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EDITORIAL

Interplay of autophagy and innate immunity in Crohn's disease: A key immunobiologic feature

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Abstract

Crohn's disease representing a clinical phenotype of inflammatory bowel disease is a polygenic immune disorder with complex multifactor etiology. Recent genome-wide association studies of susceptibility loci have highlighted on the importance of the autophagy pathway, which previously had not been implicated in disease pathology. Autophagy represents an evolutionarily highly conserved multi-step process of cellular self-digestion due to sequestration of excessive, damaged, or aged proteins and intracellular organelles in double-membranous vesicles of autophagosomes, terminally self-digested in lysosomes. Autophagy is deeply involved in regulation of cell development and differentiation, survival and senescence, and it also fundamentally affects the inflammatory pathways, as well as the innate and adaptive arms of immune responses. Autophagy is mainly activated due to sensors of the innate immunity, *i.e.*, by pattern recognition receptor signaling. The interplay of genes regulating immune functions is strongly influenced by the environment, especially gut resident microbiota. The basic challenge for intestinal immune recognition is the requirement of a simultaneous delicate balance between tolerance and responsiveness towards microbes. On the basis of autophagy-related risk genetic polymorphisms (*ATG16L1*, *IRGM*, *NOD2*, *XBP1*) impaired sensing and handling of intracellular bacteria by innate immunity, closely interrelated with the autophagic and unfolded protein pathways seem to be the most relevant immunobiologic events. Autophagy is now widely considered as a key regulator mechanism with the capacity to integrate several aspects of Crohn's disease pathogenesis. In this review, recent advances in the exciting crosstalk of susceptibility coding variants-related autophagy and innate immunity are discussed.

Key words: Crohn's disease; Innate immunity; Autophagy genes; Autophagy; Gut microbiota

Core tip: In case of Crohn's disease, on the basis of autophagy-related risk genetic polymorphisms impaired sensing and responding of intracellular bacteria by innate immunity, closely interrelated with the autophagic and unfolded protein pathways seem to be the most relevant immunobiologic events. Autophagy represents a key regulator mechanism with the capacity to integrate several aspects of Crohn's disease pathogenesis.

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis, the main clinical phenotypes of (idiopathic, relapsing-remitting) inflammatory bowel disease (IBD) are systemic disorders affect-

ing the gastrointestinal-tract with frequent extraintestinal manifestations and associated autoimmune conditions[1]. IBD is considered as a polygenic immune disorder with complex multifactor etiology. Generally, IBD is arising in susceptible individuals in whom upon environmental triggers a sustained disturbed, deleterious mucosal immune reaction is provoked towards commensal microbiota[2]. In chronic inflammatory conditions, when organs with large epithelial surfaces are affected, like in IBD the epithelial barrier function is critical for the disease onset. Since the epithelium is densely inhabited by a resident microbial flora the role of native immunity is particularly appreciated in recognizing and distinguishing commensal enteric bacteria from the invading ones, and thus, in maintaining tolerance and homeostasis[2]. Subsequently, the chronic unrestrained inflammatory response that occurs in IBD is mainly driven by a disintegrated host immune regulatory network. In IBD development the host genetic susceptibility represents an important etiologic factor. In CD the genetic component is strongly indicated by familial aggregation, and further, by an approx. Twenty-six-fold greater population-based sibling risk, and an approximately 30%-35% of concordance rate in monozygotic twins[3,4]. The introduction of genome-wide association studies (GWAS) has yielded an expansion in studying the genetic basis of IBD. Nowadays more than 70 loci are associated with CD[5]. Further, in CD pathogenesis GWAS highlighted on certain earlier not really suspected biological pathways, such as autophagy. In polygenic diseases functional variants of single genes could be identified. Indeed, many of the recently identified genetic risk loci in CD are related to various cell types and pathways, suggesting the involvement of fairly different aspects of host immune responses in the IBD phenotype. Missing heritability in CD cannot be simply explained by genetic alterations[2]. Moreover, the fact of the worldwide considerable increase in disease incidence and prevalance emphasizes the importance of additional, environmental and epigenetic contributions[6,7]. The interplay of genes regulating immune functions is strongly affected by the environment, especially gut resident microbiota. On the basis of genetic alterations in CD impaired sensing and handling of intracellular bacteria by the innate immunity, that is closely interrelated with the autophagic and unfolded protein pathways seem to be the most relevant pathophysiologic features[8].

AUTOPHAGY MACHINERY

Besides the proteasomal degradation pathway autophagy represents an additional, evolutionarily highly conserved multi-step process of cellular self-digestion due to sequestration of excessive, damaged, or aged proteins and intracellular organelles in double-membranous vesicles of autophagosomes, terminally self-digested in lysosomes[9]. Autophagy is deeply implicated in the regulation of numerous physiologic functions including cell development and differentiation, survival and senescence, and it also affects fundamentally the inflammatory process, and the innate and adaptive arms of immune responses[10]. On a basal level intact autophagy serves constantly and constitutively as a critical adaptive and surveillance mechanism in maintaining cellular homeostasis[11]. Nevertheless, autophagy is inducible in response to different cellular metabolic stress conditions, such as nutrient and growth factor deprivation in order to preserve cell viability. Further, autophagy is upregulated in cases of protein aggregation and accumulation of misfolded proteins, *i.e.,* when the structural remodeling is mandatory. In respect of innate immunity, however, autophagy plays an essential role during infections by degrading intracellular pathogens[10,12]. Different types of autophagy according to the route of delivery to lysosomes and the main physiological functions have been characterized, such as macro- and micro-autophagy, and chaperon-mediated autophagy[11]. Upon specific targeted degradation of cytosolic aggregated proteins, lipids, and organelles (ribosomes, nucleosomes, peroxisomes, mitochondria, endoplasmic reticulum), selective forms of autophagy (aggregophagy, lipophagy, ribophagy, nucleophagy, pexophagy, mitophagy, reticulophagy) can further be classified[9,11]. In addition, elimination of intracellularly infective pathogens represents another selective form of autophagy, namely xenophagy (Figure 1). Xenophagy can be considered as a substantial element of the innate immune system. Generally (macro)autophagy refers to cytoplasmatic bulk, non-selective degradation of subcellular constituents. Within this complex catabolic pathway tightly regulated by a limited number of autophagy genes (*ATGs*) various morphologic stages of the assembly process are distinguishable[12]. It is initiated with the phagophore (isolation membrane) formation around different molecules or particles to be sequestred, and is followed by elongation and maturation into the autophagosome, leading finally to fusion with lysosomes[9,12]. Subsequently, the phagophore is controlled by *Beclin1 (ATG6)* and *ATG14* genes, and both the inhibitory class1 canonical phosphatidylinositol 3'-kinase/AKT (PI3K/AKT) mammalian target of rapamycin (mTOR) and the promoting c-Jun N-terminal kinases (JNK1) pathways[13,14]. The intricate formation of autophagosome is regulated mainly by the ATG5-ATG12 complex, then stabilized by ATG16L1, and further processed by microtubule-associated protein light chain (LC3/ATG8) under the strict control of ubiquitin-like conjugation systems (ATG10, ATG7, ATG3). The engulfment of random or selective cargo, closure of the autophagosome, and fusion with the lysosomal compartment is orchestrated by LC3, and the Beclin1-UV-irradiation resistance-associated gene (UVRAG) complex[13,14]. Defects in basal autophagy may yield accumulation of cytotoxic materials, damaged DNA, and thus, genomic instability, while alterations of induced autophagy especially lead to reduced cell survival[10,12]. By compromising cellular fitness defective autophagy has been ultimately related to several chronic inflammatory disease conditions, such as inflammatory bowel disease, like CD and cancer, neurodegeneration, and infectious disorders[10,11,15]. Gener-

