same segment in the second or all subsequent colonoscopies, and the same was true for the right colon (data not shown).

The incidence of colonic adenomas was found to

Table 3 Correlation between baseline adenoma characteristics and presence of adenomas in all subsequent colonoscopies

Index colonoscopy	Subsequent colonoscopies 0 adenomas (*n*)	⩾ 1 adenoma (*n*)	χ^2 test
> 1 adenoma	31	36	*P* = 0.5
⩾ 3 adenomas	9	15	*P* = 0.2
⩾ 1 TV adenoma	7	15	*P* = 0.08
⩾ 1 villous adenoma	4	4	*P* = 0.97
⩾ 1 TV or V adenoma	11	19	*P* = 0.12
⩾ 1 adenoma > 1 cm	53	56	*P* = 0.78

TV: Tubulo-villous; V: Villous.

decline from 1.4% person-months, at the beginning of the study, to values close to 0%, 12 years after the index examination (Figure 1).

Eight years after the index colonoscopy (with 65 patients evaluated), a peak in the incidence of adenomas was observed, that approached the baseline values, which was then followed by a steady decline until the end of follow-up (with 37 patients evaluated at 10 years and 19 patients evaluated at 12 years).

There were no reports of flat lesions of the colon in any of these examinations, and there were no colorectal adenocarcinomas reported in these patients during the study period.

DISCUSSION

To explain the predominant CRC expression in the sixth and seventh decades of life, a necessary sequence of 4-7 known mutations fits a model of a stable mutation clock that ends in full-blown neoplasia at consistent time-intervals. The predominant molecular pathway responsible for CRC begins with the selection of cells with an *APC* gene loss or a β-catenin mutation, which is followed by the cumulative selection of subsequent critical events in other genes. Estimations of the time taken to acquire such a sequence of mutations suggest that full-blown neoplasia can take several decades to occur. According to recent estimations, to obtain the necessary sequence of mutations, the first event may need to occur at an early age[11-13]; most likely during the exponential phase of embryonic development, when *APC* is well known to play a key role[14].

In 2007, human colon cancer stem cells were identified by two separate research groups[15,16]. More recently, the location of normal colon stem cells, at the crypt base, has also been demonstrated[17], and these cells seem to be the origin of colon cancer stem cells[18]. It has been proposed that mutations in stem cells are much more likely to occur during the exponential phase of early growth, as opposed to later in life[19].

Accordingly, the results of the present paper strongly suggest that the sporadic formation of adenomas occurs during a limited time period and, when these adenomas are removed, virtually all of the neoplasia-prone clones may be extinguished. If this model is shown to be true,

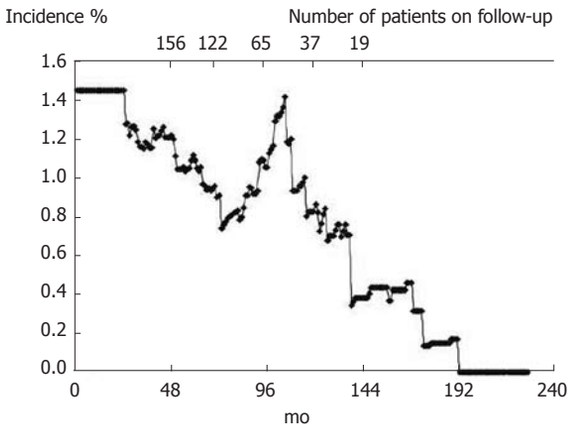

Figure 1 Variation of colonic adenomas monthly incidence (person-month percentage) with time, since the index colonoscopy.

then the concept of a field carcinogenic defect, which progresses with continuous adverse environmental exposure and/or failure of the tight controls that assure DNA replication fidelity, should be replaced by a concept of limited colonic mosaicism. When the endoscopically visible neoplastic expression of this limited mosaicism is removed, the putative carcinogenic impulse no longer compromises colonic epithelia homeostasis.

Our study was limited mainly by its retrospective nature and by the small number of patients reaching longer follow-up. However, this sample was probably representative of the population at risk for sporadic CRC, because it included both symptomatic and asymptomatic individuals who had no relevant family histories, who underwent initial colonoscopy mostly during the sixth decade of life, and who were followed for at least 4 years.

In contrast to traditional beliefs, we did not observe a correlation between baseline adenoma characteristics and the risk of recurrence at follow-up. Although the AGA still takes baseline predictors of future adenomas or cancer into consideration, in their latest Guidelines for Colonoscopy Surveillance After Polypectomy[9], several limitations of the available evidence have been raised. van Stolk *et al*[20] have reported a study in which the number of adenomas at first colonoscopy was a significant predictor of having recurrent adenomas; however, these authors noted that missed polyps were a possible explanation for this relationship. Furthermore, that previous study only included 4 years of follow-up, and the presence of a family history of CRC was not taken into account at all. In another study, by Martínez *et al*[21], multiple adenomas at baseline, large adenomas (> 1 cm) or adenomas in the proximal colon were predictors of recurrence. However, the maximum follow-up of that study was 2 years, and the baseline colonoscopy was not the first examination for several of the patients. In addition, and although this relationship was not statistically significant, a family history of CRC in first-degree relatives was associated with a higher risk of recurrence in the study population. The initial National Polyp Study also included a follow-up of only 3 years, and investigated the incidence of colorec-

tal cancer but not adenoma[2,22]. The authors included patients regardless of their family history (other than established genetic syndromes)[2] and they admitted that, as a result of the short follow-up, three of the diagnosed cancers may have been missed polyps[23]. A more recent study, by Martinez *et al*[24], has revealed that the age of the patient and the number and size of prior adenomas were associated with the risk of advanced colorectal neoplasia after polypectomy. However, that study was also limited by a median follow-up of only 4 years and a maximum follow-up of < 6 years[24]. Our study had the advantages of a minimum follow-up of 4 years and exclusion of patients with any family history of CRC in first-degree relatives. This might explain why we did not find that baseline adenoma characteristics were predictive of recurrence, and why we were able to show a decreasing incidence of adenomas with time, after an age peak and under the influence of polypectomy.

At Digestive Disease Week 2007, Zauber *et al* presented new data regarding the National Polyp Study population indicating that, after a mean follow-up of 14 years, CRC mortality was markedly reduced in all of the patients with adenomas at baseline, when compared to the general population. This was observed even when patients who refused follow-up were considered in the analysis. This finding supports the hypothesis that the major benefit is derived from the first colonoscopy and also brings into question the notion of increasing recurrence of adenomas over time.

With regard to the unexpected second peak that was observed for the incidence of adenomas in our study, after 8 years, we may speculate whether this peak is related to the type of the second hit in the *APC* gene. It has been reported that, when the first hit happens close to codon 1300, the second hit is most likely loss of heterozygosis, a faster and more efficient mutation process. In all other cases, point mutation is the most common mechanism for the second hit, thus driving a more sluggish development[25]. This confers different selective advantages to each colonic adenoma, according to the specific first hit-second hit combination and may explain why some adenomas appear earlier and why others are only apparent towards the end of the adenoma time window. We acknowledge that, during a limited period of embryonic development, a small number of stem cells may acquire different first mutations, and that these mutations are expressed along incidence waves during a restricted time window.

The results of the present study reinforce the importance of colonoscopy with polypectomy during the fifth to sixth decades of life, and the feasibility of increasing the time for re-examination after a normal examination. Furthermore, our study indirectly supports the concept of a relatively stable mutation clock, which is possibly initiated during the developmental phase of embryogenesis.

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COMMENTS

Background

Colorectal cancer (CRC) and adenomas are thought to have an increasing incidence with age, and this has led to almost lifelong surveillance colonoscopy. This view has recently started to be challenged.

Research frontiers

APC is a crucial gene in colorectal carcinogenesis. If the first hit on this gene occurs in a colonic stem cell precursor early in life, it could lead to only a few colon crypts that are prone to originate adenoma and cancer, in a restricted time window. If these clones were removed, by polypectomy, the potential for colon cancer could be erradicated in that individual.

Innovations and breakthroughs

Colon cancer stem cells have recently been identified, apparently originating from colonic stem cells, at the crypt base. Stem cells seem more prone to mutation during embryogenesis than later in life. Reports have shown that a single colonoscopy with polypectomy reduces CRC incidence. The study shows that, under the influence of polypectomy, adenoma incidence decreases with age.

Applications

If the study results are confirmed, surveillance colonoscopy intervals may safely be lengthened, and some people may even be released from surveillance after a few examinations.

Terminology

APC acts as a tumor suppressor gene by regulating the intranuclear concentration of β-catenin, a protein involved in the transcription of genes that promote proliferation. These genes and proteins are part of the Wnt pathway, which is involved in > 80% of sporadic CRC.

Peer review

The authors do a nice job to expand on a current line of questioning the need for continuing surveillance and indirectly questioning the concept of age appropriateness for surveillance as well as cost-advantageous practice. This is a very relevant study and well written.

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BRIEF ARTICLE

CCL7 and CCL21 overexpression in gastric cancer is associated with lymph node metastasis and poor prognosis

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Abstract

AIM: To investigate how a complex network of CC chemokine ligands (CCLs) and their receptors influence the progression of tumor and metastasis.

METHODS: In the present study, we used immunohistochemistry to examine the expression of CCL7, CCL8 and CCL21 in 194 gastric cancer samples and adjacent normal tissues. We analyzed their correlation with tumor metastasis, clinicopathologic parameters and clinical outcome.

RESULTS: We found that the higher expression of CCL7 and CCL21 in cancer tissues than in normal tissues was significantly correlated with advanced depth of wall invasion, lymph node metastasis and higher tumor node metastasis stage. Moreover, Kaplan-Meier survival analysis revealed that CCL7 and CCL21 overexpression in cancer tissues was correlated with poor prognosis.

CONCLUSION: These results suggest that overexpression of these two CC chemokine ligands is associated with tumor metastasis and serves as a prognostic factor in patients with gastric cancer.

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Key words: CC chemokine; Chemokine ligand 7; Chemokine ligand 21; Gastric cancer; Lymph node metastasis; Poor prognosis

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INTRODUCTION

Chemokine ligands (CCLs) belong to the small molecule chemoattractive cytokine family and are grouped into CC and CXC chemokines ligands on the basis of the characteristic presence of four conserved cysteine residues[1-3]. Chemokines mediate their chemical effect on target cells through G-protein-coupled receptors, which are characterized structurally by seven transmembrane spanning domains and are involved in the attraction and activation of mononuclear and polymorphonuclear leukocytes. CCLs and their receptors play an important role

in angiogenesis and tumor growth, however the role of CCLs in metastasis has only recently been explored[4,5]. CCL7 promoted the invasion and migration of oral squamous cell carcinoma[4]. CCL21 was significantly highly expressed in breast tumor cells with lymph node metastasis and prognosis[5].

Gastric cancer is one of the commonest malignant tumors of the alimentary tract and is characterized by late clinical presentation, rapid progression, and poor survival[6]. The reason for this poor prognosis is that, at the time of diagnosis, gastric cancer usually shows extensive local tumor invasion and frequent spread to metastatic sites, particularly lymph nodes. Spread of malignant tumors is a multistep process and many of the stages of tumor invasion require degradation or breakdown of the extracellular matrix and connective tissue surrounding tumor cells[7,8]. The matrix metalloproteinases (MMPs) are a family of zinc containing enzymes which are involved in the degradation of different components of the extracellular matrix, and there is considerable evidence to indicate that individual MMPs have important roles in tumor invasion and tumor spread[9-11]. A recent study showed that increased levels of CCL recruit immature myeloid cells that carry the chemokine ligand receptor (CCR) from the blood to the tumor invasion front. These immature myeloid cells produced MMP9 and MMP2 and help the tumor cells to migrate and invade[12].

In the present study, we used immunohistochemistry to examine the expression of CCL7, CCL8 and CCL21 in 194 gastric cancer samples and adjacent normal tissues. We analyzed their correlation with tumor metastasis, clinicopathologic parameters and clinical outcome.

MATERIALS AND METHODS

Patients and specimens

A consecutive series of 194 tissue specimens were collected from patients with gastric cancer who received subtotal or total gastroectomy resection in Chang Gung Memorial Hospital (CGMH) in Taiwan. All operations were performed between January 2001 and December 2002. Written informed consent was obtained before sample collection and this study was approved by the Institutional Review Board of CGMH. There were 114 males and 80 females with a mean age of 62 years (range, 24-90 years). The age and gender of patients, tumor location, tumor size, cell differentiation, depth of wall invasion, status of lymph node metastasis, vascular invasion, lymphatic invasion and desmoplastic reaction were obtained from histopathology records. Stage of gastric cancer was described according to the 1997 tumor node metastasis (TNM) classification of malignant tumors by the American Joint Committee on Cancer. All patients were followed until December 2007 with a minimum 5 years of follow-up. All tissue specimens were formalin-fixed and paraffin-embedded. Formalin fixed tissue sections were stained with haematoxylin and eosin and classified by a pathologist. These results were compared with the histopathology records from CGMH. Final pathology was determined by consensus and review if necessary.

Immunohistochemistry

The tissue blocks were constructed according to the method of Schraml *et al*[13] and the best representative morphological areas of tumors were used in this study. The specimen sections were deparaffinized, treated with 3% hydrogen peroxide and microwaved after pretreatment in 10 mmol/L citric acid to retrieve antigenicity. The sections were incubated with blocking solution containing phosphate buffered saline and 1% bovine serum albumin for 20 min at room temperature, and then incubated overnight at 4 ℃ with an anti-CCL7 antibody (1:100, R and D), an anti-CCL8 monoclonal antibody (1:50, R and D), or an anti-CCL21 monoclonal antibody (1:50, R and D), respectively. After washing 4 times with Tris Buffered Saline, the sections were incubated with biotinylated secondary antibody (Santa Cruz Biotechnology). The immuno-complex was visualized by the immonoglobulin enzyme bridge technique using the DAKO LSAB 2 System, HRP kit (DAKO corp. Carpinteria, CA) with 3,3' diaminobenzidine tetrachloride as a substrate. The sections were counterstained with hematoxylin, dehydrated with graded alcohols, cleared with xylene and mounted with a coverslip.

Scoring of the immunohistochemical staining

The immunostaining results were scored as follows, according to a previous report[14]. The immunostaining reaction was evaluated by subjective assessments of the median staining intensity (0, no stain; 1, weak; 2, moderate; and 3, strong stain) and by the fraction of stained cells in percentage categories (0, 0%-9%; 1, 10%-49%; 2, 50%-89%; and 3, ⩾ 90%). This scoring system was previously shown to be reproducible[15]. The scores of 0 to 3 were obtained as follows: percentage categories and staining were each ranked as indicated above. The ranks for percentage and staining intensity were multiplied by each other, divided by 3, and rounded up to the nearest whole number[15]. The results of immunostaining in tumor and normal tissues were divided into three groups, higher (rank of tumor tissue > rank of normal tissue), equal (rank of tumor tissue = rank of normal tissue), and lower (rank of tumor tissue < rank of normal tissue) (Figures 1-3).

Statistical analysis

χ^2 or Fisher's exact test was used to test for an association between CCL7, CCL8 and CCL21 expression and patient clinicopathologic parameters. Disease-free survival was defined as the time from surgery to the first relapse of cancer, occurrence of a second primary tumor, or death from any cause. Univariate survival analysis was assessed by the Kaplan-Meier method and significance of difference between groups was analysed using log rank test or log rank test for trend. Stepwise multivariate survival analysis was performed by the Cox proportional hazards model. All reported *P* values were two-sided and a *P* value < 0.05 was considered significant.