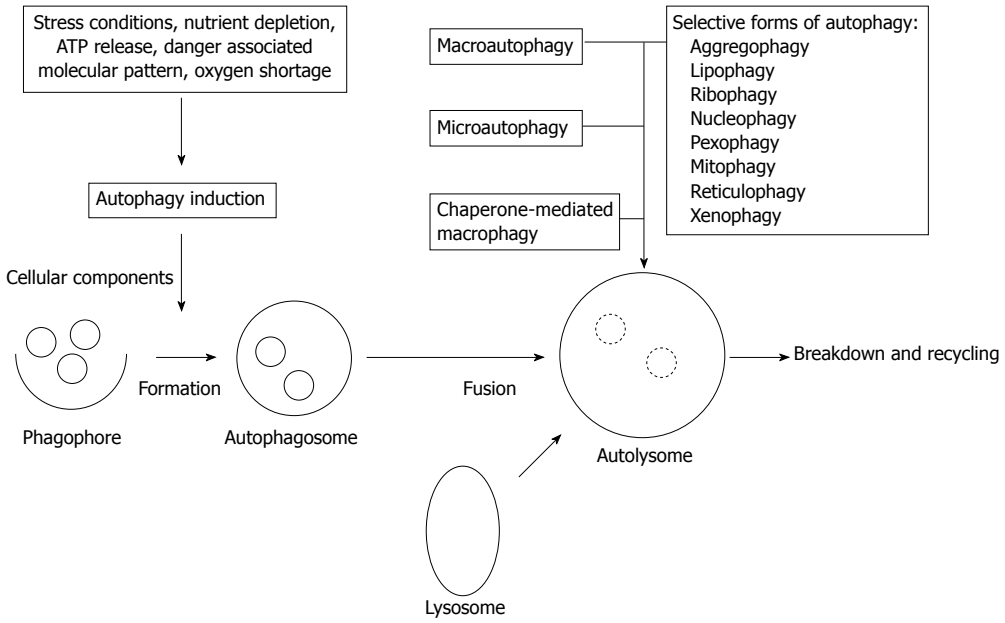

Figure 1 The autophagic process and types of autophagy.

ally autophagy deficiency is closely related to accelerated tumorigenesis. In autophagy-incompetent cells upon induced oxidative stress cell-autonomous mechanisms are exhibited in forms of accumulated DNA damage and chromatin instability[16]. However, inflammatory events as a non-cell-autonomous mechanism along with defective apoptosis could independently contribute to malignant transformation and cancer progression, partly by favoring cell necrosis[17]. Similar situation has been found in human IBD with high risk of malignancy, and in experimental cases of *Atg5*-/- or *Atg7*-/- mice displaying abnormalities resembling human IBD[18]. Autophagy and stress-responsive cellular degradation pathways of intrinsic and extrinsic apoptosis can fundamentally alter, activate or inhibit each other *via* an extensive molecular crosstalk, and in fact, cell destiny is determined by their actual functional status and interplay[19]. Their crosstalk is primarily regulated by the current status of the ATG6/Beclin-1 complex, a Bcl-2/Bcl-xL interacting element, since Bcl2 is a potent autophagy inhibitor. Dissociation of this complex can be achieved by toll-like receptor (TLR) adaptors (MyD88, TRIF), or activation of mitogen activated phosphokinase (MAPK)-JNK cascade, as well as by translocation of the damage-associated molecular pattern (DAMP) protein high-mobility-group B (HMGB)-1[13,19]. There is also diverse interaction between autophagy and the nuclear factor-κB (NF-κB) signaling pathways through positive and negative feedback regulatory loops[14]. The tumor suppressor *p53* gene exerts a typical dual role in autophagy regulation, depending primarily on its subcellular, nuclear or cytoplasmic distribution[13,14].

NOD-LIKE RECEPTORS AND CROHN'S DISEASE

NOD-like receptors (NLRs) are pattern recognition receptors (PRRs) and belong to the family of innate immune receptors sensing pathogen-associated molecular patterns (PAMPs). Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is constitutively expressed intracellularly in macrophages and dendritic cells, and to lesser extent in intestinal epithelial cells and T cells. The centrally located motifs of NLRs are referred to NOD domains that are interacted with the caspase activation and recruitment domain ones. NOD2 recognizes *N*-acetyl--muramyl-peptide (MDP), a bacterial peptidoglycan component, and upon activation the induced receptorial conformation changes result a multiprotein, the inflammasome (NLRP3). Ligation of NOD2 triggers recruiment of the adaptor protein receptor interacting protein 2 (RIP2) causing a TRAF6-mediated ubiquitination of inhibitor of κB-kinase gamma (IKKγ; NEMO), and hence results in activation of downstream signaling pathways implicating NF-κB, MAPKs and proinflammatory caspases[20,21]. The Crohn's disease-associated *NOD2* genetic variants are located in the leucine-rich repeat (LRR) region of NOD2, *i.e.*, in the ligand-binding domain of this intracellular PRR[22]. The altered amino acid sequence is related either to insertion resulting in a frame-shift mutation, or to non-synonymous SNPs resulting in amino acid exchanges. The more commonly observed genetic variants (of missense or nonsense mutations) in CD are the SNP8 (R702W), SNP12 (G908R), and SNP13 (L1007fsC), respectively, however a number of rare *NOD2* variants have also been discovered, being localized again almost exclusively to the LRR region[22,23]. Upon MDP ligation the Crohn's disease-associated "loss-of-function" NOD2 variants abrogate RIP2 binding, and so fail to activate NF-κB[24,25]. Further, NOD2 is involved in the modulation of TLR signaling, as well. Thus, in case of Crohn's disease-related gene polymorphisms the TLR2-induced NF-κB activation is also decreased[26,27].

However, yet it is difficult to correctly interpret the real functional consequences of given mutations since they may activate additional, compensatory mechanisms resulting in a definite inflammatory phenotype. On the other hand NOD2 has a pivotal role in direct antibacterial defense by the induced release of defensins. NOD2-/- mice and patients with the CD NOD2 variants display diminished expression of antimicrobial α-defensins in Paneth cells, that contributes to impaired antibacterial capacity and decreased epithelial barrier function[28,29]. In contrast to hypomorphic functions the frame-shift gene mutation variant encodes a "gain-of-function" by actively suppressing interleukin-10 (IL-10) transcription[30].