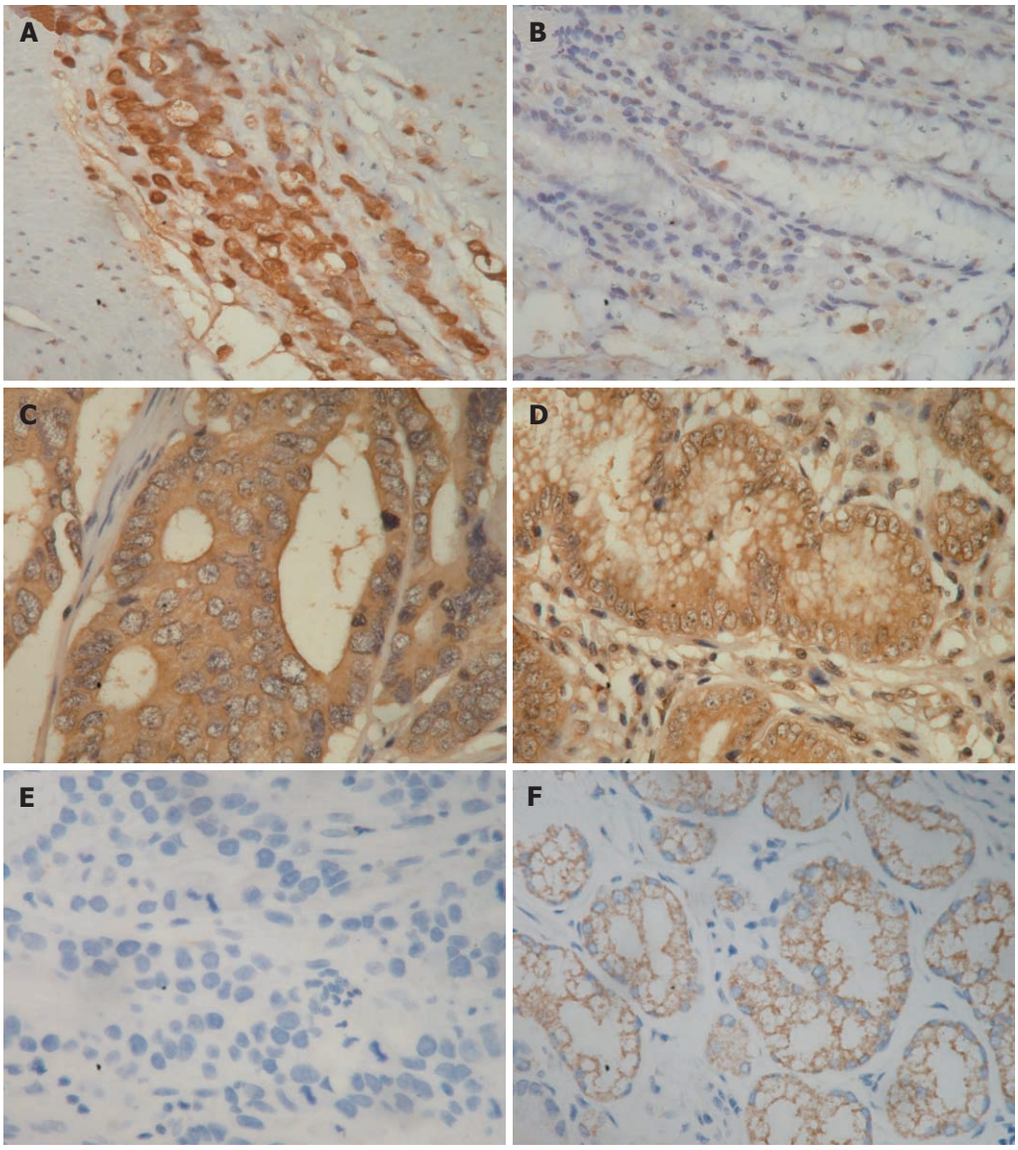

Figure 1 Immunohistochemistry of chemokine ligand 7 in gastric cancer and adjacent normal tissues. Chemokine ligand 7 (CCL7) staining is higher in the cytoplasm of gastric cancer cells (A) than in the cytoplasm of adjacent normal cells (B); CCL7 staining is equal in the cytoplasm of gastric cancer cells (C) and in the cytoplasm of adjacent normal cells (D); CCL7 staining is lower in the cytoplasm of gastric cancer cells (E) than in the cytoplasm of adjacent normal cells (F). (magnification, × 400).

RESULTS

CCL7, CCL8 and CCL21 expression in gastric cancer and adjacent normal tissues

The percentages of the higher expression of CCL7, CCL8 and CCL21 in cancer tissues than in normal tissues were 42.3% (82 of 194), 29.9% (58 of 194) and 44.8% (87 of 194), respectively (Figures 1-3 and Table 1). The percentages of the equal expression of CCL7, CCL8 and CCL21 in cancer tissues and in normal tissues were 35.6% (69 of 194), 33% (64 of 194) and 32.5% (63 of 194), respectively (Figures 1-3 and Table 1). The percentages of the lower expression of CCL7, CCL8 and CCL21 in cancer tissues than in normal tissues were 22.2% (43 of 194), 37.1% (72 of 194) and 22.7% (44 of 194), respectively (Figures 1-3 and Table 1).

CCL7, CCL8 and CCL21 overexpression in relation to clinicopathologic parameters

The overexpression of CCL7 in cancer tissues compared with normal tissues was significantly correlated with tumor location ($P = 0.025$) and tumor size ($P = 0.001$). The overexpression of CCL7 was significantly higher in gastric cancer with advanced depth of wall invasion ($P = 0.001$), lymph node metastasis ($P = 0.020$), desmoplastic reaction ($P = 0.006$) and higher TNM stage ($P = 0.008$), but was not correlated with age, gender, differentiation, vascular invasion or lymphatic invasion (Table 1).

The overexpression of CCL8 was significantly correlated with age ($P = 0.026$) and tumor location ($P = 0.004$), but not with gender, tumor size, differentiation, depth of wall invasion, lymph node metastasis, vascular invasion, lymphatic invasion, desmoplastic reaction or TNM stage.

The overexpression of CCL21 was significantly higher in females than in males ($P = 0.041$) and was correlated with tumor location ($P = 0.026$), tumor size ($P = 0.043$) and lymphatic invasion ($P = 0.006$). As with CCL7, the overexpression of CCL21 was significantly higher in gastric cancer with an advanced depth of wall invasion ($P < 0.0001$), lymph node metastasis ($P = 0.003$), desmoplastic reaction ($P < 0.0001$) and higher TNM stage ($P < 0.0001$), but was not correlated with age, differentiation or vascular

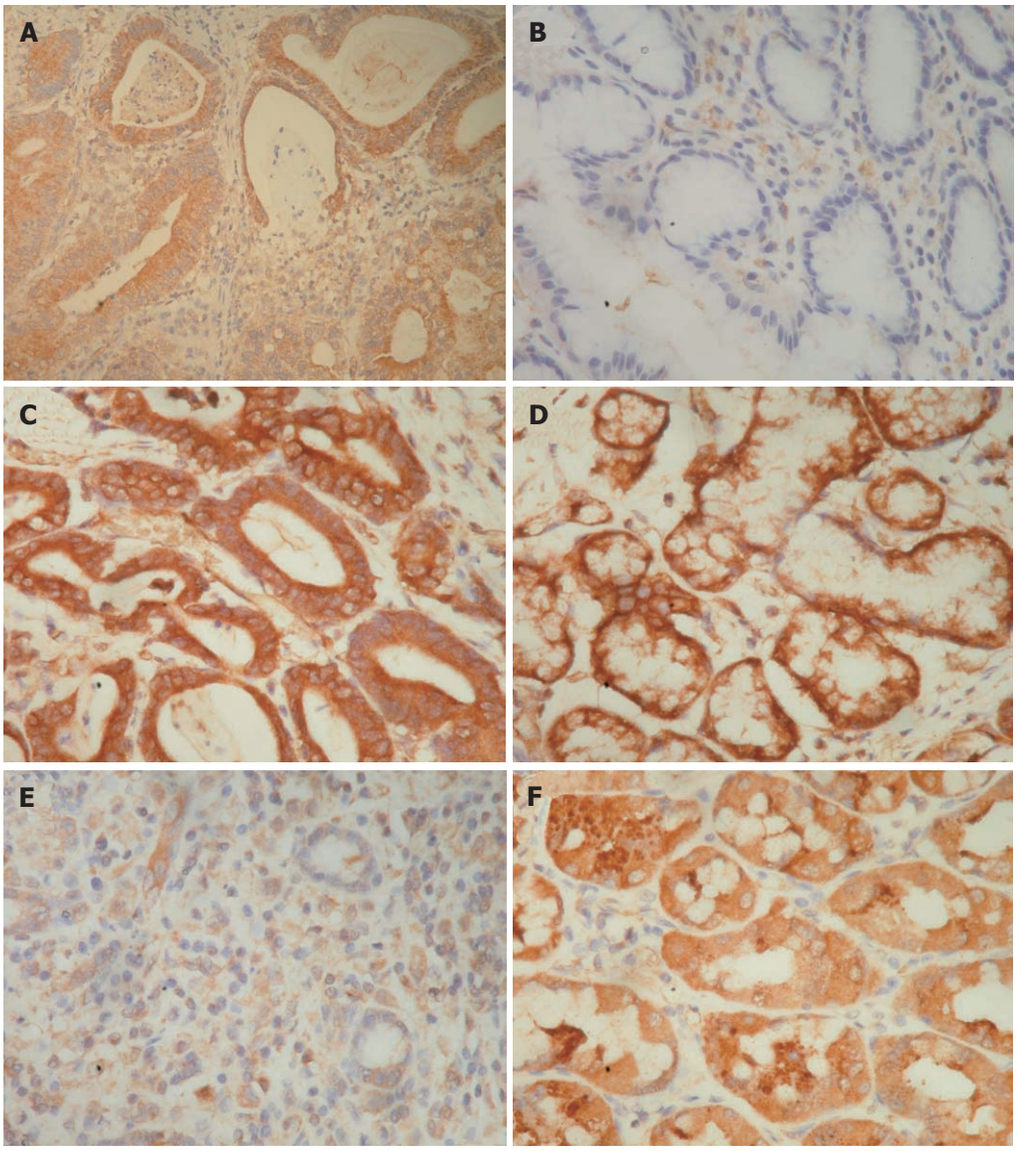

Figure 2 Immunohistochemistry of chemokine ligand 8 in gastric cancer and adjacent normal tissues. Chemokine ligand 8 (CCL8) staining is higher in the cytoplasm of gastric cancer cells (A) than in the cytoplasm of adjacent normal cells (B); CCL8 staining is equal in the cytoplasm of gastric cancer cells (C) and in the cytoplasm of adjacent normal cells (D); CCL8 staining is lower in the cytoplasm of gastric cancer cells (E) than in the cytoplasm of adjacent normal cells (F). (magnification, × 400).

invasion (Table 1).

Prognostic implications of CCL7, CCL8 and CCL21 overexpression in gastric cancer

CCL7 and CCL21 overexpression was correlated with a poor prognosis (P = 0.002 and 0.001, Table 2 and Figure 4A and C). CCL8 overexpression was not correlated with survival (Table 2, Figure 4B). Other significant prognostic factors were tumor location, tumor size, differentiation, depth of invasion, lymph node metastases, vascular invasion, lymphatic invasion, marked desmoplastic reaction and higher TNM stage. In multivariate analysis, depth of invasion, lymph node metastasis and desmoplastic reaction were independent prognostic factors (Table 3).

DISCUSSION

In this study, CCL7, CCL8 and CCL21 expression levels were examined in 194 cases of gastric cancer for correlation with patient clinicopathologic factors. We found that the higher expression of CCL7 and CCL21 in cancer tissues than in normal tissues was significantly correlated with advanced depth of wall invasion, lymph node metastasis and higher TNM stage. The mechanism for chemokine ligand promotion of tumor invasion and metastasis is not clear. Using a model of colorectal tumor progression, Kitamura *et al*[12] showed that tumor-stromal interaction could promote tumor invasion. The colonic tumor can promote the production of CCL9. Increased levels of CCL9 recruited immature myeloid cells that carry the CCL9 receptor CCR1 from the blood to the tumor invasion front. The immature myeloid cells produce MMP2 and MMP9 and help the tumor epithelium to migrate and invade into the stroma. Jung *et al*[4] also showed the importance of tumor-stromal crosstalk in invasion of oral squamous cell carcinoma (OSCC) *via* CCL7[4]. To identify key molecular regulators expressed by carcinoma-associated fibroblasts (CAF) that promote cancer cell invasion, Jung *et al*[4] used microarrays to compare cocultured OSCC and CAF with monoculture controls. Microarray and real-time polymerase chain reaction analysis identified marked upregulation of CCL7 in cocultured CAF. Enzyme-linked

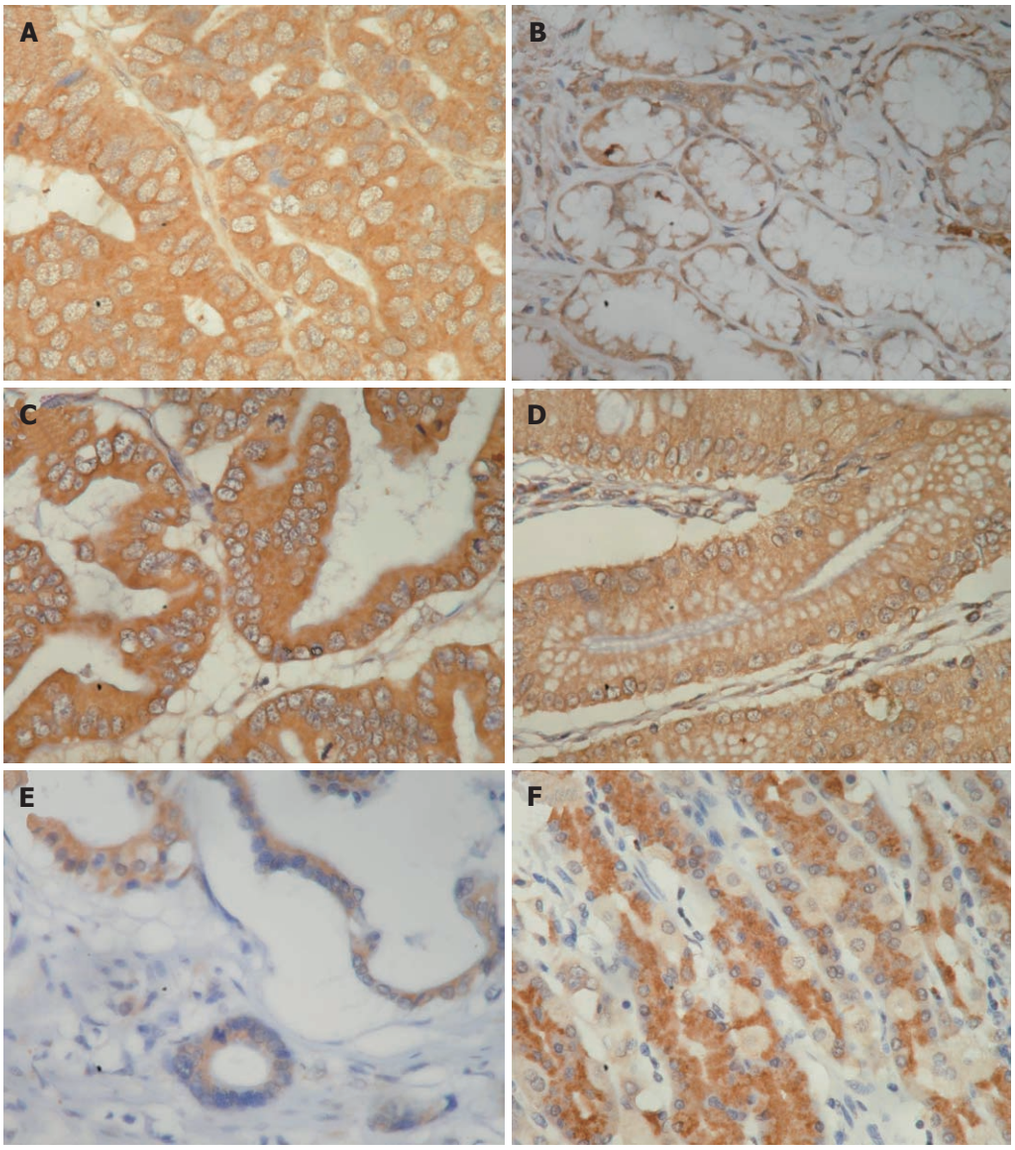

Figure 3 Immunohistochemistry of chemokine ligand 21 in gastric cancer and adjacent normal tissues. Chemokine ligand 21 (CCL21) staining is higher in the cytoplasm of gastric cancer cells (A) than in the cytoplasm of adjacent normal cells (B); CCL21 staining is equal in the cytoplasm of gastric cancer cells (C) and in the cytoplasm of adjacent normal cells (D); CCL21 staining is lower in the cytoplasm of gastric cancer cells (E) than in the cytoplasm of adjacent normal cells (F). (magnification, × 400).

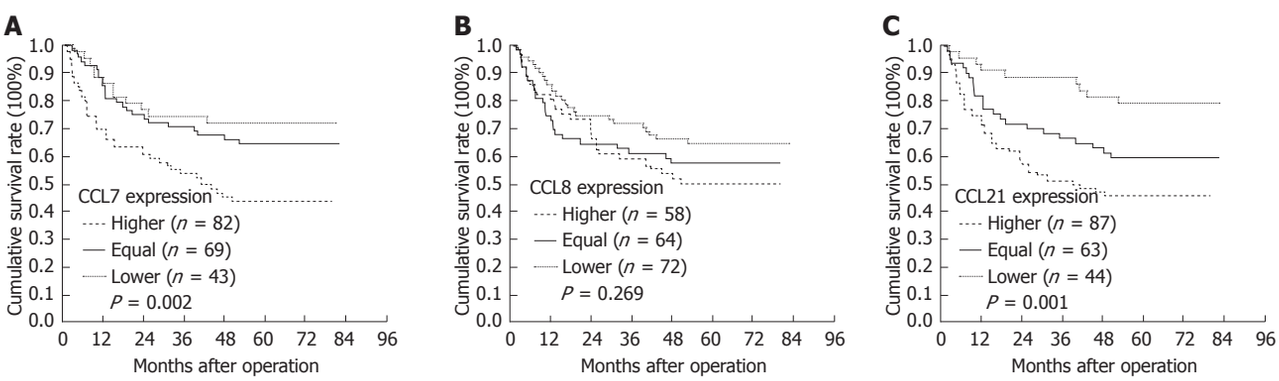

Figure 4 Kaplan-Meier survival curves for disease-free survival of 194 patients with gastric cancer. A: Categorized by chemokine ligand 7 (CCL7) expression, survival was significantly worse for patients with higher CCL7 expression than those with equal or lower CCL7 expression (P = 0.002); B: Categorized by CCL8 expression, no significant difference was observed among the three groups (P = 0.269); C: Categorized by CCL21 expression, survival was significantly worse for patients with higher CCL21 expression than those with equal or lower CCL21 expression (P = 0.001).

immunosorbent assay showed an elevated level of CCL7 secretion from CAF stimulated by coculture with OSCC cells. CCL7 promoted the invasion and migration of OSCC cells, and the invasiveness was inhibited by treatment with CCL7 neutralizing antibody.

However, other studies have shown that CC chemokine

Table 1 Association of chemokine ligand 7, chemokine ligand 8 and chemokine ligand 21 expression with the clinicopathologic parameters

Factors	Cases	CCL-7 expression				CCL-8 expression				CCL-21 expression			
		Higher n = 82	Equal n = 69	Lower n = 43	P value	Higher n = 58	Equal n = 64	Lower n = 72	P value	Higher n = 87	Equal n = 63	Lower n = 44	P value
Age (yr)													
≤ 60	83	31	31	21	0.449	17	28	38	0.026	34	24	25	0.101
> 60	111	51	38	22		41	36	34		53	39	19	
Gender													
Male	114	52	40	22	0.412	36	40	38	0.429	47	45	22	0.041
Female	80	30	29	21		22	24	34		40	18	22	
Tumor location													
Upper	22	10	6	6	0.025	4	6	12	0.004	8	9	5	0.026
Middle	40	10	17	13		10	6	24		13	11	16	
Lower	124	58	46	20		42	48	34		60	43	21	
Whole	8	4	0	4		2	4	2		6	0	2	
Tumor size (cm)													
≤ 3	95	29	36	30	0.001	24	28	43	0.068	34	35	26	0.043
> 3	99	53	33	13		34	36	29		53	28	18	
Differentiation													
Well	18	6	8	4	0.177	5	6	7	0.422	7	7	4	0.130
Moderate	55	27	22	6		17	23	15		30	18	7	
Poor	52	25	15	12		19	13	20		27	12	13	
Signet ring cell	69	24	24	21		17	22	30		23	26	20	
Depth of wall invasion													
T1	47	12	19	16	0.001	8	15	24	0.070	9	18	20	< 0.0001
T2	37	8	18	11		13	12	12		9	18	10	
T3	101	58	29	14		35	31	35		64	24	13	
T4	9	4	3	2		2	6	1		5	3	1	
Lymph node metastasis													
N0	88	30	36	22	0.020	21	29	38	0.488	28	33	27	0.003
N1	47	16	16	15		16	17	14		20	17	10	
N2	23	13	7	3		7	6	10		13	5	5	
N3	36	23	10	3		14	12	10		26	8	2	
Vascular invasion													
No	168	70	60	38	0.800	50	58	60	0.561	71	57	40	0.163
Yes	26	12	9	5		8	6	12		16	6	4	
Lymphatic invasion													
No	104	37	39	28	0.086	27	34	43	0.325	36	38	30	0.006
Yes	90	45	30	15		31	30	29		51	25	14	
Desmoplastic reaction													
None	28	6	14	8	0.006	5	6	17	0.071	7	9	12	< 0.0001
Mild	64	22	20	22		18	21	25		17	26	21	
Moderate	75	43	25	7		23	30	22		44	24	7	
Marked	27	11	10	6		12	7	8		19	4	43	
TNM stage													
Ⅰ	64	16	27	21	0.008	14	20	30	0.427	13	26	25	< 0.0001
Ⅱ	44	17	17	10		13	14	17		18	18	8	
Ⅲ	38	20	12	6		13	14	11		24	8	6	
Ⅳ	48	29	13	6		18	16	14		32	11	5	

CCL: Chemokine ligand; TNM: Tumor node metastasis.

ligands promote T cells to kill the tumor cells. Wu *et al*[16] investigated the effect of exogenous CCL21 expressed in breast cancer MCF-7 cells on human monocyte-derived dendritic cells (DCs). Stimulation of CCL21-transfected MCF-7 cells prompted DC function: migration, antigen uptake and presentation. The stimulated DCs facilitated Th 1 type cytokine production, perforin-forming $CD8^+$ T cell transformation and final T cell-associated clearance of MCF-7 cells. Wetzel *et al*[17] showed that human CCL7 can reduce tumorigenicity and augment infiltration of dendritic cells and neutrophils toward mouse mastocytoma; it also inhibits mouse melanoma growth through activation of T lymphocytes and natural killer cells.

The differences between the expression of CCL7 and CCL21 correlated with clinicopathologic parameters were gender and lymphatic invasion. The overexpression of CCL7 in gastric cancer was not correlated with gender and lymphatic invasion, but that of CCL21 was correlated with these two parameters. The overexpression of CCL21 was significantly higher in females than in males. The reason for the significance is not clear and more studies are necessary to clarify the significance. The overexpression of CCL21 was also correlated with lymphatic invasion. Recently, metastatic gastric carcinoma cells have been shown to express the receptor for chemokine CCL21, chemokine receptors CCR7, a prop-

Table 2 Univariate analysis of the clinicopathologic parameters influencing the disease-free survival of 194 gastric cancer patients undergoing gastrectomy

Factors	Cases	Mean survival (mo)	95% CI of mean	5-year survival (%)	*P* value
Age (yr)					
≤ 60	83	57.33	50.22-64.44	60.2	0.516
> 60	111	53.34	46.93-59.76	56.1	
Gender					
Male	114	57.90	51.74-64.05	62.2	0.174
Female	80	51.09	43.61-58.56	51.7	
Type of gastrectomy					
Total	41	31.85	23.28-40.43	33.5	< 0.0001
Subtotal	153	60.21	55.14-65.48	64.5	
Tumor location					
Upper	22	26.01	15.81-36.21	27.3	< 0.0001
Middle	40	69.22	61.15-71.29	77.0	
Lower	124	56.56	50.70-62.43	59.5	
Diffuse	8	23.41	7.46-39.36	25.0	
Margin					
Negative	173	57.71	52.73-62.68	62.3	0.0001
Positive	21	27.41	17.26-37.56	12.6	
Tumor size (cm)					
≤ 3	95	70.94	65.97-75.92	80.7	< 0.0001
> 3	99	39.02	32.23-45.81	34.6	
Differentiation					
Well	18	65.58	59.43-71.73	94.4	< 0.0001
Moderate	55	43.11	35.16-51.06	54.4	
Poor	52	37.91	30.28-45.54	33.6	
Signet ring cell	69	62.16	54.89-69.43	67.9	
Depth of invasion					
T1	47	79.53	75.46-83.60	95.7	< 0.0001
T2	37	71.92	64.56-79.28	80.6	
T3	101	40.04	33.53-46.54	35.4	
T4	9	10.57	5.94-15.21	0.0	
Lymph node metastasis					
N0	88	73.64	69.17-78.11	83.8	< 0.0001
N1	47	57.67	47.75-67.59	64.9	
N2	23	31.61	21.57-41.65	23.9	
N3	36	15.39	10.83-19.95	0.0	
Vascular invasion					
No	168	60.36	55.48-65.23	65.4	< 0.0001
Yes	26	17.74	11.39-24.08	4.4	
Lymphatic invasion					
No	104	70.58	65.84-75.33	79.2	< 0.0001
Yes	90	36.48	29.43-43.53	32.1	
Perineural invasion					
No	109	67.57	62.27-72.88	76.3	< 0.0001
Yes	84	37.36	30.70-44.02	34.1	
Desmoplastic reaction					
None	28	67.55	56.87-78.24	78.6	< 0.0001
Mild	64	70.20	64.03-76.37	78.7	
Moderate	75	41.99	34.35-49.62	38.8	
Marked	27	36.67	24.49-48.85	36.7	
TNM stage					
Ⅰ	64	78.86	75.66-82.07	92.1	< 0.0001
Ⅱ	44	66.55	58.17-74.92	74.2	
Ⅲ	38	46.52	36.06-56.98	45.7	
Ⅳ	48	14.71	10.94-18.48	0.0	
CCL-7					
Higher	82	45.62	37.97-53.28	43.6	0.002
Equal	69	60.09	52.62-67.57	64.7	
Lower	43	63.28	54.24-72.32	71.8	
CCL-8					
Higher	58	51.05	42.41-59.70	50.0	0.269
Equal	64	51.89	43.39-60.39	57.4	
Lower	72	59.97	52.60-67.34	64.6	
CCL-21					
Higher	87	44.96	37.83-52.10	45.8	0.001
Equal	63	56.60	48.30-64.90	59.5	
Lower	44	70.58	63.24-77.93	79.2	

CCL: Chemokine ligand; TNM: Tumor node metastasis.

Table 3 Cox's proportional hazards analysis

Factors	Hazard ratio	95% CI upper-lower	*P* value
Depth of invasion			
T2/T1	7.850	1.454-42.390	0.017
T3/T1	23.200	4.733-113.716	0.000
T4/T1	65.052	10.830-390.730	< 0.0001
Lymph node metastasis			
N1/N0	1.856	0.865-3.982	0.112
N2/N0	3.520	1.597-7.758	0.002
N3/N0	7.227	3.349-15.596	< 0.0001
Desmoplastic reaction			
Mild/none	3.663	1.272-10.638	0.016
Moderate/none	3.623	1.304-10.101	0.014
Marked/none	4.926	1.590-15.152	0.006
CCL-7			0.801
CCL-8			0.620
CCL-21			0.084

CCL: Chemokine ligand.

erty that may allow them to access the lymphatic system and spread to regional lymph nodes[18]. Thus the "chemoattraction" theory of metastasis may be reflected by malignant cells expressing functional chemokine receptors that can respond to organ-specific chemoattractant molecules and migrate directionally along chemokine gradients to set up site-specific metastases in the target organs. Such chemotactic migration of tumors would mirror the physiologic mechanisms of lymphocyte homing into lymphoid organs.

Kaplan-Meier survival analysis revealed that CCL7 and CCL21 overexpression in cancer tissues was correlated with poor prognosis. If tumor-infiltrating leukocytes are able, in some instances, to promote cancer, then the local production of chemokines that attract leukocytes could be a poor prognostic sign. This is the case in human breast cancer, where levels of CCL5 and CCL2 correlate with tumor progression and there is a positive correlation between the extent of the macrophage infiltrate, lymph-node metastasis and clinical aggressiveness[19-21]. In esophageal squamous cell carcinoma, CCL2 expression has been associated with the extent of macrophage infiltration, tumor cell invasion and tumor vascularity[22].

In conclusion, the higher expression of CCL7 and CCL21 in gastric cancer tissues than in normal tissues was significantly correlated with advanced depth of wall invasion, lymph node metastasis and higher TNM stage. Moreover, Kaplan-Meier survival analysis revealed that CCL7 and CCL21 overexpression in cancer tissues was correlated with poor prognosis. These results suggest that overexpression of these two CC chemokine ligands is associated with tumor metastasis and serves as a prognostic factor in patients with gastric cancer.

COMMENTS

Background

Gastric cancer is one of the commonest malignant tumors of the alimentary tract and is characterized by late clinical presentation, rapid progression, and

poor survival. The reason for this poor prognosis is that, at the time of diagnosis, gastric cancer usually shows extensive local tumor invasion and frequent spread to metastatic sites, particularly lymph nodes. Spread of malignant tumors is a multistep process and many of the stages of tumor invasion require degradation or breakdown of the extracellular matrix and connective tissue surrounding tumor cells.

Research frontiers

The matrix metalloproteinases (MMPs) are a family of zinc containing enzymes which are involved in the degradation of different components of the extracellular matrix, and there is considerable evidence to indicate that individual MMPs have important roles in tumor invasion and tumor spread. A recent study showed that increased levels of chemokine ligand (CCL) recruit immature myeloid cells that carry chemokine ligand receptor from the blood to the tumor invasion front. These immature myeloid cells produced MMP9 and MMP2 and help the tumor cells to migrate and invade.

Innovations and breakthroughs

In the present study, the authors used immunohistochemistry to examine the expression of CCL7, CCL8 and CCL21 in 194 gastric cancer samples and adjacent normal tissues. The authors analyzed their correlation with tumor metastasis, clinicopathologic parameters and clinical outcome. They found that the higher expression of CCL7 and CCL21 in cancer tissues than in normal tissues was significantly correlated with advanced depth of wall invasion, lymph node metastasis and higher tumor node metastasis (TNM) stage. Moreover, Kaplan-Meier survival analysis revealed that CCL7 and CCL21 overexpression in cancer tissues was correlated with poor prognosis.

Applications

These results suggest that overexpression of CCL7 and CCL21 is associated with tumor metastasis and serves as a prognostic factor in patients with gastric cancer.

Peer review

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BRIEF ARTICLE

Bacterial flora concurrent with *Helicobacter pylori* in the stomach of patients with upper gastrointestinal diseases

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Abstract

AIM: To investigate the non-*Helicobacter pylori* (*H. pylori*) bacterial flora concurrent with *H. pylori* infection.

METHODS: A total of 103 gastric biopsy specimens from *H. pylori* positive patients were selected for bacterial culture. All the non-*H. pylori* bacterial isolates were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

RESULTS: A total of 201 non-*H. pylori* bacterial isolates were cultivated from 67 (65.0%) of the 103 gastric samples, including 153 isolates identified successfully at species level and 48 at genus level by MALDI-TOF MS. The dominant species were *Streptococcus, Neisseria, Rothia* and *Staphylococcus*, which differed from the predominantly acid resistant species reported previously in healthy volunteers. The prevalence of non-*H. pylori* bacteria was higher in non-ulcer dyspepsia group than in gastric ulcer group (100% *vs* 42.9%, $P < 0.001$). Six bacterial species with urease activity (*Staphylococcus epidermidis, Staphylococcus warneri, Staphylococcus capitis, Staphylococcus aureus, Brevibacterium spp.* and *Klebsiella pneumoniae*) were also isolated.

CONCLUSION: There is a high prevalence of the non-*H. pylori* bacteria concurrent with *H. pylori* infection, and the non-*H. pylori* bacteria may also play important as-yet-undiscovered roles in the pathogenesis of stomach disorders.

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Key words: Non-*Helicobacter pylori*; Bacterial flora; Gastrointestinal diseases; Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

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INTRODUCTION

Stomach is generally regarded as an environment that is not conducive to bacterial colonization. A notable exception is *Helicobacter pylori* (*H. pylori*), which may cause chronic gastritis, peptic ulcer and is correlated with gastric adenocarcinoma[1,2]. However, gastritis also occurs in *H. pylori* negative human subjects or mouse models, suggesting that *H. pylori* is just one of the organisms causing gastritis.

With the reduced level of acid induced by *H. pylori* dissipates or antiulcer medications, the stomach becomes more susceptible to the colonization of other organisms[3], which may complicate the development of gastric disorders. In recent years, considerable interest has emerged in the interactions among *H. pylori*, non-*H. pylori* bacteria and acid-suppressive therapy. Some studies have shown that the co-infection of *H. pylori* and non-*H. pylori* bacteria enhances the development of atrophic corpus gastritis[4]. But up till now, there are only a limited number of studies regarding the stomach bacterial flora and all are based on a small size of a few patients or volunteers. The aim of this study was to investigate the non-*H. pylori* bacterial flora concurrent with *H. pylori* infection.

Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) is a new technology for bacterial identification[5]. Its performance has been evaluated in different studies, and a higher accuracy was obtained compared with phenotypic methods[6,7].

MATERIALS AND METHODS

Patients and materials

A total of 104 patients, who underwent upper gastrointestinal endoscopy because of dyspeptic symptoms and yield positive results in rapid urease test (RUT), were selected for the further *H. pylori* detection and bacterial examinations. Among the 104 patients, 103 were *H. pylori* positive and ultimately entered into this study, including 63 from Jiangxi Province and 40 from Beijing, China. No subject had received anti-secretory drugs, antibiotics, or probiotics two wk prior to entry into this study and no one had *H. pylori* eradication history. Diseases included gastritis (n = 23), gastric ulcer (n = 21), duodenal ulcer (n = 42), reflux esophagitis (n = 4), and non-ulcer dyspepsia (NUD) (n = 13). There were 81 (78.6%) patients aged less than 50 years. The study was reviewed and approved by the Ethics Committee of the National Institute for Communicable Disease Control and Prevention. Each subject gave informed oral consent before entering into the study.

Upper gastrointestinal endoscopy was performed after an overnight fast in all patients. Mucosal samples were taken from the greater curvature of gastric antrum and corpus using sterile disposable biopsy forceps. All the samples were placed into sterile brain-heart infusion broth for transport to the cultural laboratory.

Bacterial culture and polymerase chain reaction detection for H. pylori

After biopsies were taken, the samples were dispersed using a homogenizer. Each homogenate was inoculated onto three plates, two Columbia blood agars (CM0331, OXOID) and a Campylobacter agar (CM0935, OXOID) supplemented with 5% sheep blood, polymyxin B, vancomycin, trimethoprim, and amphotericin B. One of the Columbia blood agar was incubated under aerobic condition at 37 ℃, the other one and the Campylobacter agar were incubated under microaerophilic condition at 37 ℃.