AUTOPHAGY AND CROHN'S DISEASE

The autophagy machinery in IBD represents a recently developed pathway fundamentally contributing to the pathogenesis[10]. Functional polymorphisms of the autophagy genes *ATG16L1* (T300A) and immunity-related GTPase family M protein *(IRGM*; C313T) have been found as definite risk factors for CD[31-34]. The ATG16L1 protein is widely expressed in intestinal epithelial cells, and also in macrophages and lymphocytes. The ubiquitous *ATG16L1* seems to be fundamental in selective autophagy, *i.e.,* in xenophagy, nonetheless its defect has only been described within the gut[35].

In CD patients homozygous for the risk *ATG16L1* allele the "loss-of-function" deficiency due to failures of autophagosome formation results in impaired engulfment and degradation of cytoplasmic content (microbes), defective presentation of bacterial antigens to $CD4^+$ T cells, and further, in alterations of Paneth cell granule formation causing a disrupted granule exocytosis[18,36-38]. Additionally, ATG16L1-deficient Paneth cells in CD display a "gain-of-function" defect by increasing expression of inflammatory cytokines[18,38]. Moreover, upon stimulation with NOD2 ligands or with lipopolysaccharides (LPS) through TLR4, macrophages and myeloid cells with the ATG16L1 risk variant generate high levels of reactive oxygen species (ROS), and respond with inflammasome overactivation leading to enhanced IL-1β and IL-18 production *via* Myd88 and TRIF-dependent activation of caspase-1[36-38]. Generally, aberrant activation of PRR signaling pathways may result critically severe inflammation. *IRGM* is the only human gene representative for innate immunity-related GTPases, necessary for γ-interferon-mediated resistance to intracellular pathogens[39]. During initiation of autophagy IRGM expression is essentially required for the proper clearance of bacteria. The risk polymorphism of *IRGM* due to the impaired protein expression can lead to functional abnormalities in xenophagy[32,33]. Since IRGM is possibly regulated in a cell specific manner the CD risk allele may cause cell specific phenotypes.

NOD2 and autophagy

Functionally NOD2 is closely associated with autophagy, and yet interacts mechanically (*i.e.,* immunoprecipitated) with ATG16L1, therefore autophagy seems to be a key factor in CD[37,40,41]. Autophagy is mainly activated due to sensors of the innate immunity, *i.e.,* by PRR signaling upon recognition of PAMPs (MDP, LPS, ss/ds RNA, methylated DNA/CpG), but it could also be induced by DAMPs (like ATP, ROS, and misfolded proteins), pathogen receptors (like CD46), IKK, JNK and HMGB proteins[10,12,19,38]. Sensory PRR-molecules include TLRs, NLRs and RIG-I-like receptors (RLRs). Induction of NOD2 in dendritic and epithelial cells by bacterial ligands and leaving bacteria results in ATG16L1-dependent formation of autophagic vacuoles. However, the NOD2 variants of CD lack this activity, and further MDP-induced autophagy is also absent in cells with the ATG16L1 risk variant, suggesting that both NOD2 and ATG16L1 co-localized on plasma membrane are required for an optimal innate immune signaling[40-42]. In addition, a NOD2-dependent failure in autophagy-induction and consequently a diminished bacterial killing was found for *Salmonella typhimurium*, *Shigella flexneri*, and enteroadherent invasive *Escherichia coli* (*E. coli*)(AIEC)[40,41,43]. The normal NOD2, but not the CD-associated variants recruits ATG16L1 to the plasma membrane preferentially at the bacterial entry side, so physiologically NOD2 is critical for engulfing invading pathogens by autophagosomes[41,42]. Furthermore, in dendritic cells NOD2-dependent autophagy is also essential for the appropriate antigen processing and presentation and a subsequent induction of $CD4^+$ T-cells[41]. Dendritic cells from CD patients with either NOD2 or ATG16L1 variants display a failure to translocate bacteria to lysosomes and relocate MHC II to cell surface, as well[40]. When the disease-associated *ATG16L1* and *NOD2* alleles are present in combination, a synergistic genetic epistasis, *i.e.,* an increase in CD susceptibility was observed, underscoring the importance of a signaling crosstalk regarding the inflammasome and autophagy[44].

Endoplasmic reticulum stress and autophagy

The unfolded protein response (UPR) induced by endoplasmatic reticulum (ER) stress represents another pathway in IBD pathophysiology[45]. Genetically ER stress is associated with both forms of IBD and occurs upon excessive accumulation of misfolded or unfolded proteins in the ER, leading to UPR especially in cells with high secretory capacity, like goblet cells and Paneth cells[35,46]. UPR is regulated by different pathways (and related transcription factors) with the preference of the inositol-requiring enzyme 1/X-box binding protein 1 (IRE1/XBP1) axis[47]. *Via* this axis there is a conserved link between innate immunity (TLR and NOD signaling) and the UPR[47]. GWAS-based candidate gene studies revealed the role of *XBP1* SNPs in IBD-related ER-stress[48,49]. Decreased or absent XBP1 function in the intestinal epithelial cell (IEC) compartment through IRE1 hyperactivation results in uncontrolled ER-stress, *i.e.,* a proinflammatory overactivation, and further in dysfunction and premature apoptotic depletion of Paneth cells, with