The *H. pylori* status was assessed with culture and polymerase chain reaction (PCR) detection. Small (0.5 to 2 mm) translucent colonies were selected to gram-strain and tested for urease, catalase and oxidase activity. Microscopy of gram-stained smears with curved gram-negative rods resembling *Helicobacter,* together with positive in all the tree enzyme activity tests were identified as *H. pylori.*

H. pylori culture-negative samples were further detected by *ureA*, *vacA* and *cagA* PCR analysis, using the primers described previously[8,9]. *H. pylori* status was defined as negative only if all the three PCR detections were negative.

Mass spectrometry identification of non-H.pylori bacteria

Preparation of samples: Appropriate amount (5-10 mg) colony was scraped by inoculating loop and suspended in 300 μL distilled water, and 900 μL ethanol was added and mixed. Then the sample was centrifuged at 12 000 r/min for 2 min, and the pellets were dried. Fifty μL formic acid (70% in water) was added to the dried bacterial pellet and mixed thoroughly, and then added with 50 μL acetonitrile. After centrifugation at 12 000 r/min for 2 min, 1 μL of the supernatant containing the bacterial extract was transferred onto the MSP 96 target ground steel plate (Bruker Daltonics, Bremen, Germany) and dried, then 1 μL of matrix solution (satured solution of a cyano-4-hydroxycinnamic acid in 50% acetonitrile + 2.5% trifluoroacetic acid) was added and crystallized by air-drying at room temperature. Each isolate was analyzed on the day of isolation.

Measurement with spectrometer: Measurement was performed with Microflex LT mass spectrometer (Bruker Daltonics) equipped with a 200 Hz smart-beam laser. The parameter settings were as follows: delay 320ns; ion source 20 kV; ion source 18.5 kV; lens voltage 8.5 kV; and mass range 2-15 kDa. Each run was validated with an *E. coli* control sample where the presence of 10 specific proteins insured that the spectrometer was set properly. Raw spectra of the strains were analyzed by MALDI Biotyper 2.0 software (Bruker Daltonics) using the default settings. A list of peaks up to 100 was generated. The threshold for peak acceptance was a signal-to-noise (S/N) ratio of 3. After alignment, peaks with a mass-to charge (m/z) ratio difference of less than 250 ppm were considered to be identical. The peak lists generated were used for matches against the reference library, by directly

Table 1 Non-*Helicobacter pylori* bacterial cultures of gastric biopsies

Micro-organism	Culture condition[1]			Colonized patients (*n*)[2]	Urease activity[3]	Gram stain
	Ⅰ	Ⅱ	Ⅲ			
Streptococcus				(54)		G+
Streptococcus pneumoniae	3	10		11		
Streptococcus salivarius	10	9		16		
Streptococcus anginosus		3		3		
Streptococcus oralis		2		2		
Streptococcus cristatus	1			1		
Streptococcus gordonii	2			2		
Streptococcus vestibularis	1			1		
Streptococcus spp.	10	13		18		
Neisseria				(32)		G-
Neisseria flavescens	11	16	11	23		
Neisseria perflava	2	1	1	3		
Neisseria macacae		1		1		
Neisseria sicca	1			1		
Neisseria spp.	3	1		4		
Rothia				(29)		G+
Rothia mucilaginosa	6	10		15		
Rothia dentocariosa		3	1	4		
Rothia aeria	1			1		
Rothia spp.	4	7		9		
Staphylococcus				(21)		G+
Staphylococcus epidermidis	3	3		5	5	
Staphylococcus aureus	3	4		5	3	
Staphylococcus capitis		3		3	1	
Staphylococcus warneri	2	1		3	3	
Staphylococcus cohnii		2		2		
Staphylococcus haemolyticus		1		1		
Staphylococcus spp.		2		2		
Lactobacillus				(9)		G+
Lactobacillus salivarius			4	4		
Lactobacillus oris			2	2		
Lactobacillus fermentum			1	1		
Lactobacillus spp.		1	1	2		
Corynebacterium				(4)		G+
Corynebacterium argentoratense	2	1		3		
Corynebacterium propinquum	1	1		1		
Kingella				(3)		G-
Kingella denitrificans			2	2		
Kingella spp.			1	1		
Others						
Capnocytophaga ochracea			3	3		G-
Haemophilus parainfluenzae		1	1	2		G-
Acinetobacter lwoffii	2			2		G-
Klebsiella pneumoniae	1			1	1	G-
Cardiobacterium sp			1	1		G-
Actinomyces spp.		2		2		G+
Micrococcus luteus	1			1		G+
Weissella confusa			1	1		G+
Aerococcus spp.	1			1		G+
Bacillus spp.	1			1		G+
Brevibacterium spp.		1		1	1	G+
Total	72	99	30	168		

[1]The number of bacterial strains isolated in Ⅰ: Columbia blood agar incubated under aerobic condition; Ⅱ: Columbia blood agar incubated under microaerophilic condition; Ⅲ: Campylobacter agar under microaerophilic condition; [2]The number of patients colonized by the same genus is shown in parentheses; [3]The number of bacterial isolates with urease activity.

using the integrated pattern matching algorithm of the software. All parameters were the same regardless of the bacteria analyzed. Spectra were obtained in the positive linear mode after 500 shots. A score was attributed to each identification. When this score was > 2.00, the identification was considered correct at the species level; between 1.7 and 1.999, the identification was considered correct at the genus level; and < 1.7, the identification was not similar enough to draw a conclusion.

Urease activity test of non-H. pylori bacteria

Each non-*H. pylori* bacterial culture was subjected to urease test. The medium used for the test was urea broth containing dipotassium hydrogen phosphate, 9.5 g/L; potassium dihydrogen phosphate, 9.1 g/L; urea 20 g/L; phenol red, 0.01 g/L; and yeast extract, 0.1 g/L, final pH 6.8. Any change in the indicator from pale yellow to pink in 24 h was taken as positive.

Statistical analysis

Continuous data were described with mean (minimum, maximum), categorical data with number and proportions. Difference between the groups for age and sex was analyzed using analysis of variance and χ^2 test. Fisher exact test was conducted to compare non-*H. pylori* prevalence data. *Post hoc* tests were conducted by the Bonferroni method. $P < 0.05$ was considered as statistically significant. All analyses were performed with SPSS 12.0 software.

RESULTS

A total of 92 (88.5%) samples were *H. pylori* culture positive. Only one of the 12 culture-negative cases was PCR negative. As result, 103 *H. pylori* positive patients were eventually enrolled into the analysis.

Non-H. pylori bacteria isolation

A total of 201 non-*H. pylori* bacterial isolates were cultivated from gastric samples of 67 (65.0%) of the 103 *H. pylori* positive patients. All the isolates were identified by mass spectrometry, including 153 identified at species level and 48 at genus level (Table 1). Some species isolated from more than two culture plates were shown in Table 1. A total of 168 isolates was obtained with the exception of the repeat identification of the same patient, and 39 patients harbored more than 2 non-*H. pylori* species. Overall, a total of 18 non-*H. pylori* bacterial genera (43 species) were isolated from the stomach biopsy specimen (Table 1), most of them were gram-positive bacteria (73.8%). The dominant bacterial species were *Neisseria flavescens* (13.7%), *Streptococcus salivarius* (9.5%), *Rothia mucilaginosa* (8.9%) and *Streptococcus pneumonia* (6.6%). Twelve gram-negative bacilli isolates (7.14%) belonged to 6 genera obtained in this study, which were discovered from 10 patients (9.7% of all the patients and 14.9% of the non-*H. pylori* positive patients). Concurrent colonization by gram-positive and/or gram-negative

Table 2 Demographic, non-*Helicobacter pylori* bacterial colonization and clinical characteristics of 103 patients

Diseases	Enrolled patients (*n*)	Age (yr)[1]	Gender (male:female)	Colonized patients
Duodenal ulcer	42	39 (20-56)	2:1	28 (66.7%)
Gastric ulcer	21	42 (20-62)	0.75:1	9 (42.9%)
Gastritis	23	39 (10-75)	0.44:1	13 (56.5%)
Non-ulcer dyspepsia	13	34 (22-57)	0.63:1	13 (100%)[a]
Reflux esophagitis	4	52 (41-63)	1:1	4 (100%)
Total	103	39 (10-75)	0.96:1	67 (65.0%)
P value		0.079[2]	0.074[3]	0.0028[4]

[1]Data are expressed as median (range); [2]By analysis of variance; [3]By χ^2 test; [4]By Fisher exact test; [a]P = 0.00006 *vs* gastric ulcer group by Bonferroni method.

coccus occurs in 10 of them.

The demographic and relevant clinical characters of the patients are described in Table 2. The patients were subdivided into five groups based on the clinical diseases. There were no significant intergroup differences in age and sex ratio, but the prevalence of non-*H. pylori* flora was significantly different (P = 0.0028) among different clinical diseases, as shown in Table 2. *Post hoc* tests were conducted and statistical difference was found between the gastric ulcer group (42.9%) and the NUD group (100%) (P = 0.00006). In addition, significant difference was also presented between the 50-plus age group and the younger age (< 50 years old) group (45.5% *vs* 69.1%, χ^2 test, P = 0.048).

Urease activity of non-H. pylori isolates

All the 201 non-*H. pylori* isolates were screened for urease activity, 14 isolates belonged to 6 species (*S. epidermidis*, *S. warneri*, *S. capitis*, *S. aureus*, *Brevibacterium spp.* and *K. pneumoniae*) and were urease positive (Table 1), which could benefit in the bacterial overgrowth in the stomach. Interestingly, not all the isolates of the 6 species were urease-positve except *S. warneri*. Especially in a 26-year-old patient with non-ulcer dyspepsia, two *Staphylococcus epidermidis* isolates were isolated under aerobic and microaerobic conditions, respectively, but only the microaerobic isolate was urease-positive.

DISCUSSION

It has been suggested that non-*H. pylori* bacteria and their by-products may act as a persistent antigenic stimulus, and thereby augment the inflammatory response induced by the *H. pylori* infection[4]. The current study documented a high prevalence (65.0%) of the non-*H. pylori* bacterial flora in the *H. pylori* positive patients, which indicate that the upper gastrointestinal disorders may enable non-*Helicobacter* bacteria to survive and colonize in the human stomach. Further studies should be done to elucidate the role of the co-infection of the stomach with *H. pylori* and non-*H. pylori* bacteria in the pathogenesis process.

Most of the non-*H. pylori* bacteria isolated in this study were upper respiratory tract microflora, which was in agreement with other researches[10]. The major species were *Streptococcus, Neisseria, Rothia* and *Staphylococcus*, that was different from the previous reports of healthy volunteers, which was predominantly acid resistant species - *Veillonella sp*, *Lactobacillus sp*, and *Clostridium sp*[11].

Gram-negative bacilli are uncommon in the upper gut of healthy individuals, but in patient populations with gastric hypochlorhydria, gram-negative bacilli are recovered in a minor proportion[12,13]. Gram-negative bacilli were discovered in 9.7% of the patients in this study. Concurrent colonization by gram-positive and/or gram-negative coccus occurs in 10 of the 12 gram-negative bacilli. *Acinetobacter lwoffii* was the only gram-negative bacilli species that was found as the only non-*H. pylori* bacteria in the patients. The two *A. lwoffii* carriers were a 42-year-old male patient with duodenal ulcer and a 25-year-old female patient with non-ulcer dyspepsia. *A. lwoffii* has been proved to cause the same histologic gastritis as *H. pylori* infection in a murine model[14]. And outer membrane protein (OmpA-like protein) of *A. lwoffii* was reported to activate interleukin 8 and gastrin promoter activity *in vitro*[15]. Moreover, a case of *A. lwoffii* bacteremia associated with acute gastroenteritis was reported recently[16], which stimulate more interest in the contribution of *A. lwoffii* to gastrointestinal diseases.

It has been well known that gastric acid secretion declines with age which may increase the sensitivity of the stomach to the bacterial colonization. Surprisingly, a higher non-*H. pylori* prevalence was found in the younger age group (less than 50 years old, P = 0.048). The result must be interpreted with caution.

The pathogenesis of NUD was not well understood and a number of hypotheses have been proposed. The role of *H. pylori* infection in NUD remains controversial too[17]. A high prevalence of non-*H. pylori* bacteria in NUD group observed in this study gave us a new clue that non-*H. pylori* bacteria may play a role in the pathogenesis of NUD.

Urease is a major virulent factor for *H. pylori*, which aids in neutralizing hydrochloric acid and allows *H. pylori* to colonize the gastric mucosa. Some rapid methods for *H. pylori* diagnosis, [13C]urea breath test (UBT) and RUT, were also based on the urease activity. But *H. pylori* is not the only bacteria which can produce urease. It has been reported that the colonization and overgrowth of urease-producing bacteria other than *H. pylori* induced false-positive UBT results[18,19]. All the 104 patients were RUT-positive, but one of them was *H. pylori*-negative based on the further detection and no urease-positive non-*H. pylori* bacteria was isolated. So role of the urease-positive non-*H. pylori* bacteria to cause false-positive results in RUT was not definite in this study.

Overall, a high prevalence of non-*H. pylori* bacteria obtained in this study, the major of which were *Streptococcus, Neisseria, Rothia* and *Staphylococcus*, differed from the previous report of healthy volunteers. It should be noted that this study only examined cultured bacteria. But not all flora can be cultivated due to the harsh culture condi-

tion, so the prevalence of the non-*H. pylori* bacteria may underestimate here. The remained question is whether these non-*H. pylori* species contribute to the pathogenesis of gastric disorders. Further studies should be done to elucidate it in the future.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection has been known to trigger a variety of gastric disorders. Recent years, non-*H. pylori* bacteria have also been reported to play roles in the development of upper gastrointestinal disease, but little is known about the stomach bacterial flora. Analysis of stomach bacterial flora was performed in order to stimulate future research in this area.

Research frontiers

The pathogenesis of gastric disorders is complicated. Besides *H. pylori*, considerable interest has emerged in the co-infection in the stomach with non-*H. pylori* bacteria.

Innovations and breakthroughs

To date, there has only a limited number of studies regarding the stomach bacterial flora and all are based on a few patients or volunteers. In this study, the authors enrolled 103 *H. pylori* positive patients with upper gastrointestinal diseases and employed forefront matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) bacterial identification technique, which is more accurate and rapid than the routine methods or genotypic identification. Furthermore, the authors confirmed a high prevalence of the non-*H. pylori* bacteria concurrent with *H. pylori* in the patients.

Applications

A high prevalence of the non-*H. pylori* bacteria and the evaluation of the bacterial species found in gastric tissues from *H. pylori* infected patients would stimulate future research to elucidate the role of the mixed infection of the stomach in the pathogenesis of the upper gastrointestinal diseases.

Terminology

MALDI-TOF MS fingerprinting is a fast and reliable method for the classification and identification of micro-organisms. The BioTyper™ MALDI-TOF MS fingerprinting system allows researchers to perform this process for the unambiguous identification of bacteria, yeasts and fungi in minutes by measuring the exact sizes of peptides and small proteins, which are assumed to be characteristic for each species.

Peer review

The study provided an interesting evaluation of the bacterial species found in gastric tissues from *H. pylori* infected persons.

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BRIEF ARTICLE

Gastric cancer incidence and mortality in Zhuanghe, China, between 2005 and 2010

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Abstract

AIM: To investigate the incidence and mortality of gastric cancer (GC) in Zhuanghe region, northeast China and the influencing factors for their changing trends.

METHODS: All new cancer cases and deaths registered from 2005 to 2010 in Zhuanghe County were reviewed. The annual GC cases, constituent ratio, crude rates, age-standardized rates, their sex and age distribution and temporal trends were assessed. The method of annual percentage change (APC) was used to estimate the trends of GC.

RESULTS: Altogether 2634 new cases of GC and 1722 related deaths were registered, which accounted for 21.04% and 19.13% of all cancer-related incidence and deaths, respectively. The age-standardized incidence rate steadily decreased from 57.48 in 2005 to 44.53 in 2010 per 10^5 males, and from 18.13 to 14.70 per 10^5 females, resulting in a APC of -5.81% for males and -2.89% for females over the entire period. The magnitude of APC in GC mortality amounted to -11.09% and -15.23%, respectively, as the age-standardized mortality rate steadily decreased from 42.08 in 2005 to 23.71 in 2010 per 10^5 males, and from 23.86 to 10.78 per 10^5 females. Females had a significantly lower incidence (a male/female ratio 2.80, $P < 0.001$) and mortality (a male/female ratio 2.30, $P < 0.001$). In both genders, the peak incidence and mortality occurred in the 80-84 years age group. The age-standardized mortality/incidence ratio also decreased from the peak of 0.73 in 2005 to 0.53 in 2010 for males, and from 1.32 to 0.73 for females.

CONCLUSION: Encouraging declines of incidence and mortality of GC were observed in Zhuanghe region between 2005 and 2010, possibly due to the economic development and efficient GC control strategies.

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Key words: Gastric cancer; Incidence; Mortality; Trend; High-risk areas

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INTRODUCTION

Gastric cancer (GC) is one of the most frequently occurring cancers globally; a total of 989 600 new GC cases and 738 000 deaths are estimated to have occurred in 2008, accounting for 8% of the total cases and 10% of total cancer-related deaths, respectively[1]. The geographical distribution of GC exhibits wide international variation and over 70% of new cases and deaths occur in developing countries[1,2]. Although a notable decreasing trend has been recently observed in the developed countries, the incidence of GC is still high[3] and it remains an important public health burden in China. Moreover, there is a significant regional disparity in the distribution of GC in China. People in rural areas are supposed to have a higher risk of GC than those in cities[4], thus, strategy of GC control in China should be implemented specifically based on the different distribution patterns.

Zhuanghe is a county of northern China with a total population of approximate 900 000, of whom approximately 87.2% are agricultural residents. It is located along the coast of the Huanghai Sea and occupies an area of approximately 4034 km^2[5]. Zhuanghe has been recognized for its high GC mortality since 1984, the mortality rate being $49.55/10^5$ in males and $22.23/10^5$ in females[6]. The high risk of GC in this region aroused the interest of researchers, and a series of investigations regarding the potential risk factors and corresponding etiological intervention methods and screening measures were conducted in representative villages[7]. To date, the population in Zhuanghe County has been surveyed through census for 28 years. Various risk factors, such as salted pork, *Helicobacter pylori* (*H. pylori*) infection with a specific strain, and genetic polymorphisms in the etiology of GC in local residents have been found to play a role[8-12]. In addition, several pilot surveys regarding incidence and mortality were also conducted. The GC mortality rates fluctuated between $63.29/10^5$ and $38.98/10^5$ during the period of 1996-2003 and the incidence rate was $35.42\text{-}41.03/10^5$ from 1998 to 2004[7,13,14].

Despite some epidemiological surveys in pilot areas, a population-based investigation concerning the incidence and mortality of GC in Zhuanghe has not been previously reported. Monitoring and studying the incidence as well as mortality provides important information, enabling effective assessment of potential cancer prevention and control. Therefore, the aim of the present study is to examine the GC patterns and temporal trends of incidence and mortality at the population level in Zhuanghe region and to elucidate the factors that influence the changing trends, in an attempt to establish future general and specific strategies for the prevention and control of cancers.

MATERIALS AND METHODS

Cancer registry

The preliminary cancer surveillance was conducted in Zhuanghe as a high-risk GC research base in 1984. However, previous cancer data were obtained from limited populations and sample surveys. Over the past two decades, a cancer registry was developed along with an expanding research scope, with coverage of only several villages initially in the 1980s which increased to 70 villages in the 1990s. Since 2001, a population-based cancer registry has been gradually established, in collaboration with the Cancer Registration Office of Liaoning Province, China that has collected information on all deaths and all cancer cases in Zhuanghe County and has functioned well since 2005.

The framework of this registry consisted of three parts: village clinics that served as basic units, rural administration units, and Center of Disease Prevention and Control (CDC) of Zhuanghe County. Village clinic doctors were required to report each new case of cancer using a standard card to the rural administration unit. The cards were then submitted to the CDC of Zhuanghe, registered and processed on a regular basis. These cards were checked, analyzed, coded and stored at the CDC. All data were coded according to the manual "National Criteria of Cancer Registry in China", which describes the cancer inclusion and exclusion criteria, and provides definitions and coding for cancer cases. The accuracy of the data was assured through the comprehensive training of doctors and registrars by the Cancer Registration Office of Liaoning Province, and by computerized consistency checks. At the end of each year, a sample survey was conducted to check the quality of the registration. The cancer occurrence data were compared one by one with the death registry database data to supplement cases of cancer death which were not registered for cancer occurrence.

Data collection

Annual GC cases data were provided by the CDC of Zhuanghe County. All data were coded and checked for eligibility and validity prior to analysis. Data collected on each patient included demographics and native origins, primary cancer site/type, morphology, and pathological diagnosis confirmation, stage of the disease, and active patient follow-up. The 10th revision of International Classification of Diseases (ICD-10) was used for cancer classification. Data concerning age and sex in the Zhuanghe population were provided by the local police station.

Statistical analysis

All of the data were checked and analyzed by SPSS 13.0 software (SPSS, Chicago, Illinois, United States). Incidence and mortality rates were calculated as the mean annual number per 100 000 residents. Age-standardized

Table 1 The top ten malignant tumors for incidence in Zhuanghe area, 2005-2010

Rank	Total				Males				Females			
	Cancer	ICD-10	Cases	CR %	Cancer	ICD-10	Cases	CR%	Cancer	ICD-10	Cases	CR %
1	LC	C33-34	2673	21.35	GC	C16	1950	26.41	LC	C33-34	1068	20.80
2	GC	C16	2634	21.04	LC	C33-34	1605	21.74	GC	C16	684	13.32
3	HCC	C22	1488	11.89	HCC	C22	1093	14.80	BC	C50	617	12.02
4	CRC	C18-21	1211	9.67	CRC	C18-21	719	9.74	CRC	C18-21	492	9.58
5	BC	C50	636	5.08	EC	C15	322	4.36	TC	C73	234	4.56
6	EC	C15	364	2.91	BLC	C67	246	3.33	CC	C53	212	4.13
7	TC	C73	317	2.53	PC	C25	151	2.05	EMC	C54	135	2.63
8	BLC	C67	286	2.28	CNC	C70-72	135	1.83	CNC	C70-72	111	2.16
9	PC	C25	253	2.02	PRC	C61	85	1.15	OC	C56	105	2.05
10	CNC	C70-72	246	1.97	KC	C64	83	1.12	PC	C25	102	1.99
	Total		10108	80.74	Total		6389	86.53	Total		3760	73.24

CR: Constituent ratio; ICD-10: The 10th revision of International Classification of Diseases; GC: Gatric cancer; LC: Lung cancer; HCC: Liver cancer; CRC: Colon/rectum cancer; BC: Breast cancer; EC: Esophageal cancer; TC: Thyroid cancer; BLC: Bladder cancer; PC: Pancreatic cancer; CNC: Cancers of brain and nervous system; PRC: Prostate cancer; KC: Renal cancer; CC: Cervical cancer; EMC: Endometrial carcinoma; OC: Ovarian cancer.

incidence rates (ASRs) and age-standardized mortality rates (ASMRs) were calculated using a direct method[15] by weighting age-specific incidence and mortality rates in accordance with the World Standard Population (World Health Organization, published in 2000) and the China Standard Population (2000). The Chi-squared test was used to determine whether the differences between sexes were statistically significant, and the test was also used to examine trends of annual change in crude rates.

The average annual percent change (APC)[16,17] was estimated by fitting a regression line to the natural logarithm of the rates using the calendar year as the regression variable, i.e., y = mx + b in which y = ln (rate) and x = calendar year. The estimated APC = 100 (e^{m}-1) and 95% CI of APC = 100 ($e^{m \pm SEm}$-1), where the standard error of m (SEm) was obtained according to the fit of the regression line. To test the hypothesis that the APC equals zero is equivalent to the hypothesis that the slope of the regression line is zero, the t-distribution of m/SEm was used. The number of degrees of freedom is equivalent to the number of calendar years minus two. This calculation assumes that the rate increased/decreased at a constant rate over the entire period. Statistical significance was assessed by the two-sided Student's *t* test, and $P < 0.05$ was considered statistically significant.

Ethical consideration

Ethical approval for this study was obtained from the Human Ethics Review Committee of China Medical University (Shenyang, China). Written informed consents were obtained from the participants in accordance with the Declaration of Helsinki and its later revisions.

RESULTS

Incidence

From 2005 to 2010, a total of 2634 new cases of GC were registered in Zhuanghe, including 1950 males and 684 females. We analyzed the constituent ratios for incidence according to various types of cancer (Table 1). GC ranked first among all types of cancers in males, whereas it ranked the second among females and both sexes combined. The ten most common types of cancer in the Zhuanghe population were lung, stomach, liver, colon/rectum, breast, esophageal, thyroid, bladder, pancreatic cancers, and cancer of brain and nervous system. These cancers comprised 80.74% of all cases of cancer. The five predominant primary cancer sites in males were stomach, lung, liver, and esophagus; whereas in females, the leading sites were lung, stomach, breast, colon/rectum, and thyroid.

The incidence rate of GC occurring over different years was analyzed. We initially calculated the annual crude incidence rate according to sex. The average crude incidence rate of GC in males was $70.23/10^5$, which was 2.80-fold higher than in females ($25.12/10^5$). The difference was statistically significant ($\chi^2 = 584.31$, $P < 0.001$). Over the past 6 years, no obvious increasing or decreasing trend was observed concerning crude incidence (P = 0.404 in males, 0.061 in females and 0.101 in both sexes) (Table 2).

To adjust for the effect of age differences over different time periods, age-standardized incidence rates were calculated in accordance with the China Standard Population (ASR1) and World Standard Population (ASR2). As shown in Figure 1, over the past 6 years, the incidence rate of GC has gradually decreased. For both genders, the ASR2 decreased from 37.67 to 29.83 per 10^5 with an APC of -4.82% (95% CI: -3.58% to -6.04%; P = 0.019) and for males the rate decreased from 57.48 to 44.53 per 10^5 with an APC of -5.81% (95% CI: -4.67% to -6.94%; P = 0.008). The ASR2 decreased from 18.13 to 14.70 per 10^5 with an APC of -2.89% (95% CI: 0.06% to -5.75%) in females; however, the change had no statistical significance (P = 0.383) (Table 2).

In both genders, GC was very rare among populations younger than 20 years, but the rate rose sharply to a peak at approximately 80 years of age, and it declined afterwards. The median age of GC patients was 67 years and the average age was 65.78 years. The cases above 50

Table 2 Time trends for incidence rates of gastric cancer in Zhuanghe population, 2005-2010 (/100 000)

Year	Total				Males				Females			
	Cases	Crude	ASR1	ASR2	Cases	Crude	ASR1	ASR2	Cases	Crude	ASR1	ASR2
2005	409	44.74	33.41	37.67	312	67.51	47.92	57.48	97	21.46	16.97	18.13
2006	437	47.62	34.89	40.12	323	69.63	48.01	60.06	114	25.12	19.30	20.66
2007	411	44.60	32.88	37.77	314	67.42	46.89	57.86	97	21.28	16.68	18.17
2008	474	51.36	31.65	35.30	346	74.26	42.69	51.05	128	28.01	18.53	19.60
2009	476	51.98	30.55	34.04	337	72.97	39.58	47.66	139	30.63	19.53	20.30
2010	427	47.03	26.84	29.83	318	69.55	38.46	44.53	109	24.18	13.67	14.70
Total	2634	47.89	31.25	35.10	1950	70.23	43.28	51.95	684	25.12	17.17	18.26
χ^2 trend test	χ^2 = 2.693, P = 0.101				χ^2 = 0.696, P = 0.404				χ^2 = 3.519, P = 0.061			
APC	APC = -4.82%, P = 0.019				APC = -5.81%, P = 0.008				APC = -2.89%, P = 0.383			

Crude: The crude incidence rate; ASR1: The age standardized incidence of Chinese population in 2000; ASR2: The age standardized incidence of the world population in 2000; APC: Average annual percent change of incidence rates.

Table 3 The top ten malignant tumors for mortality in Zhuanghe area, 2005-2010

Rank	Total				Males				Females			
	Cancer	ICD-10	Cases	CR %	Cancer	ICD-10	Cases	CR %	Cancer	ICD-10	Cases	CR%
1	LC	C33-34	2683	29.81	LC	C33-34	1560	27.89	LC	C33-34	1123	32.95
2	GC	C16	1722	19.13	GC	C16	1207	21.58	GC	C16	515	15.11
3	HCC	C22	1623	18.03	HCC	C22	1180	21.10	HCC	C22	443	13.00
4	CRC	C18-21	544	6.04	CRC	C18-21	305	5.45	CRC	C18-21	239	7.01
5	EC	C15	290	3.22	EC	C15	245	4.38	BC	C50	179	5.25
6	CNC	C70-72	289	3.21	CNC	C70-72	158	2.82	CNC	C70-72	131	3.84
7	PC	C25	252	2.80	PC	C25	143	2.56	LK	C91-95	118	3.46
8	LK	C91-95	251	2.79	LK	C91-95	133	2.38	PC	C25	109	3.20
9	BC	C50	185	2.06	BLC	C67	95	1.70	EMC	C54	101	2.96
10	LNC	C77	151	1.68	LNC	C77	93	1.66	LNC	C77	58	1.70
	Total		7990	88.77	Total		5119	91.52	Total		3016	88.48

CR: Constituent ratio; ICD-10: The 10th revision of International Classification of Diseases; GC: Gastric cancer; LC: Lung cancer; HCC: Liver cancer; CRC: Colon/rectum cancer; BC: Breast cancer; EC: Esophageal cancer; BLC: Bladder cancer; PC: Pancreatic cancer; CNC: Cancers of brain and nervous system; EMC: Endometrial carcinoma; LK: Leukemia; LNC: Secondary malignant tumors of lymph nodes.

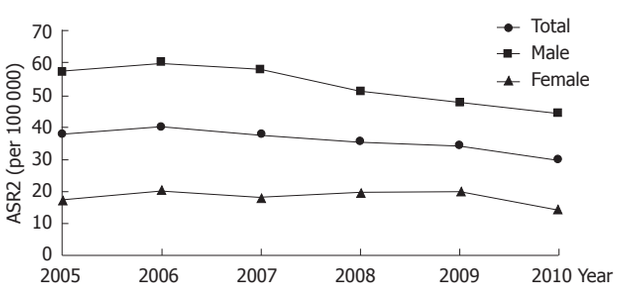

Figure 1 World standardized incidence rates of gastric cancer in Zhuanghe County during 2005-2010. ASR2: The age standardized incidence of the world population in 2000.

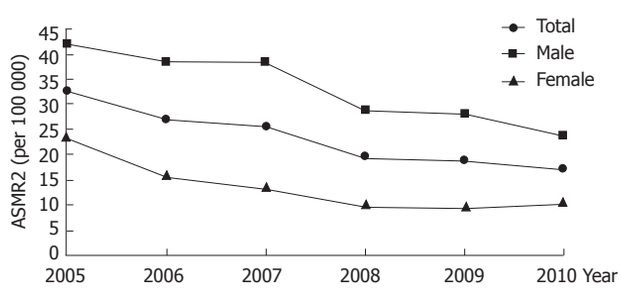

Figure 2 World standardized mortality rates of gastric cancer in Zhuanghe County during 2005-2010. ASMR2: The age standardized mortality of the world population in 2000.

years old accounted for 88.65% of all GC cases.

Mortality

There were a total of 1722 GC deaths between 2005 and 2010 in Zhuanghe, including 1207 males and 515 females. Table 3 shows the constituent ratio of malignant tumors leading to death, in which GC accounted for 19.13% (21.58% in males and 15.11% in females) of the total cancer deaths, making it the second leading cause of cancer death.

The crude mortality rate during this period due to GC in Zhuanghe was 31.31 per 10^5; 43.47 per 10^5 in males and 18.91 per 10^5 in females. The difference in sex-specific incidence rates was statistically significant (χ^2 = 264.82, P < 0.001); a higher rate was observed in males than in females with a ratio of 2.30. A decreasing trend in crude mortality rate was observed over the past 6 years (P < 0.001 in both sexes; P = 0.046 in males, P < 0.001 in females) (Table 4).

A progressive decrease in the world age-standardized mortality rate was noted in both genders (Figure 2 and Table 4): ASMR2 decreased steadily from 42.08/10^5 in

Table 4 Time trends for mortality rates of gastric cancer in Zhuanghe population, 2005-2010 (/100 000)

Year	Total				Males				Females			
	Cases	Crude	ASMR1	ASMR2	Cases	Crude	ASMR1	ASMR2	Cases	Crude	ASMR1	ASMR2
2005	352	38.51	27.94	32.74	224	48.47	33.31	42.08	128	28.32	21.75	23.86
2006	293	31.93	23.11	27.00	204	43.97	30.16	38.67	89	19.62	14.55	15.91
2007	276	29.95	21.79	25.78	202	43.37	29.26	38.55	74	16.24	12.50	13.54
2008	268	29.04	17.06	19.61	199	42.71	23.22	29.02	69	15.10	9.54	10.32
2009	272	29.71	16.20	18.74	202	43.74	21.82	28.07	70	15.42	8.99	9.52
2010	261	28.74	14.92	17.28	176	38.50	18.86	23.71	85	18.86	9.92	10.78
Total	1722	31.31	19.54	22.65	1207	43.47	25.42	32.18	515	18.91	12.35	13.32
χ^2 trend test	χ^2 = 12.949, P < 0.001				χ^2 = 3.965, P = 0.046				χ^2 = 12.736, P < 0.001			
APC	APC = -12.23%, P = 0.001				APC = -11.09%, P = 0.002				APC =-15.23%, P = 0.015			

Crude: The crude mortality rate; ASMR1: The age standardized mortality of Chinese population in 2000; ASMR2: The age standardized mortality of the world population in 2000; APC: Average annual percent change of mortality rates.