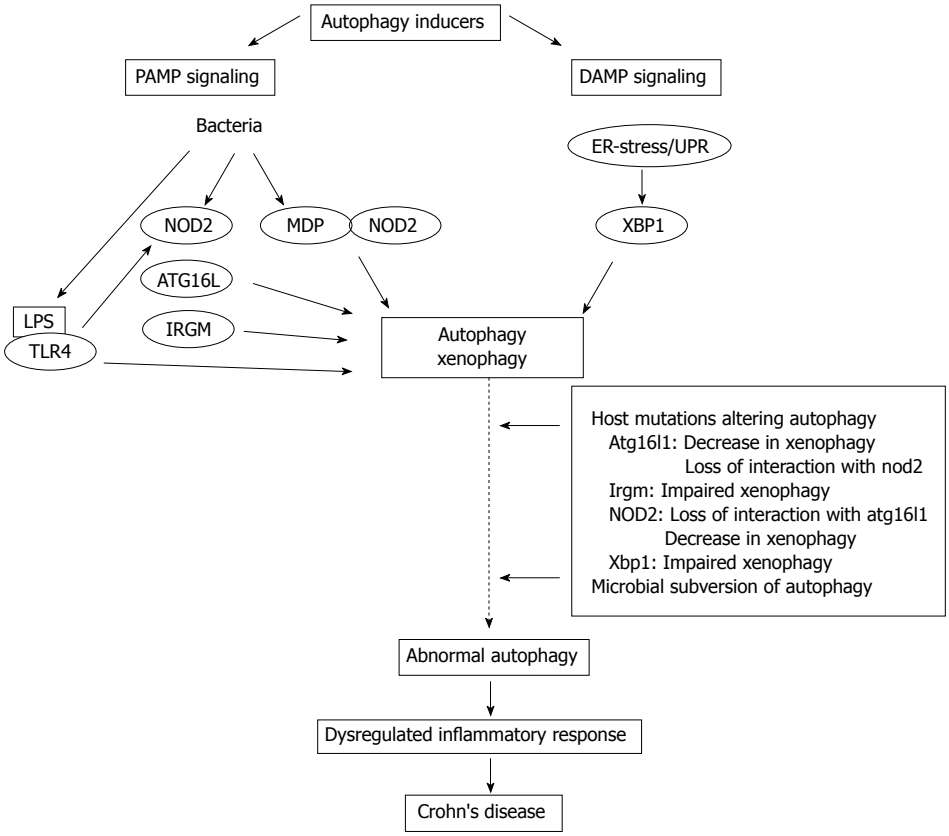

Figure 2 Schematic illustration of the crosstalk between autophagy and innate immunity in Crohn's disease. PAMP: Pathogen-associated molecular patterns; DAMP: Damage-associated molecular pattern; ER: Endoplasmic reticulum; UPR: Unfolded protein response; NOD: Nucleotide-binding oligomerization domain-containing protein; MDP: *N*-acetyl-muramyl-peptide; LPS: Lipopolysaccharide; TLR: Toll-like receptor; XBP1: X-box binding protein 1; IRGM: Immunity-related GTPase family M protein.

the consequent impaired handling of the microbiota[49]. Under ER stress autophagy is induced *via* JNK (downstream of IRE1), which is overactivated by the hypomorphic XBP1[50,51]. However, even defective autophagy *per se* is able to provoke ER stress, especially when the ATG7 protein involved in regulation of autophagosome formation is also depressed[52]. Regarding PI3K there is an antagonistic action, since in UPR it is responsible for the activation of XBP1, but in the contrary autophagy is suppressed by the canonical AKT-TOR pathway[53,54]. In IBD, IECs presumably are affected both by impaired UPR signaling and aberrant autophagy, but their exact interplay needs to be further clarified.

GUT MICROBIOTA AND CROHN'S DISEASE

The intestinal microbiota, which normally colonize mucosal surfaces in symbiotic mutualism with the host is unique and quite stable over time[55]. The basic challenge for the intestinal immune recognition is the requirement of a simultaneous delicate balance between tolerance and responsiveness towards microbes[56]. Several data suggest the existence of immune tolerance to antigens of the individual own bacterial flora, whereas its breakdown definitely contributes to IBD pathogenesis[37,57]. In CD there is a profound and complex host defect in sensing and responding intestinal (lumenal and mucosal) microbiome. Accordingly, reprogramming in the microbial composition, *i.e.,* a significant decreased load of commensal, protective resident bacteria (like *Bifidobacteria, Lactobacilli* and *Firmicutes*) along with the impaired immunity against the putative pathogenic (harmful) ones (such as *Bacteriodetes*, and *Proteobacteria*, including *E. coli*) provoke a deleterious inflammatory condition, corresponding to CD[58]. The exact nature of the distinct mucosal flora (dysbiosis), however has not yet been fully elicited. Specific strains of *E. coli* (termed AIEC) in CD affect especially the epithelial layer with the ability to adhere, invade and replicate in IECs, and further, a subpopulation even resides and survives within macrophages, and thereby induces increased production of tumor necrosis factor-α[43,59]. ATG16L1 and IRGM-deficient autophagosomes promote the AIEC survival as well[43]. Moreover, in the presence of CD-associated NOD2 variants or hypomorphic XBP1 dendritic cells exhibit diminished intracellular bacterial killing[41]. It is hypothesized, that AIEC possesses the capacity to circumvent innate immune responses leading to activation of NF-κB[60]. Thus, regarding the host interactions with microbes genetic risk factors of CD functionally render pathways of the innate immunity to converge to a deeply impaired autophagic process.

CONCLUSION

Overall, there is no doubt that autophagy can be considered as an apparently difficult regulatory network, being in close connection with several signal transduction pathways and cellular programs. Principle elements of immunological autophagy include the direct cell-autonomous pathogen elimination, the regulation of PRRs, and inflammasome activation, and the cytoplasmic antigen processing for MHC presentation to T cells. Recently significant advances have been achieved in understanding the importance of autophagy in CD, which previously had not been implicated in IBD pathology. In CD functional consequences of the underlying autophagy-related gene defects (*ATG16L1, IRGM, NOD2, XBP1*), in particular the inappropriate stimulation of antimicrobial and inflammasome pathways eventually result in uncontrolled inflammation (Figure 2). Therefore, autophagy in CD is predicted as a key regulator mechanism with the capacity to integrate several aspects of disease pathogenesis. Theoretically the complex autophagy signaling in CD offers a promising novel therapeutic target, since due to its induction potentially not only the load of cytoinvasive bacteria, and the perturbed immune responses, but the resulting inflammatory process, as well may simultaneously be reduced. Thus, autophagy boosting would represent an efficient biologic manipulation, and could provide an alternative therapeutic option. Several candidate pathways, *e.g.*, inhibition of mTOR, decrease of ER-stress, lowering of inositol triphosphate (IT3), *etc.*, could be considered. On the other hand, however, much cautiousness is required regarding its pleiotropic physiological repertoire, since pharmacologic autophagy modulation can initiate additional biologic effects not expected in CD. Further detailed functional analyses of the Crohn's disease-associated genetic polymorphisms are needed to explore and define more precisely the subcellular and molecular basis of the crosstalk between autophagy and the innate immune axis, hopefully allowing the introduction of selective new therapeutic approaches into daily practice.

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REVIEW

Autoimmune hepatitis in childhood: The role of genetic and immune factors

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Abstract

Autoimmune hepatitis (AIH) is a rare chronic inflammatory disease of the liver, which affects a group of patients who lost their immunological tolerance to antigens of the liver. It is clinically characterized by hypergammaglobulinemia, elevated liver enzymes, presence of autoantibodies and histological changes. Although being rare in children, it represents a serious cause of chronic hepatic disease that can lead to cirrhosis and hepatic failure. Clinical findings, exclusion of more common liver disorders and the detection of antibodies antinuclear antibodies, smooth muscle antibodies and anti-LKM1 are usually enough for diagnosis on clinical practice. The pathogenic mechanisms that lead to AIH remain obscure, but some research findings suggest the participation of immunologic and genetic factors. It is not yet knew the triggering factor or factors that stimulate inflammatory response. Several mechanisms proposed partially explain the immunologic findings of AIH. The knowledge of immune factors evolved might result in better markers of prognosis and response to treatment. In this review, we aim to evaluate the findings of research about genetic and immune markers and their perspectives of application in clinical practice especially in pediatric population.

Key words: Autoimmune hepatitis; Genetics; Clinical practice; Immunophenotype

Core tip: In this review article, we reported recent data on autoimmune hepatitis in pediatric patients highlighting the importance of genetic and immune markers. We also discuss the perspectives of the application of these new biomarkers in clinical practice.