Table 5 Age-specific incidence and mortality of gastric cancer in Zhuanghe population (/100 000)

Age (yr)	Incidence			Mortality		
	Males	Females	Total	Males	Females	Total
0-4						
5-9						
10-14						
15-19				0.68	0	0.33
20-24	0.61	0.57	0.59	0	0	0
25-29	2.49	1.97	2.23	0.99	0.99	0.99
30-34	2.15	5.01	3.54	2.15	3.19	2.65
35-39	12.08	5.49	8.85	3.02	3.53	3.27
40-44	23.34	9.93	16.65	11.32	4.97	8.15
45-49	35.80	12.46	24.15	14.61	8.43	11.52
50-54	70.78	28.85	50.01	29.64	21.18	25.45
55-59	130.08	50.15	91.74	57.05	29.10	43.65
60-64	169.90	62.55	119.25	85.33	35.50	61.82
65-69	280.88	87.94	186.18	169.55	56.15	113.89
70-74	369.88	123.30	245.56	245.76	80.57	162.48
75-79	474.45	128.06	296.01	373.28	116.57	241.04
80-84	573.56	183.05	367.92	453.11	206.25	323.12
85+	341.65	129.97	224.60	414.00	175.46	282.10

2005 to 23.71/10^5 in 2010 for males, and from 23.86/10^5 to 10.78/10^5 for females, with an average reduction of -11.09% for males (95% CI: -9.65% to -12.50%; P = 0.002) and -15.23% for females (95% CI: -11.71% to -18.62%; P = 0.015) per year. In general, ASMR2 decreased from 32.74 to 17.28 per 10^5 with an APC of -12.23% (95% CI: -10.94% to -13.90%; P = 0.001).

The minimum age group for GC death was 15-20 years. With increasing age, the mortality rate increased sharply, reaching a peak at approximately 80 years of age and declining thereafter. The median age was 71.00 years and the average age was 69.17 years. The cases in subjects over 50 years of age accounted for 91.70% of all deaths (Table 5).

In addition, we calculated the age-standardized mortality-incidence ratio (M/I ratio: ASR2/ASMR2) during different time periods. The M/I ratio decreased from 0.87 to 0.58 over a 6-year period. The worst ratio was recorded in 2005: 0.73 for males and 1.32 for females, followed by a steady decrease over the subsequent years with the M/I ratio falling to 0.53 for males and 0.73 for females in 2010 (Table 6).

Table 6 Mortality to incidence ratio of gastric cancer

Year	Age-standardized mortality to incidence ratio[1]		
	Total	Male	Female
2005	0.87	0.73	1.32
2006	0.67	0.64	0.77
2007	0.68	0.67	0.75
2008	0.56	0.57	0.53
2009	0.55	0.59	0.47
2010	0.58	0.53	0.73
Total	0.65	0.62	0.73

[1]The age-standardized mortality to incidence ratio was computed according to ASR2 and ASMR2. ASR2: The age standardized incidence of the world population in 2000; ASMR2: The age standardized mortality of the world population in 2000.

DISCUSSION

GC is one of the most common cancers worldwide. There is substantial geographic variation regarding the incidence and mortality of GC. Studies specifically examining the population in high-risk areas should be very useful in potentially controlling this disease. Zhuanghe County, which is situated in the coastal area of the eastern Liaoning peninsula, is a rural area exhibiting a high GC mortality in north China. A series of epidemiological investigations and comprehensive preventive measures for GC have been conducted since 1984. Surveillance of cancer incidence and mortality rates has provided a guideline to identify the etiology and aid in the evaluation of the impact of intervention on the population. Our previous sampling data indicated the initial effects of GC intervention. However, the lack of an overall picture of GC remains due to the unavailability of cancer registry data covering the entire region prior to 2005. In this study, we reviewed the data regarding the incidence and mortality of GC in Zhuanghe County during the period of 2005-2010 based on the population-based registry in order to analyze the changing trends and to elucidate the underlying causes.

The results about the organ distribution of all can-

cers in Zhuanghe indicated that 80.74% of new cases originated from ten primary organ sites, the two major sites being lung (21.35%) and stomach (21.04%). Moreover, the ten major cancers accounted for 88.77% of all cancer-related deaths, with lung and stomach as the top two positions (29.81% and 19.13%). GC was the most common type of cancer in males and the second most common in females in the Zhuanghe region, and was the second leading cause of cancer death for both genders. All of the data indicated that GC remained a major health burden for the local population.

Globally, the incidence rate of GC is nearly twice as high in males as in females[1]; the male-to-female incidence ratio in Zhuanghe county was 2.80 (χ^2 = 264.82, P < 0.001), and the mortality of GC in males was 2.30 times higher than in females (χ^2 = 584.31, P < 0.001). There were gender differences in many types of cancer, which may be related to the different risk level for exposure to diet, smoking, drinking and occupation[18]. Investigation of genetic factors together with other intrinsic and extrinsic differences between the genders could potentially help reveal the relevant factors in oncogenesis, and lead to more effective prevention measures and disease control.

Regarding age-specific incidence and mortality, we found an overall increasing trend that corresponded with increasing age, peaking at age 80-84 years, and a declining trend for older men and women during this period. According to the data collected from 2001 to 2003, the peak in GC incidence and mortality occurred earlier[13] and the median and the average age were also increased. These differences were hypothesized to be related to preventive measures as well as the change of population composition such as population growth and aging.

In our study, we specifically analyzed the changing trends in GC rates in Zhuanghe, China, and found encouraging declines in both incidence and mortality. Between 2005 and 2010, GC incidence decreased with an APC of 4.82% in Zhuanghe. Moreover, a similar but more noticeable trend was observed in mortality (APC = -12.23%). The decreasing trends were in concordance with the results from various other reports in China. Wang *et al*[19] found that the estimated annual percentage change in GC incidence in Yangzhong was -2.96% in males and -2.86% in females. The APC change for GC incidence in Changle was reported to be -3.44% in men and -2.21% in women[20]. The report by Cui *et al*[21] demonstrated an APC of GC mortality in Kaifeng of approximately -2.92% in males and -3.37% in females. Although similar trends were observed, the amplitudes of the declines were not similar to those demonstrated by our data, which indicated a far more significant decrease among the comparable studies.

The descending trends of GC rates in other regions were mostly reported to be attributed to the "unplanned triumph" of improvement in sanitation, fresh fruit and vegetable consumption, and reduced *H. pylori* infection. It is commonly accepted that the incidence and mortality of GC in a region are related to both the economic status of the population and dietary habits[22-24]. In the past, residents in Zhuanghe had a low standard of living and were accustomed to eating highly salted food. However, currently, with economic development and increased knowledge regarding tumor prevention, residents have changed their dietary habits, and this potentially plays an important role in the descending trends of the disease. In 2010, Gross Domestic Product of Zhuanghe average per capita reached 54 348 RMB *yuan* - a 5.2-fold increase since 2000. Meanwhile, the rural health investment including environmental sanitation for water and housing has been increased by 23.8 times in the past decade[25], and the consequentially improved sanitary conditions may help reduce the prevalence of *H. pylori* infection, and the risk of GC development. Therefore, further research regarding preventive factors, such as eating habits, foods, prevalence of *H. pylori* infection, environmental factors, *etc*.[26-28] is warranted.

In addition to the above causes for the decreasing trends in GC, prevention and control measures were also taken in Zhuanghe, and these measures also play an important role in the encouraging declines of GC incidence in our study. In Zhuanghe, comprehensive control and etiological intervention through mass behavior interference and chemoprophylaxis in the high-risk populations have been conducted in pilot villages for many years by a research group from the First Affiliated Hospital of China Medical University (FAHCMU). With professional guidance by the researchers, knowledge of prevention and treatment of GC was disseminated for general residents by means of broadcasting, video, brochures and face-to-face conversation. In addition, the targeted high-risk population was treated promptly with antibiotics, Chinese herbal medicine and nutritional therapy based on *H. pylori* detection and gastroscopic and pathological examinations[29]. Our previous pilot survey demonstrated that these strategies are feasible and cost-effective for the treatment of gastric premalignant lesions and carcinoma in early stages, and a decrease in the mortality rate was observed in the intervened population at the initial period of prevention. The average mortality rate in the intervention groups ($34.97/10^5$) during the post-intervention period (1997-2000) was significantly lower than ($59.31/10^5$) in the pre-intervention period (1996) (P < 0.05)[30]. In the present study, we focused on trends at the population level. Interestingly, the same downward trend seen in the intervention groups was observed in the entire population. This study demonstrates that the preventive activities potentially contributed to alleviating the adverse effects of GC on the health and life of patients, even in the population as a whole.

Despite declines in both incidence and mortality of GC, we noted a dissociation phenomenon, i.e., an APC decrease was greater for mortality compared with incidence. It was hypothesized that early diagnosis and early treatment would play an important role in the dissociation phenomenon between incidence and mortality. The data suggested the need for further promotion of GC screening to enable early diagnosis. In this study,

we also analyzed the M/I ratio in Zhuanghe. The M/I ratio, which compares the number of deaths attributed to a specific cancer and the number of incident cases in the same time period, can be interpreted as an indirect indicator of general survival if registration is complete and there are no marked temporal changes in incidence rates[31]. The age-standardized M/I ratio steadily decreased in both genders, which indicated a prolonged survival; hence the decrease in mortality was not caused by the fall in incidence rate alone, but by other factors, including earlier diagnosis or improved treatment. Since 1997, more than 15 000 people have received GC screening by the research group from FAHCMU through a two-round GC screening strategy, and this large-scale population screening program extensively covered 70 villages of Zhuanghe region. Through unremitting efforts for years, the detection rate of early GC reached 60%-80% and has been increasing yearly. The GC patients screened with pathological confirmation will be sent to hospitals for treatment, and their postoperative five-year survival rate was above 90%[7]. Thus, such a population screening program may contribute to the above-mentioned dissociation phenomenon and prolonged survival of the patients. Furthermore, the age-standardized M/I ratio was lower in the male population, suggesting that the survival rate was higher in males than in female patients after treatment.

This study describes the changes in incidence and mortality rates in the entire population of Zhuanghe over the past 6 years, which may not fully reflect broader population trends; however, our observations of the short-term changes potentially form the basis for future analysis. In concert with our previous studies, we described a general picture of GC patterns and changing trends in Zhuanghe. In addition, despite a significant declining trend, the age-standardized incidence (ASR1: $26.84/10^5$ *vs* $17.06/10^5$) and mortality (ASMR1: $14.92/10^5$ *vs* $11.67/10^5$) remained higher than the national level in China[32,33]. Therefore, in future studies, continuous monitoring over a longer period of time is needed to not only identify whether the declining trends persist, but also to acquire a more comprehensive understanding of GC in this region. Accordingly, effective prevention and treatment strategies would be further developed, which will greatly benefit the residents in high-risk areas. Moreover, researches regarding the impact of genetics, the environment, and their interaction are warranted to fully elucidate the disease etiology.

COMMENTS

Background

Gastric cancer (GC) is one of the most common cancers worldwide. The geographical distribution of GC is characterized by wide regional disparity. Monitoring and studying the incidence as well as mortality at the population level in high-risk areas should be very helpful in controlling this disease in the future.

Research frontiers

Surveillance of cancer incidence and mortality provides important epidemiological information, enabling development of cancer prevention and control strategies. Several population-based cancer registries provided data on cancer rates in China, however, most of which were established in the affluent big cities rather than in the poor rural counties. In this study, the data regarding the incidence and mortality of GC in Zhuanghe County, a rural area of China, were reviewed based on the population-based registry, which provided a general picture of the GC distribution in a rural high-risk area.

Innovations and breakthroughs

The distribution of GC has substantial geographic variation, thus, studies concerning the population in high-risk areas should be more meaningful in potentially controlling this disease. The present study, for the first time, examined all-cancers distribution and temporal trends of GC in Zhuanghe and explored the possible influencing factors. Based on the findings in the registry studies, the control strategies of GC could be further developed in local areas, which may be also instructive and meaningful to cancer prevention of other similar areas in China.

Applications

There is a significant global variation in the distribution of GC. The findings of the study would be helpful for the development of general and specific cancer prevention and control strategies to benefit the residents in the high- risk areas both locally and nationally.

Terminology

Age-standardized incidence rates and age-standardized mortality rates are used to compare the incidence or mortality rates of places without being skewed by the difference in age distributions from place to place.

Peer review

The authors studied the patterns and time trends of gastric cancer in terms of incidence and mortality in Zhuanghe region at population level and investigated the influencing factors of the changing trends. Findings from this study showed a downward trend of both incidence and mortality that may be due to the raised economic level along with implementation of prevention and control measures. It enriched epidemiological data of GC and offered reference to the other high-risk areas of China to control the disease based on population-level evidences.

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CASE REPORT

Esophageal duplication cysts: Endosonographic findings in asymptomatic patients

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Abstract

Esophageal duplication cysts are rare inherited lesions usually diagnosed in early childhood. Most of them are found in the mediastinum and manifest themselves as separate masses along or in continuity with the native esophagus. Their prevalence remains unknown and they are treated either surgically or endoscopically. In this report we describe a series of four adult patients in whom esophageal duplication cysts were localised intramurally as masses pressing on the esophageal lumen and who were diagnosed with endoscopic ultrasonography. All patients were initially referred to other centres for upper gastroduodenoscopy due to non-specific dyspeptic symptoms. After finding suspicious lesions in the esophagus their endoscopists referred them for endoscopic ultrasound examination at our centre. In two of the cases lesions mimicked esophageal varices and the other two submucosal tumours. In all four patients endoscopic ultrasonography has shown esophageal duplication cysts. Patients had no symptoms suggesting disease of the esophagus and required no treatment. As the true prevalence of esophageal cysts is unknown, it is very likely that in many patients, like in these four described by us, they may cause no symptoms, remain undetected and require no intervention. Increasing availability of new diagnostic modalities such as endoscopic ultrasonography may change the current view regarding the prevalence of esophageal duplication cysts and prove that they may, in fact, not be such rare findings.

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Key words: Esophageal cyst; Endoscopic ultrasonography; Endovascular surgery

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INTRODUCTION

Esophageal duplication cysts are congenital anomalies of the foregut resulting from aberration of the posterior division of the embryonic foregut at 3-4 wk gestation[1]. They represent either simple epithelial-lined cysts, or true esophageal duplication, which is a duplication of the muscularis mucosa and externa without epithelial duplication.

Esophageal cysts are considered rare, usually described in single case reports, and their true prevalence remains

unknown[2]. Up to 80% of cases are diagnosed during childhood[1-5]. Most of them are found in the mediastinum and manifest themselves as separate masses along or in continuity with the native esophagus. Intramural esophageal cysts are considered very uncommon in adults[6,7].

Symptoms are caused by compression or displacement of surrounding mediastinal structures and comprise dysphagia, respiratory distress, failure to thrive and retrosternal pain[7,8]. Malignant progression is considered extremely rare[9]. Diagnosis is usually made by computed tomography scan or endoscopic ultrasonography (EUS).

Treatment of symptomatic esophageal cysts can be either surgical or endoscopic. Surgical resection of the cyst is usually carried out in childhood or in adults, in cases of lesions impossible to treat endoscopically. Surgical treatment is currently moving from thoracotomy to less-invasive procedures, such as video-assisted thoracoscopic surgery[7] and to endoscopic treatments which, however, still remain challenging interventions[10].

We present a series of 4 patients with esophageal cysts which caused no symptoms and required no intervention.

CASE REPORT

All patients were initially referred to other centres for upper gastroduodenoscopy due to non-specific, dyspeptic symptoms. They gave no history of dysphagia. After finding suspicious lesions in the esophagus their endoscopists referred them for EUS examination. Written informed consent was obtained from each patient.

Case 1

A 60-year old female: On endoscopy, multiple impressions, covered with normal mucosa and localized to the middle and lower third of the esophagus were seen (Figure 1A). They could be easily passed by the endoscope. No other abnormality was found on endoscopy. On EUS multiple cystic fluid-filled intramural lesions were detected (Figure 1B). They were avascular on Doppler study and their size varied from 3 mm × 5 mm to 11 mm × 25 mm.

Case 2

A 51-year old male: Endoscopy revealed multiple impressions, covered with normal mucosa and localized in the lower third of the esophagus (Figure 1C). The prepyloric area showed features of atrophic gastritis subsequently confirmed with histology. On EUS, multiple cystic, fluid-filled, intramural lesions were detected (Figure 1D). Their size varied from 4 mm × 6 mm to 8 mm × 16 mm. They showed no vascular pattern on Doppler examination.

Case 3

A 57-year old female: Endoscopy showed a solitary impression, localized 25 cm from the incisor teeth and covered with unchanged mucosa (Figure 1E). Examination was otherwise unremarkable. EUS revealed a cluster of unechoic fluid-filled intramural lesions showing no vascular pattern on Doppler examination (Figure 1F).

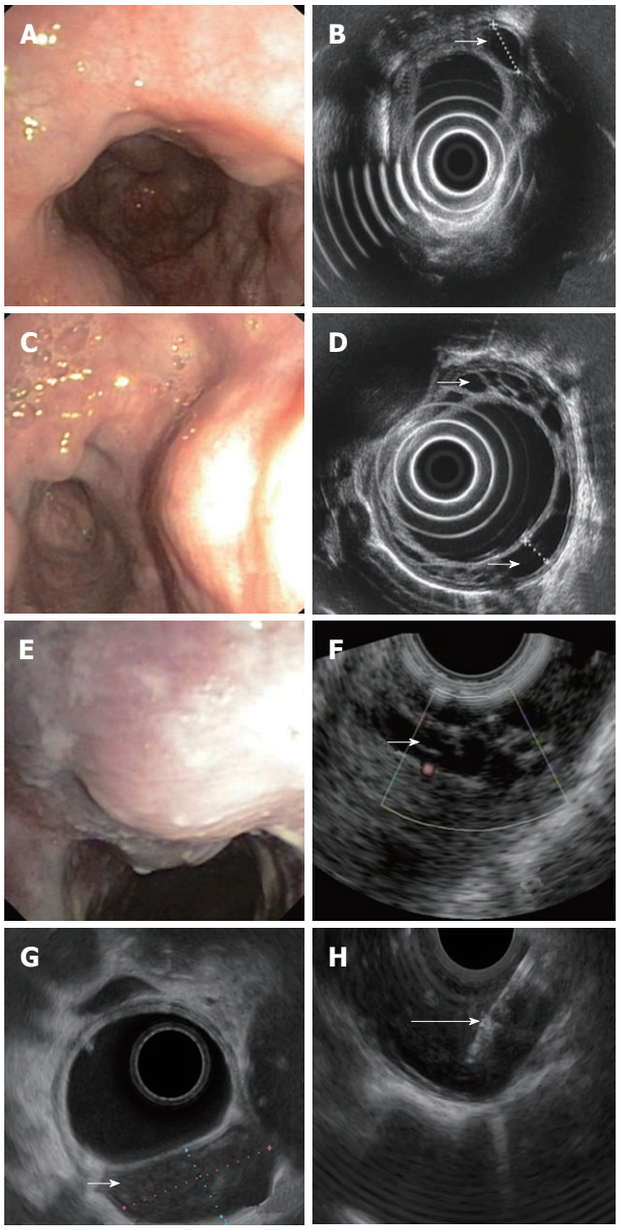

Figure 1 Endoscopic and endosonographic images of described patients. A: Multiple impressions to middle and lower third of esophagus seen in endoscopy; B: Multiple cystic fluid-filled intramural lesions detected on endoscopic ultrasonography (EUS); C: On endoscopy multiple impressions, covered with normal mucosa, localized in the lower third of esophagus; D: On EUS multiple cystic, fluid-filled, intramural lesions; E: Solitary impression, localized 25 cm from the incisor teeth and covered with unchanged mucosa seen in endoscopy; F: On EUS a cluster of unechoic fluid-filled intramural lesions; G: On EUS an unechoic fluid-filled intramural lesion 24 x 12 mm large; H: Aspiration of the fluid with 19G needle. Small arrows show duplication cysts in D, F and G and big arrow shows the biopsy needle in H.

Case 4

A 47-year old male: Endoscopy revealed a solitary lesion, size approximately 25 mm localized 30 cm from the incisor teeth and covered with unchanged mucosa (Figure 1G). No other abnormality was found on endoscopy. EUS demonstrated an unechoic fluid-filled intramural lesion

24 mm × 12 mm large. The lesion showed no vascular pattern on Doppler examination (Figure 1H). Aspiration with 18G needle revealed a clear fluid with no malignant or suspicious cells.

DISCUSSION

In this paper we describe a series of 4 patients who were referred to our centre for further investigation of esophageal lesions found on routine gastroduodenoscopy. In two cases, where there were multiple lesions, referring doctors suspected unusual looking varices or vascular lesions. In view of our interest in EUS of portal hypertension[11-13] a more detailed study of both the lesions and intrinsic para- and peri-esophageal circulation was performed. However, these patients had no clinical, laboratory or imaging features suggesting liver cirrhosis or portal hypertension. In the other two cases the initial diagnosis was submucosal tumours. These findings were expected to be confirmed using EUS. All these patients turned out to have esophageal duplication cysts.

Esophageal cysts do not usually communicate with the esophageal wall but occur as a separate malformation along or in continuity with the native esophagus. In the presented cases they were localized intramurally as a mass pressing on the esophageal lumen and this is why they resembled esophageal varices or submucosal tumours.

Treatment of esophageal duplication cysts in asymptomatic patients is controversial with no clear clinical guidelines. As all our patients remained asymptomatic we arbitrarily decided to schedule them for follow up EUS/gastroduodenoscopy in a period of 2 years. An earlier intervention will be performed if they become symptomatic.

As the true prevalence of esophageal cysts is unknown[2], it is very likely that in many patients, like in these four described by us, they cause no symptoms, remain undetected and require no intervention at all. Increasing availability of new diagnostic modalities such as EUS may change the current view regarding the prevalence of esophageal duplication cysts and prove that they are not rare findings.

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CASE REPORT

Tumors with macroscopic bile duct thrombi in non-HCC patients: Dynamic multi-phase MSCT findings

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Abstract

Non-hepatocellular carcinoma (non-HCC) with macroscopic bile duct tumor thrombus (BDTT) formation is rare, few radiological studies have been reported. In this case report, we retrospectively analyzed the imaging findings of three cases of non-HCC with macroscopic BDTT on dynamic enhanced multislice computed tomography (MSCT) scan. One case of primary hepatic carcinosarcoma was presented as a solitary, large well-defined tumor with significant necrotic changes. One case of liver metastasis from colon cancer was presented as a lobulated, large ill-defined tumor. One case of intraductal oncocytic papillary neoplasm involved the entire pancreas, presented as a cystic and solid mass with multilocular changes (the individual loculi were less than 5.0 mm in diameter). The bile duct was dilated due to expansible growth of the BDTT in all three patients. The BDTT was contiguous with hepatic or pancreatic tumor, and both of them showed the same enhancement patterns on dynamic contrast-enhanced computed tomography scan: early enhancement in the hepatic arterial phase and a quick wash-out of contrast agent in the portal and equilibrium phases. Macroscopic BDTT in non-HCC patient is rare, dynamic enhanced MSCT scan may be valuable in the diagnosis of non-HCC with BDTT.

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Key words: Liver neoplasms; Carcinosarcoma; Metastasis; Pancreatic neoplasms; Oncocytic papillary neoplasm; Bile ducts tumor thrombus; Computed tomography; X-ray

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INTRODUCTION

Hepatocellular carcinoma (HCC) associated with bile duct invasion and subsequent bile duct tumor thrombus (BDTT) formation occurs in only 0.79%-4% of patients with primary HCC[1,2]. Because HCC is one of the most prevalent malignant tumors, HCC with BDTT is not uncommonly encountered in clinical practice and its

clinical characteristics have been well described[3]. Non-HCC liver tumor can also invade the biliary tree, such as combined hepatocellular and cholangiocellular carcinoma and liver metastasis[4-10]. However, only few cases of non-HCC with BDTT have been reported in the radiological literatures[5-7]. In this study, we report three cases of macroscopic BDTT: one case of primary hepatic carcinosarcoma, one case of liver metastasis from colon cancer, and one case of intraductal oncocytic papillary neoplasm (IOPN) of the pancreas. The cases of primary hepatic carcinosarcoma or IOPN of the pancreas associated with macroscopic BDTT have not been previously reported.

CASE REPORT

Patients

All three non-HCC patients with macroscopic BDTT were treated in our hospital. They had jaundice on admission. Liver function tests showed that aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transpeptidase and alkaline phosphatase were elevated. Total serum bilirubin (TBil) was significantly increased mainly due to direct bilirubin (DBil). Serum markers of hepatitis B and hepatitis C and tumor markers alpha-fetoprotein (AFP), carcino-embryonic antigen (CEA) and CA125 were all normal. However, CA19-9 was elevated in a patient with primary hepatic carcinosarcoma and a patient with IOPN of the pancreas. All three cases of non-HCC with macroscopic BDTT were pathologically confirmed. We retrospectively analyzed the dynamic enhanced multislice computed tomography (MSCT) findings in these three cases.

Computed tomography imaging protocols

The computed tomography (CT) examination was performed using a 64-slice spiral CT scanner (Sensation 64, Siemens Medical Solutions), the technical parameters were as follows: 120 kVp tube voltage, 200 effective mAs, 64 mm × 0.6 mm beam collimation, pitch of 0.9, rotation time of 0.5 s, 5 mm reconstruction slice thickness. After acquisition of unenhanced images, nonionic iodinated contrast agent (iodipamide, 370 mg I/mL, Bracco) was injected through a dual-head injector at a rate of 3.5 mL/s and followed by a 20-mL saline flush, with a dose of 2.0 mL/kg body weight. To determine the timing for the hepatic arterial phase (HAP) scanning, a bolus-tracking technique was used with a region of interest in the descending aorta. After achieving enhancement of the descending aorta up to 100 HU, the HAP images were acquired with the scanning delay of 5 s. Portal venous phase (PVP) and equilibrium phase (EP) images were obtained with a delayed time of 30 s and 120 s, respectively.

Case 1

A 41-year-old man was admitted to our hospital on November 18, 2010 with a history of jaundice and right upper abdominal discomfort for one month. Laboratory examination showed that serum TBil was 282.1 μmol/L (normal range: 5-24 μmol/L), DBil was 221.4 μmol/L (normal range: ≤ 11 μmol/L), and tumor marker CA19-9 was 953.4 U/mL (normal range: ≤ 35 U/mL). A well-defined tumor with a size of 10.8 cm × 6.9 cm in the middle hepatic lobe (Segments Ⅳ, Ⅴ and Ⅷ) was revealed on CT scan. The tumor showed hypo-attenuation (relative to normal liver parenchyma) on pre-contrast CT scan, early rim enhancement in the HAP images and hypo-attenuation in the PVP and EP images. A large necrotic area was noted in the tumor, and showed no enhancement after contrast administration. A tumor thrombus was revealed in the dilated left and right hepatic duct and the common hepatic duct, which was contiguous with the hepatic tumor. No bile duct wall invasion was noted. The enhancement pattern of the intraductal mass was consistent with intrahepatic tumor on dynamic enhanced CT scan. Biliary tree dilation was seen distal to the intraductal mass. The patient underwent left trisectionectomy and right posterior hepatic bile duct-jejunum anastomosis, and intraductal mass was removed through resection of the extrahepatic bile duct. The intrahepatic tumor was not encapsulated. The intraductal mass was not adhered to the bile duct wall, and was easily removed. Primary hepatic carcinosarcoma with BDTT was pathologically confirmed. The carcinomatous and sarcomatous elements were HCC and fibrosarcoma, respectively. The immunohistochemical results revealed that the sarcomatous elements were Vimentin positive, and negative results for cytokeratin, epithelial membrane antigen, hepatocyte, CD34, AFP, AAT, S-100, actin and CD117 (Figure 1). The patient died two months after surgery due to hepatorenal syndrome.

Case 2

A 75-year-old woman was admitted on November 19, 2010 with epigastric pain and progressive jaundice for seven days. The patient underwent radical resection of colon cancer six years ago. Laboratory results showed significantly elevated serum TBil (196.5 μmol/L) and DBil (131.3 μmol/L). The tumor markers AFP, CEA, CA125 and CA19-9 were all in the normal range. A large lobulated tumor of 8.7 cm × 9.5 cm was displayed on CT scan in the left hepatic lobe with an ill-defined margin. The tumor demonstrated hypo-attenuation on precontrast CT scan, early mild enhancement in the HAP images and inhomogeneous hypo-attenuation in the PVP and EP images. An intraductal tumor thrombus was seen extending from the dilated left intrahepatic bile duct to the upper common bile duct, and was contiguous with intrahepatic tumor. The intraductal thrombus showed similar enhancement patterns with intrahepatic tumor on dynamic enhanced CT scans. Intrahepatic ductal dilation was found distal to the intraductal mass. Tumor thrombi were also seen in the inferior vena cava, the middle hepatic vein and the left hepatic vein. No enlarged lymph node was observed. The patient received percutanoeus transhepatic biliary drainage instead of surgery due to poor liver reserve. However, jaundice was not effectively relieved, thus palliative thrombectomy through choledochotomy was performed. The intraductal tumor thrombus was easily removed as it was not adhered to the bile duct wall. Resection specimen

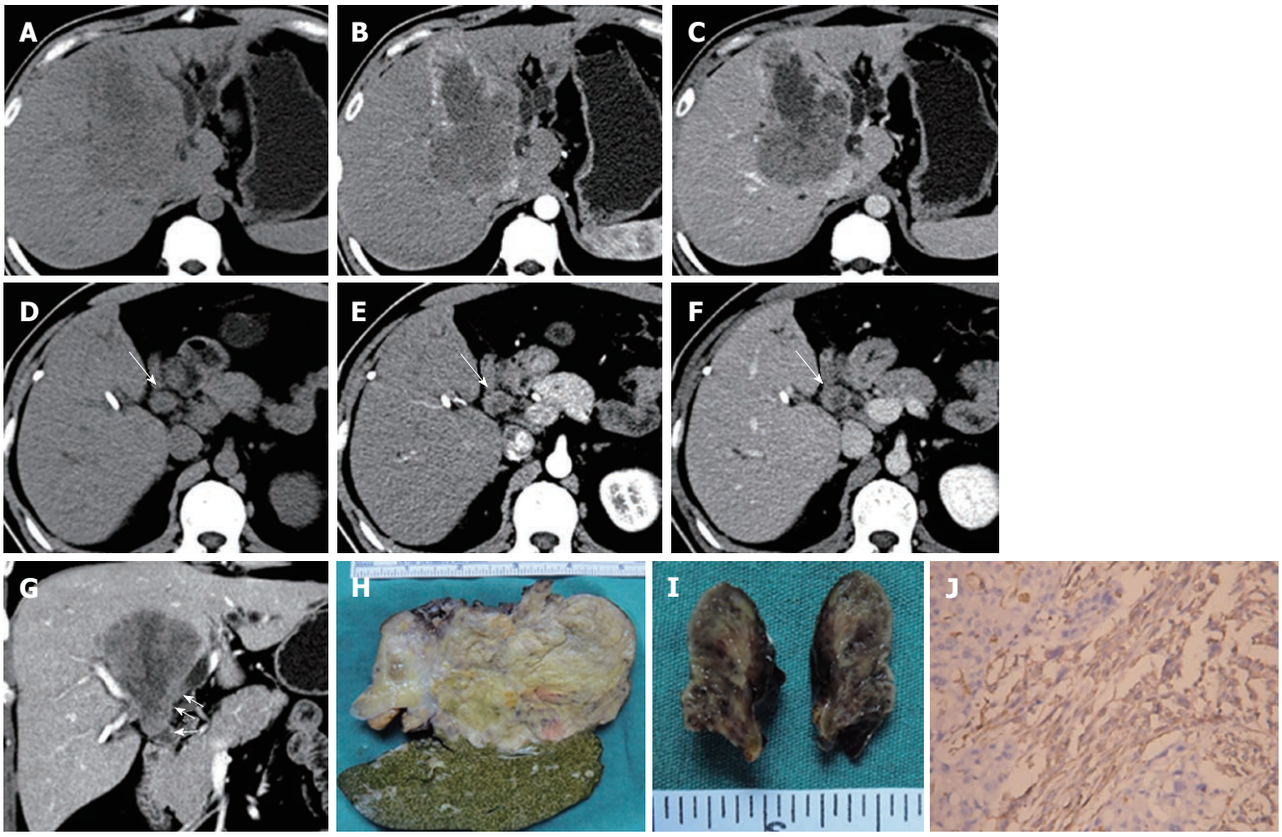

Figure 1 A 41-year-old man with primary hepatic carcinosarcoma. A large hypodense tumor in the hepatic middle lobe appears on precontrast computed tomography (CT) scan (A), which shows early rim enhancement in the hepatic artery phase (B) and a low density in the portal phase with large areas of necrosis (C). The bile duct tumor thrombus (BDTT) (arrow) shows similar enhancement patterns with the intrahepatic tumor on pre-contrast CT scan (D), hepatic artery phase (E) and portal phase scan (F). Coronal reconstruction image in the portal phase shows that BDTT (arrows) is contiguous with the intrahepatic tumor (G). The resected hepatic tumor (H) and BDTT (I) specimens. The sarcomatous component of the primary hepatic carcinosarcoma is vimentin positive on the immunohistochemical staining (J), × 200.

revealed to be metastatic adenocarcinoma pathologically, and was positive for CDX2 and CEA, and negative for hepatocyte, AFP, HBsAg, and CD34 on immunohistochemical staining (Figure 2). The patient was still alive 10 months after the operation.

Case 3

A 43-year-old woman was admitted on December 29, 2009 with a history of jaundice for 10 d. The laboratory results showed a high level of serum TBil (174.6 μmol/L) and DBil (141.3 μmol/L). The tumor marker CA19-9 was slightly elevated, with a level of 36.5 U/mL. CT scan showed enlargement of the pancreas which consisted of a mixed solid and cystic tumor. The solid components showed hypo-attenuation on precontrast CT scan, early enhancement on the HAP images and hypo-attenuation on the PVP and EP images. The cystic components were multilocular, with the loculi less than 5.0 mm in diameter, and showed no enhancement after contrast administration. The main pancreatic duct was mildly dilated and communicated with pancreatic tumor. An intraductal tumor thrombus appeared in the dilated right hepatic duct and the common bile duct on CT scan, and it was contiguous with the pancreatic tumor. The enhancement patterns of the intraductal thrombus were consistent with pancreatic tumor on dynamic enhanced CT scans. No enlarged lymph node was detected. Thrombectomy through choledochotomy and pancreatic-duodenal resection was performed in this patient. IOPN of the pancreas with BDTT formation was confirmed pathologically. Tumor invasion was only observed in the lower segment of the common bile duct wall (Figure 3). The patient was still alive 21 mo after the operation.