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INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic inflammatory disease of the liver that is rarely found in children and adolescents. AIH affects a group of patients who have lost their immunological tolerance to antigens of

the liver[1-4]. It is more frequent in female patients and is characterized by hypergammaglobulinemia, elevated liver enzymes, the presence of autoantibodies and histological changes[4-7]. The age of onset usually ranges from months to 75 years old, but it is very rare before the age of two years old, and the highest incidence occurs between 10 and 30 years old[2]. In addition to being considered rare in children, AIH represents a serious cause of chronic hepatic disease, which can result in cirrhosis and its complications. Immunosuppressive treatment results in a good response, but a delay in or absence of treatment can result in cirrhosis and liver failure[2,6]. This condition can also be complicated by association with autoimmune cholangitis, in which bile duct disease is present together with hepatitis, particularly in children[2,7,8].

Clinical and laboratory diagnosis

Because histological activity index (HAI) is a rare disorder, one crucial point for diagnosis is the exclusion of more common pathologies. The diagnosis is confirmed by clinical findings, laboratory and histopathology tests and the exclusion of other causes of chronic liver disease[4,6,7,9]. The clinical spectrum is broad, ranging from asymptomatic laboratory abnormalities to clinical symptoms similar to fulminant acute viral hepatitis. The classical presentation is jaundice, dark urine, fever, asthenia, anorexia and increased abdominal volume in an acute or insidious presentation[6,10]. Hepatomegaly, splenomegaly and signs of chronic liver disease, such as spider veins, collateral circulation and abdominal ascites, might be present. Approximately 20% of cases are associated with other autoimmune disorders[8].

According to the presence of autoantibodies, AIH can be classified into two forms: type 1 autoimmune hepatitis, in which antinuclear antibodies (ANAs) and/or anti-smooth muscle antibodies (SMAs) are detected; and autoimmune hepatitis type 2, in which anti-liver-kidney (anti-LKM1) autoantibodies are detected[9,11-13]. In adult patients, the presence of anti-soluble liver-kidney antigen and anti-liver-pancreas might be understood as a third form of AIH (AIH type 3), despite clinical features similar to type 1[14]. Type 1 is the most common type of AIH in any age group, while type 2 usually occurs in younger patients, with courses having a greater likelihood of acute liver failure[2,3].

During treatment, ANA and SMA levels can decrease, but neither level seems to have a correlation with prognosis[15-17]. Therefore, 10%-15% patients are negative for ANAs, SMAs and LKM-1 at clinical presentation but later show detectable levels of these autoantibodies, with only five percent remaining negative over time[15,18]. Other autoantibodies could facilitate in diagnosis and/or act as prognostic markers, and their possible clinical applications are listed in Table 1[19-38].

Most services do not perform routine assessment of the autoantibodies shown in Table 1, which remain reserved for research situations. The antibodies ANA, SMA and anti-LKM1 are usually sufficient for diagnosis in clinical practice. More research is needed to establish the clinical use of these autoantibodies and to investigate the presence of these autoantibodies in pediatric patients, thereby elucidating their role in this group of patients.

Diagnostic criteria

The International Autoimmune Hepatitis Group diagnostic criteria for AIH, published in 1993 and revised in 1999, guide diagnosis and facilitate early treatment[39-41]. A simplified scoring system, created in 2008, considers transaminases levels, autoantibodies, immunoglobulin G levels, liver biopsy, exclusion of Wilson disease and of viral hepatitis and cholangiogram[41,42]. The use of these criteria could also be helpful in children, but limitations must be recognized[43]. In children, it is difficult to differentiate AIH from primary sclerosing cholangitis or to identify autoimmune cholangitis overlap syndrome. The diagnosis of fulminant hepatitis cases has not been well determined because the use of 1/40 as a titer for autoantibodies is high to use in children (1/20 for ANA and SMA and 1/10 for anti-LKM1 are considered positive in this age group)[3,43]. For these reasons, histology is often included in the diagnostic criteria for HAI in children[3,44]. On histological examination, characteristic findings include the presence of piecemeal necrosis (interface hepatitis), lymphoplasmocytic infiltrates with numerous plasmocytes, and rosette formation[44,45]. Histology is a powerful tool for diagnosis, with high specificity (81%-99%) and predictability (62%-91%) but low sensitivity (36%-57%)[45]. Some cases also demonstrate biliary duct alterations, such as inflammatory infiltration of duct cells, cholestasis and ductopenia, which might represent an overlapping syndrome[46].

Genetic and immunologic markers

Some studies have unveiled the association of AIH with genetic markers, and the impact of immunophenotyping on clinical practice has been described.

Although the pathogenesis of AIH is not fully understood, susceptibility is partly determined by the presence of genes related to major histocompatibility complex Ⅱ (MHC Ⅱ) and most directly to human leukocyte antigen (HLA)[7,47]. The main associations are with HLA-DR3 and HLA-DR4 (DRB1*03 and DRB1*04) in Europeans and North Americans[48]. In children, HLA-DRB1*1301 is related to susceptibility to HAI, determining the prognosis and response to treatment[47,49]. The findings of the immunophenotyping in HAI are shown in Table 2.

Some conclusions can be drawn from these studies, in addition to some controversial findings[48-57]. Fortes Mdel *et al*[50] showed that patients presenting the HLA-DRB1*1301 allele were associated with a higher likelihood of developing cirrhosis. Czaja *et al*[56] concluded that patients with -DRB1*03 were younger at disease onset than patients with -DRB1*04, and they also had worse responses to corticotherapy. Patients expressing HLA

Table 1 Autoantibodies studies and their findings

Type of AIH	Autoantibodies	Antigen	Meaning
AIH type 1	Anti-actin	Actin	Poor response to treatment with corticosteroids[19-21]
AIH types 1 and 2 (80%-90% of cases)	Anti-asialoglycoprotein receptor	Asialoglycoprotein receptor	Liver specific antigen and indicative of prognosis[22,23]
AIH types 1 and 2 (8%-20% of cases)	Antimitochondrial antibody-M2	Mitochondria	Favorable response to corticosteroids[24,25]
AIH type 1 (39% of cases)	Anti-chromatin	Chromatin	High titers of immunoglobulin G and shows disease activity[26,27]
AIH type 2 (32% of cases)	Anti-liver-cytosol type 1	Enzyme formiminotransferase cyclodeaminase	Diagnostic tool and marker of liver inflamation[28-30]
AIH type 1	Antibody to histone and dsDNA	dsDNA	High titers of immunoglobulin G and poor-immediate response to corticosteroids[26]
AIH type 1 (47.5% of cases)	Anti-soluble liver antigen	t-RNAs	Presence of severe forms, associated with fatal outcome[31-35]
AIH type 2 (5%-19% of cases)	LKM-3	Uridinediphosphateglucuronyl tranferase	Allows diagnosis, being sometimes the only marker identified[36]
AIH type 1	Perinuclear antinuclear neutrophil cytoplasmic antibodies	Peripheral nuclear and perinuclear antigen	Presence of severe forms; Most frequent in primary sclerosing cholagitis and primary biliary cirrhosis[36-38]

AIH: Autoimmune hepatitis; dsDNA: Double-stranded DNA.