DISCUSSION

Macroscopic BDTT secondary to liver tumors is not commonly seen clinically and may occur in HCC, combined hepatocellular and cholangiocellular carcinoma and hepatic metastasis[4-10]. The mechanism of tumor thrombus formation in the bile duct is unclear. It is widely accepted that hepatic tumors (primary or secondary) can directly invade the bile duct, even extrabiliary malignant tumors can directly metastasize to the bile duct[10]. Consequently, tumors can develop along the bile duct and form intraductal thrombus. However, Peng *et al*[11] indicated that the tumor cells in HCC with BDTT might be originally derived from the canals of Hering and hepatic stem cells or primitive progenitor cells. We retrospectively analyzed three cases of BDTT secondary to primary hepatic carcinosarcoma, hepatic metastasis from colon cancer and IOPN of the pancreas and reviewed their

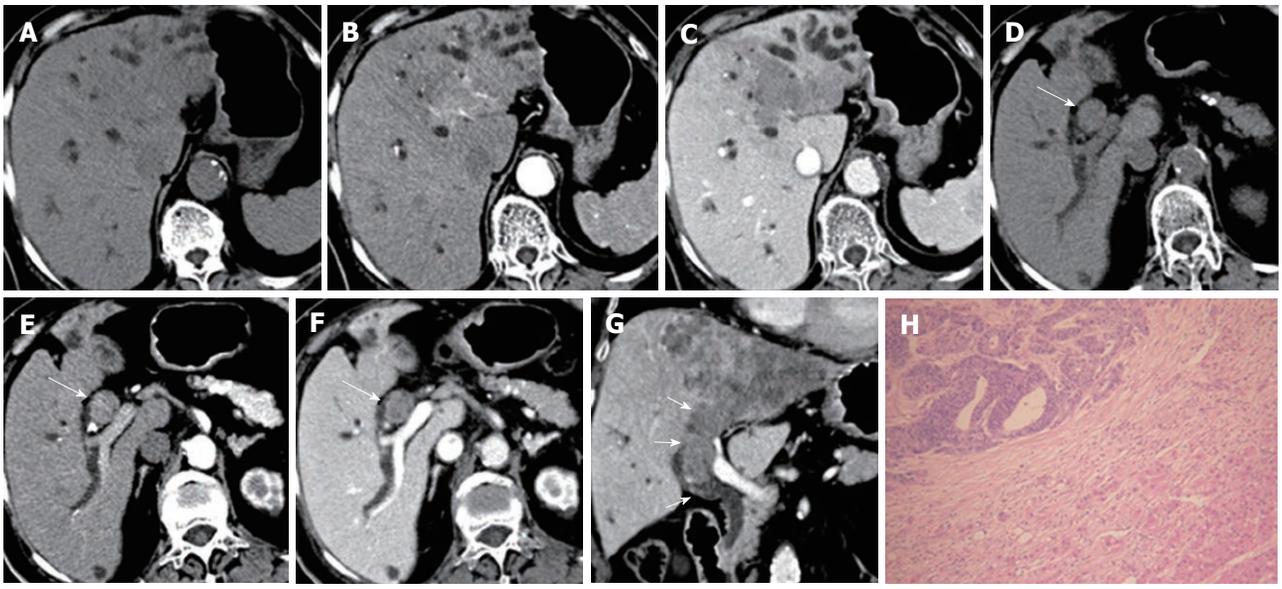

Figure 2 A 75-year-old woman with liver metastasis from colon cancer. A lobulated, ill-defined hypodense tumor in the left hepatic lobe is noted on pre-contrast computed tomography (CT) scans (A), which shows early enhancement in the hepatic artery phase (B) and a low density in the portal phase (C). The bile duct tumor thrombus (BDTT) (arrow) shows similar enhancement patterns with the intrahepatic tumor on precontrast CT scan (D), hepatic artery phase (E) and portal phase scan (F). The coronal reconstruction image in the portal phase shows that BDTT (arrows) is contiguous with the intrahepatic tumor (G). Intraductal tumor thrombus is proved to be metastatic adenocarcinoma pathologically (H), hematoxylin and eosin stain, × 100.

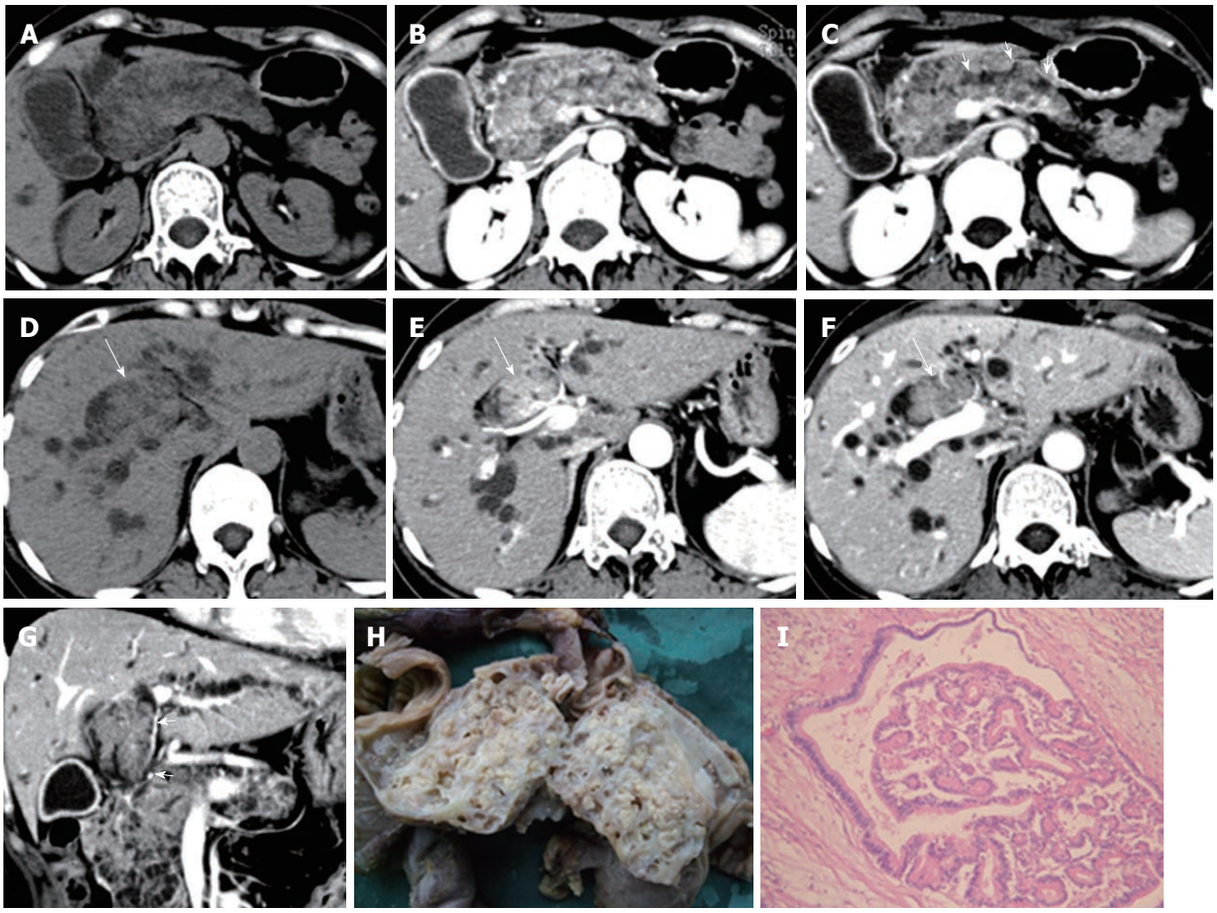

Figure 3 A 43-year-old woman with intraductal oncocytic papillary neoplasm of the pancreas. An inhomogeneous hypodense tumor involving the entire pancreas is noted on precontrast computed tomography (CT) scan (A), which shows early enhancement in the hepatic artery phase (B) and a low density in the portal phase with mild dilation of the pancreatic duct (arrows) and multilocular changes (C). The bile duct tumor thrombus (BDTT) (arrow) shows similar enhancement patterns with the pancreatic tumor on precontrast CT scans (D), hepatic artery phase (E) and portal phase scan (F). The coronal reconstruction image in the portal phase shows that BDTT (arrows) is contiguous with the pancreatic tumor (G). Resected pancreatic tumor specimen shows multilocular changes with papillary growth in the loculi (H). Pancreatic tumor is proved to be intraductal oncocytic papillary neoplasm pathologically (I), hematoxylin and eosin stain, × 100.

clinical and imaging findings.

World Health Organization defined primary hepatic carcinosarcomas as tumors containing both carcinomatous (either hepatocellular or cholangiocellular) and sarcomatous components and without obvious transition zone between them[12]. Primary hepatic carcinosarcoma is extremely rare and aggressive. To our knowledge, only about 20 cases have been reported in the English literature, and the pathogenesis is still controversial[12,13]. This tumor occurs between 34 and 84 years of age and presented no specific clinical manifestations[12,13]. More than half of the patients had a background of hepatic cirrhosis or fibrosis[12-14]. Elevated serum AFP and CA19-9 could be observed in some cases[14]. Patients usually have poor prognosis due to the highly invasive and metastatic features of the tumors[14].

The CT characteristics of primary hepatic carcinosarcomas reported in the literature include a solitary mass with central necrosis and myxoid changes, round, oval or lobulated in shape, and without capsule formation. They show iso-attenuation or hypo-attenuation on precontrast CT scan. Dynamic enhanced CT scan revealed early rim or peripheral ring enhancement in the HAP images, hypo-attenuation in the PVP images and iso-attenuation in the EP images[12,13,15]. If the sarcomatous components were osteosarcoma or chondrosarcoma, dense rocky calcifications or bone formations may be presented within the mass radiologically[15]. The case of primary hepatic carcinosarcoma described here has similar CT findings as reported in the literature. Interestingly, this case was also associated with BDTT, which has not been previously reported. The BDTT grew expansively without invasion of the bile duct wall, and was contiguous with the hepatic carcinosarcoma. Both of them showed the same enhancement pattern on dynamic enhanced CT scan as they shared the same blood supply. We found similar phenomena in HCC with BDTT, i.e., the enhancement pattern of BDTT was the same as HCC on dynamic enhanced CT scan, intraductal tumor thrombus was continuous with the main intrahepatic HCC mass, no thickened bile duct wall adjacent to the thrombus was observed[16]. The differential diagnosis of primary hepatic carcinosarcoma with BDTT from HCC with BDTT is difficult. In the presence of large amounts of tumor necrosis, tumor calcification or ossification and negative serum AFP, hepatic carcinosarcoma should be considered in the differential diagnosis.

Macroscopic intrabiliary tumor growth is a peculiar mode of intrahepatic spread in patients with colorectal liver metastasis. Metastatic hepatic tumors from colorectal cancer demonstrated macroscopic BDTT in approximately 5.8%-12.1% of resected tumors[6,8,9]. The growth of BDTT included two components: intraluminal and intraepithelial extension[8]. The diagnosis of metastatic hepatic tumors from colorectal cancer associated with macroscopic BDTT is important because macroscopic BDTT is an independent indicator of favorable prognosis in such patients. Aggressive surgical treatment can improve the chances of long-term survival[8,9]. Currently, there are few radiological studies about hepatic metastasis from colorectal cancer with bile duct invasion[5-7]. Okano *et al*[6] reported the following radiological findings on CT scan in detecting the presence of intrabiliary tumor growth in patients with liver metastases from colorectal cancer: intrahepatic bile duct dilatation, thickened portal tract and wedge-shaped area with enhancement. A thickened portal tract around the tumor corresponded to intrabiliary thrombus itself, and the presence of this sign depended on the length of the intraductal thrombus. If the length of the thrombus is larger than 30 mm, thickened portal tract will likely be observed[6]. However, in our cases and in 8 cases of hepatic metastasis with BDTT from colon cancer reported by Lee *et al*[5], no such sign was observed. This discrepancy was likely due to the use of an obsolete CT scanner as reported by Okano *et al*[6], which was limited by low spatial resolution and thicker slice scanning (7-10 mm), thus the intraductal thrombus itself could not be clearly detected. The wedge-shaped area with enhancement is assumed to be caused by reduced portal flow and a compensatory increase in arterial blood flow caused by tumor compression or arterioportal shunt in the involved portal venous branch[6]. Jinzaki *et al*[7] reported four cases of liver metastasis from colorectal cancer with intraductal invasion. Two of the cases revealed hypo-dense hepatic tumors with bile duct dilation on an enhanced CT scan without presence of intraductal thrombus, while in the other two cases, intraductal thrombus itself appeared on CT scans, which were contiguous with the main intrahepatic tumors. The intraductal thrombus was isodense on unenhanced and enhanced CT compared with the main intrahepatic tumor. In our case of liver metastasis from colon cancer with macroscopic BDTT, the BDTT showed similar expansible growth pattern, and was contiguous with the hepatic metastatic tumor. Both of them showed the same enhancement pattern on CT scan as they shared a common blood supply.

In clinical practice, it is important to differentiate metastatic tumor from primary intraductal cholangiocarcinoma in patients with a history of colorectal cancer when an intraductal mass is presented in the bile duct. The expansible growth of intraductal mass and its direct connection with hepatic tumors are very valuable features in the differential diagnosis[5]. The diagnosis of liver metastasis with BDTT should also be differentiated from HCC with BDTT. Hepatic cirrhosis background and a rise in AFP level may aid in the diagnosis of HCC[16].

The term "intraductal oncocytic papillary neoplasm" was proposed by Adsay *et al*[17] in 1996 to describe a rare type of pancreatic tumor. Currently, only about 20 cases of IOPN of the pancreas have been reported, but the accurate incidence and etiology of these cases are still unknown[18,19]. One of the distinguishing features of IOPN of the pancreas is the abundant, finely granular, eosinophilic cytoplasm of the cells. The tumors usually form complex, branching, and arborizing papillaries[17,18]. IOPN of the pancreas is often found in the elderly (mean age, 63.9 years), with no gender predilection and specific clinical features[18]. Tumors mainly involve the main pancreatic duct. They are more commonly observed in the head of the pancreas although they can occur in any part of the pancreas. Multiple sites involved have also been reported

in the literature[17-20]. In general, IOPN of the pancreas usually presents as either unilocular or multilocular cystic tumor with individual loculi ranging from 0.2 to 4.0 cm. The cystic loculi are filled with mucin and have prominent papillary growth. Cystic tumors usually communicate with the pancreatic duct system[17-20]. Most IOPNs of the pancreas exhibit severe dysplasia histopathologically to warrant the term intraductal oncocytic papillary carcinoma, and therefore they should be surgically resected completely[17].

Radiologically IOPN of the pancreas can be presented as a well-defined mass with unilocular or multilocular cystic changes and with mural or solid nodules. The tumors always communicated with the dilated pancreatic duct[18-20]. Mural nodules or solid tumor components may show enhancement on CT or magnetic resonance imaging imaging[19,20]. Adsay *et al*[17] reported one case of IOPN of the pancreas with common bile duct involvement, and Fischer *et al*[19] reported one case of IOPN of the pancreas with dilation of the common bile duct and intrahepatic ducts. However, no further description has been mentioned in both cases. Currently, no case of IOPN of the pancreas with macroscopic BDTT has been reported. The case we reported here has the following features: tumor involved the entire pancreas with multilocular, mixed cystic and solid changes, and the individual loculi were less than 5.0 mm in diameter. The pancreatic tumor invaded the lower segment of the common bile duct, and hence intraductal thrombus was formed, extending upward along the bile duct with expansible growth pattern. On dynamic enhanced CT scans, pancreatic tumor and BDTT have the same enhancement patterns, which might be due to direct connection of BDTT with main pancreatic tumor, and both sharing the same blood supply. Intraductal papillary mucinous neoplasm (IPMN) of pancreas has similar radiological features as IOPN. A fluorodeoxyglucose (FDG) positron emission tomography scan can help differentiate between IOPN and IPMN of the pancreas. A strong uptake of FDG was reported in IOPN of the pancreas, which suggested high glucose metabolic activity of tumor cells[19,20].

In conclusion, non-HCC with macroscopic BDTT is a rare clinical entity. All three cases in this study shared the following imaging features: expansible growth of the BDTT; the intrahepatic tumor or pancreatic tumor contiguous with the BDTT, and both showing the same enhancement patterns on CT scans; and intrahepatic bile duct dilation distal to the BDTT. MSCT may be a valuable modality in the diagnosis of non-HCC with BDTT.

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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

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Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

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The Liver Meeting
San Francisco, CA 94101, United States

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American Association for the Study of Liver Diseases
Boston, MA 02298, United States

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

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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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