DRB1*04 are more often women, with a greater risk of comorbidity with other immune diseases and with good responses to corticosteroids[56,58].

In contrast, MHC Ⅱ antigens have shown significant heterogeneity among different ethnicities. Patients with HLA-DRB1*13 and -DRB1*03 have an earlier onset of disease compared to other patients, possibly because their ethnic groups that have a tendency toward AIH onset at younger ages. Moreover, certain ethnic groups have low prevalences of these immunophenotypes, such as the populations of Mexico and Japan, where HLA-DRB1*04 is more common, and these low rates seem to establish increased susceptibility to the disease in older people[50-52]. Few studies have demonstrated the role of immunophenotypes in HAI in children; to apply these markers as indicators of response to treatment and prognosis, more studies are needed.

The known physiopathological mechanism in AIH consists of an inflammatory response with T-lymphocyte cells, principally helpers, and B lymphocytes, macrophages and natural killer cells. The triggering factor or factors that stimulate this inflammatory response are not yet known. Several mechanisms have been proposed that would partially explain the immunologic findings of AIH[7,59].

Studies in adults and children have identified some potential pathways for the damage observed in AIH, such as the deregulation of immunoregulatory mechanisms. Some of the studies have shown that AIH patients have reductions in the number and function of T lymphocytes $CD4^+CD25^+$, which is one of the regulatory cells (T-regs) that normally represent 5%-10% of CD4 T cells in healthy humans[7,59-66]. These cells suppress the proliferation and cytokine responses of effectors CD4 and CD8 T cells, and they down-regulate the functions of macrophages, dendritic cells, natural killer cells, and B lymphocytes[62].

All immune findings are more pronounced in the initial presentation than after remission with treatment[61,62,66,67]. T-reg immunosuppressive functioning causes the production of anti-inflammatory cytokines, such as interleukin-4 (IL-4), interleukin-10 (IL-10) and transforming growth factor (TGF)-beta[68,69]. The surface markers involved in anti-inflammatory mechanisms are glucocorticoid-induced tumor necrosis factor receptor (CD62L), cytotoxic T lymphocyte-associated protein-4 (CTLA-4) and fork head/winged helix transcription factor (FOXP3)[62,70]. If the mechanisms of failure become known, new treatments, based on recuperation of the function of T-regulation, could be used in AIH[70-72].

Natural killer T cells ($CD3^+$ and $CD56^+$) are found in reduced numbers, producing lower levels IL-4 and IL-2 in AIH patients. These lower levels result in reduction of the surface expression of CTLA-4 in $CD4^+$T cells, playing a pivotal role in liver autoaggression, especially during the active phase of the disease[61,72]. Kurokohchi *et al*[73] also found that the levels of CTLA-4 were reduced in inflammatory cells from the peripheral blood of AIH patients, compared with controls, while levels of $CD80^+$ and $CD86^+$ were increased in liver-infiltrating cells. Other research has shown that the CCR5 cytokine receptor was preferentially expressed on Th1 cells. This cytokine plays a pivotal role in the recruitment of interferon-gama (IFN-γ) (a pro-inflammatory cytokine), producing $CD4^+$ T cells at inflammatory sites, such as hepatic tissue, and promoting hepatocyte damage in AIH[73,74]. Another possibility involves the presence of CD4 and/or CD8 self-reactive T cells, which could damage liver cells. These cells are found in healthy people, but in AIH patients, they are 10-fold higher in number[68,75].

Studies have also suggested that mutations in these genes act as precursors of the surface markers of immune cells and might also have significance in autoimmune diseases because changes in HLA (MHC) are absent in some patients. Mutations of several lympho-

Table 2 Major histocompatibility complex class Ⅱ human leukocyte antigen and its association with autoimmune hepatitis patients

Ref.	Total No. of patients/ controls (No. of children)	What was evaluated	Conclusions
Donaldson *et al*[48]	96/100 (no)	HLA-DR	HLA-DR3 and DR4 genes independently confer susceptibility to autoimmune hepatitis
Fortes Mdel *et al*[50]	41/111 (13)	HLA-A, -B, -C, -DR and DQ	Regarding HLA-A and -C there were no significant differences between groups; For HLA class Ⅰ, an increase in the frequency of B*08, B*18, B*45 and B*50 was observed. HLA B*40 was more frequent in healthy controls; For HLA class Ⅱ, an increase in the frequency of HLA-DQB1*02, -DQB1*04, HLA-DRB1*03, DRB1*13 and DRB3 was observed. HLA-DRB1*1301 and -DRB1*0301 were more frequent in children
Ota *et al*[51]	51/no (no)	HLA-DR and -DQ	Increased frequency of all HLA-DRB1*04 alleles, principally -DRB1*0405. Secondary association with -DRB1*15 and DRB1*16
Vázquez-García *et al*[52]	30/175 (not cited)	HLA-A, -B, -C, -DR and -DQ	A significant association with HLA-DRB1*0404 was found. It was present in patients with average age onset. DQB1*0301 had a low frequency in patients and may represent a protective factor; No association was found with any class Ⅰ antigen
Fainboim *et al*[53]	52/197 (all)	HLA-A, -B, -C, -DR and -DQ	No significant associations with HLA class Ⅰ antigens were found; HLA-DR6 group (HLA-DRB1) showed increased frequency, principally HLA-DRB1*1301; The analyses of HLA-DQ group showed an associations of HLA-DQB1*0603
Pando *et al*[54]	206/208 (122)	HLA-DR and -DQ	The frequencies of HLA-DRB1*1301, -DRB1*0301, -DQA1*0103, -DQB1*0603 were significantly increased on AIH patients; HLA-DRB1*1301 was associated with younger age at disease onset, being the allele associated with AIH in children and HLA-DRB1*1302 worked as a protective factor
Bittencourt *et al*[55]	139/129 (74)	HLA-DRB and -DQB1	In AIH type 1, there was significant increase in the HLA-DRB1*13, -DRB1*03, -DRB3 and -DQB1*06 alleles in patients. HLA-DRB1*13 was more frequent in children than adults. The low frequency of HLA-DQB1*0301 may indicate a protective role of this allele; In AIH type 2, a significant increase in DRB1*07, DRB1*03, DRB4 and DQB1*02 was observed
Czaja *et al*[56]	86/102 (not cited)	HLA-A, -B, -C, -DR and -DQ	DRB4*0103 is associated with immune diseases, DRB1*0301 with a poor treatment response, and DRB1*0401 with a lower frequency of hepatic death or transplantation
Czaja *et al*[57]	210/396 controls with other chronic liver disease/102 healthy controls (no)	HLA-DR B1*03, -DRB1*04 and -DRB1*13	The frequency of HLA DRB1*13 was higher in patients without -DRB1*03 and -DRB1*04; Primary sclerosing cholangite patients showed a similar frequency of HLA-DRB1*13 when compared with AIH patients

HLA: Human leukocyte antigen; MHC: Major histocompatibility complex; AIH: Autoimmune hepatitis.

cyte surface markers studied could represent molecular markers of autoimmunity in AIH. Among them is the CTLA-4 (CD152) gene mutation, which has appeared in controversial reports of the phenotypes that represent susceptibility to AIH[76-81]. For instance, in the Brazilian study by Bittencourt *et al*[77] no association was established between exon 1 *CTLA-4* gene polymorphisms at position 49 and AIH susceptibility, contradicting findings in a North American population[78].

CTLA-4, which is expressed on the surface of T cells, induces peripheral tolerance by biding CD80 and CD86 on antigen-presenting cells. In doing so, CTLA-4 competes with the co-stimulatory molecule CD28, reducing the immune response[47]. CTLA-4 is considered a critical coordinator in immune regulation. Based on this finding, some researchers have attempted to find a drug that simulates its mechanism and that could be used in the treatment of autoimmune conditions; one such drug is an immunoglobulin G-CTLA-4 (Abatacept), which was recently approved by the FDA for use in rheumatoid arthritis[82,83].

Furthermore, some studies have aimed to evaluate whether a *Fas* gene polymorphism or its increased expression on lymphocyte surfaces could be key mechanisms for autoimmunity in AIH. Fas (CD95) is part of the tumor necrosis factor family, and it induces receptor-mediated programmed cell death (apoptosis) through engagement with its ligand (FasL/CD95L). It indirectly controls the number of antigen-activated lymphocytes[84]. Ogawa *et al*[85] showed that AIH patients show an increase in CD95 (Fas)-positive $CD4^+$ and $CD8^+$ T cell numbers. These individuals show disease courses with high levels of conversion of naive $CD45RO^-$ to primed $CD45RO^+$ $CD4^+$ T cells. This course could indicate that constant activation of T lymphocytes and/or the persistent presence of activated lymphocytes requires continuous work from regulation cells, such as $CD95^+$ T $CD4^+$[85]. Tsikrikoni *et al*[86] also found a greater number of Fas^+ and $FasL^+$ cells in the mononuclear cells of AIH patients and increased TNF-α and IFN-γ production in cultured cells, suggesting that these cytokines could be involved in accelerating apoptosis. They also showed an increase in $CD14^+$ monocyte cell numbers, in accordance with the increased

expression of apoptotic markers, such as $CD14^+$ cells, responding to the clearance of apoptotic cells[87]. Concomitantly, the results of genetic studies have shown that some mutations can affect the function of Fas receptors, but more research is needed to determine these receptors' relationship with AIH[88-91].

A lack of consistent evidence has persisted for studies evolving genes of cytokines, such as tumor necrosis factor; TGF-beta1, and TBX21, (a regulator of T lymphocyte lineage development and a controller of the expression of IFN-γ)[91-98].

TREATMENT

An important feature of AIH is response to treatment with corticosteroids and immunosuppressants[2,6,99]. Prednisone alone or in combination with azathioprine is the main form of treatment[99]. This treatment has the goal of reducing hepatic inflammation, the induction of clinical remission, relief of symptoms and improvement of survival. The treatment response characterizes clinical improvement and a reduction of aminotransferases to normal or to no more them two times of the maximum of the reference value, while remission lies in clinical improvement, normalization of aminotransferases and gamma globulin, autoantibody reduction or extremely low titers of autoantibodies and histological resolution of inflammation with a reduction in fibrosis[3,44]. Moreover, relapse is characterized by increased transaminases after remission has been achieved, as shown by Ferreira *et al*[44,100]. Relapse is common during treatment and occurs in up to 40% of patients, requiring a temporary increase in the dose of corticosteroid[3,99]. Noncompliance play a prominent role in a percentage of relapses[44,100]. Some medications offer alternative treatment, such as cyclosporine, tacrolimus and mycophenolate mofetil. These drugs are reserved for patients who fail to respond to the first treatment choice[2,6]. In cases of autoimmune sclerosing cholangitis and autoimmune cholangitis, the use of ursodeoxycholic acid can be necessary to control bile duct disease[2].

Liver transplantation is the last-line treatment indicated for patients who have not responded to medication. The need for transplantation is present in 8.5% of children with HAI[8]. The total duration of immunosuppressive therapy has not been established, but in the face of the possible side effects with medication, discontinuation of treatment should be considered when the remission criteria are met in patients with type 1 AIH[3]. To meet this goal, the patient must present histological resolution of inflammation after at least two years of clinical and laboratory remission (normal liver enzymes, liver function and gamma globulin and autoantibodies in low or undetectable titers)[3]. Approximately 20% of patients with type 1 AIH can remain in remission after discontinuation of treatment, but relapses are frequent after the suspension of treatment[6,8,100]. In type 2 AIH, treatment discontinuation is not recommended because relapses are more frequent, and failure of remission upon suspension is almost certain in this condition[8].

The prognosis of patients who respond to immunosuppressive treatment is good, even when there is cirrhosis at baseline; there is a good quality of life and, in general, use of low doses of medication[2-4]. Except for the changed autoantibodies that were initially detected, no markers are currently used in clinical practice to choose and follow treatment.

CONCLUSION

In conclusion, recent studies have shown new possibilities for the diagnosis and prognostic evaluation of AIH, except for in the pediatric age group, which remains unrepresented in these assessments. Susceptibility to autoimmune diseases is multifactorial, but genetic and immunological factors play pivotal roles. MHC Ⅱ antigens could represent a susceptibility marker for AIH, considering the differences between ethnic groups, or they might predict treatment response and prognosis. Finally, in pediatric populations, the prevalence and titers of autoantibodies can be different from in adults, such as for the MHC Ⅱ HLA-DRB1*1301, which can be a marker of susceptibility in the pediatric population.

Perhaps in the future, knowledge of autoimmune mechanisms will reveal better markers for the diagnosis, monitoring and treatment of AIH and other autoimmune diseases, but there are still only few available studies with good suggestions for markers.

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ORIGINAL ARTICLE

Antioxidant properties of glutamine and its role in VEGF-Akt pathways in portal hypertension gastropathy

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Abstract

AIM: To investigate the effects of glutamine on oxidative/nitrosative stress and the vascular endothelial growth factor (VEGF)-Akt-endothelial nitric oxide synthase (eNOS) signaling pathway in an experimental model of portal hypertension induced by partial portal vein ligation (PPVL).

METHODS: Portal hypertension was induced by PPVL. The PPVL model consists of a partial obstruction of the portal vein, performed using a 20 G blunt needle as a guide, which is gently removed after the procedure. PPVL model was performed for 14 d beginning treatment with glutamine on the seventh day. On the fifteenth day, the mesenteric vein pressure was checked and the stomach was removed to test immunoreactivity and oxidative stress markers. We evaluated the expression and the immunoreactivity of proteins involved in the VEGF-Akt-eNOS pathway by Western blotting and immunohistochemical analysis. Oxidative stress was measured by quantification of the cytosolic concentration of thiobarbituric acid reactive substances (TBARS) as well as the levels of total glutathione (GSH), superoxide dismutase (SOD) activity, nitric oxide (NO) production and nitrotyrosine immunoreactivity.

RESULTS: All data are presented as the mean ± SE. The production of TBARS and NO was significantly increased in PPVL animals. A reduction of SOD activity was detected in PPVL + G group. In the immunohistochemical analyses of nitrotyrosine, Akt and eNOS, the PPVL group exhibited significant increases, whereas decreases were observed in the PPVL + G group, but no difference in VEGF was detected between these groups. Western blotting analysis detected increased expression of phosphatidylinositol-3-kinase (PI3K), P-Akt and eNOS in the PPVL group compared with the PPVL + G group, which was not observed for the expression of VEGF when comparing these groups. Glutamine administration markedly alleviated oxidative/nitrosative stress, normalized SOD activity, increased levels of total GSH and blocked NO overproduction as well as the formation of peroxynitrite.

CONCLUSION: Glutamine treatment demonstrated to reduce oxidative damage but does not reduce angiogenesis induced by PH in gastric tissue, demonstrating a beneficial role for the PI3K-Akt-eNOS pathway.

Key words: Partial portal vein ligation; Oxidative stress; Glutamine; Portal hypertension; Rats

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INTRODUCTION

Portal hypertension (PH) is a clinical syndrome that is usually secondary to obstruction of the intra- or extra-hepatic portal flow. It is considered the main complication of liver disease, being responsible for the development of other liver diseases, such as portal hypertensive gastropathy, ascites, spontaneous bacterial peritonitis, hepatorenal syndrome, hepatopulmonary syndrome, portopulmonary hypertension, hyperkinetic syndrome and hepatic encephalopathy[1].

PH is characterized by an increase in pressure above 5 mmHg in the portal venous system. When the pressure reaches 8 to 10 mmHg, in the esophagus and stomach, gastroesophageal varices arise, which develop from a network of collateral circulation through the vessels that form the splanchnic circulation. Bleeding from gastroesophageal varices can occur when the portal pressure gradient reaches values above 12 mmHg[2-4].

Numerous veins dilate, including the hemorrhoidal plexus, abdominal wall and esophagogastric junction. The umbilical vein communicates with and dilates the superficial veins of the abdominal wall, and the presence of abdominal collateral circulation is an important clinical sign of portal hypertension, which is characterized by dilated and tortuous veins radiating from the navel to the upper abdomen and lower chest[5,6]. Collateral circulation of the left gastric vein to the azygos vein is responsible for esophagogastric varicose veins and increased circulation in the gastric mucosa, which characterize the complications of portal hypertension referred to as portal hypertension gastropathy (PHG)[5].

The vascular endothelium releases vasodilators, including nitric oxide (NO) and prostacyclins, and vasoconstrictors, including endothelin, angiotensin and thromboxane. The function of vascular tone is maintained by balancing these agents. The increased peripheral resistance is maintained by elevation of vasoconstrictors or vasodilators or by reducing the levels of both. The blood exerts a force against endothelial cells, which are the main agonist in the release of NO[7].

Increases in NO synthesis have also been reported in the liver of rats with PH. Moreover, NO production has been implicated in the pathogenesis of PHG, with increases in NO serum levels being detected in patients with PHG[5]. When present in high concentrations, NO acts as a free radical, forming two molecules of dinitrogen trioxide (N_2O_3) or peroxynitrite (ONOO), which are responsible for cytotoxic effects such as inflammation and septic shock. Recently, NO has been presented as an important signal of the maintenance of homeostasis, as well as a cytotoxic agent involved in numerous diseases[8-10].

Increased formation of blood vessels in the splanchnic region occurs through the process of angiogenesis, which is involved in the maintenance of hyperdynamic circulation in portal hypertension. This hypothesis is based on recent studies that demonstrate the presence of increased splanchnic angiogenesis and neovascularization, which are responsible for the formation of portosystemic collaterals in experimental models of PH[11]. The stimulus for the proliferation of new blood vessels occurs through a complex cascade of angiogenic events, and it is the main modulator of this mechanism, vascular endothelial growth factor, vascular endothelial growth factor (VEGF), that stimulates the proliferation and migration of endothelial cells. Overproduction of NO is stimulated by endothelial nitric oxide synthase (eNOS), which can be stimulated by both VEGF and phosphatidylinositol-3-kinase (PI3K). In turn, PI3K-Akt also receives stimulation through the VEGF pathway, and the shear stress that occurs in PH can be a factor in stimulation *via* PI3K-Akt as well[12-14]. The Akt protein directly stimulates eNOS by increasing the capacity of eNOS to generate NO. There are several ways to stimulate the Akt pathway, including through growth factors, cytokines and the mechanical force of shear stress in a blood vessel, which activates the NO release mechanism in a PI3K-dependent manner[15,16]. Overproduction of vascular NO plays a central role in both systemic and splanchnic vasodilations, which are characteristics of portal hypertension that cause it to be recognized as a major complication of liver cirrhosis. The increased expression and activity of eNOS are well-established events in chronic models of portal hypertension[17,18].

Glutamine, a nonessential amino acid, has received increasing attention because it becomes essential during stress and catabolic conditions[19]. Glutamine administration can result in an enhanced antioxidant capacity in various situations, such as critical illness or sepsis[20]. In the stomach, glutamine is able to protect against peptic ulceration and improves the healing of ulcers[21]. The present study was designed to investigate the potential beneficial effects of glutamine administration on gastric oxidative stress and to evaluate the role of the VEGF-PI3K-Akt-eNOS pathway in NO overproduction in an experimental model of PHG.

MATERIALS AND METHODS

Ethics

All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23, revised 1985).

Animals and experimental groups

Male Wistar rats with a mean weight of 250 g were used. The animals were obtained from the Center for Breeding

of Laboratory Animals of the Federal University of Rio Grande do Sul. The rats were held in cages at 20-24 ℃ with a 12 h light/dark cycle and given free access to food and water. They were randomly divided into the following four groups of fourteen animals each: (1) [sham-operated (SO) rats receiving only NaCl as a vehicle]; (2) SO + G (SO rats receiving glutamine); (3) PPVL (PPVL rats receiving vehicle); and (4) PPVL + G (PPVL rats receiving glutamine).

Partial portal vein ligation and sham operation

During the procedure, rats were anesthetized with a ketamine (Ketalar, Parke Davis, 100 mg/kg) and xylazine 2% (Rompun, Bayer, 50 mg/kg) cocktail *ip*. PH was induced by partial portal vein ligation (PPVL) as described by Moreira *et al*[22]. Briefly, the portal vein was isolated, and a 3-0 silk ligature was tied around both the portal vein and an adjacent 20 gauge blunt-tipped needle. The needle was subsequently removed, and the vein was allowed to re-expand. The abdomen was then closed, and the animal was allowed to recover. Control rats underwent a similar operation but without ligature of the portal vein. Sham-operated animals received only vehicle (NaCl, 1 mL/kg, *ig*). Glutamine was administrated daily (14 mg/kg, daily, *ig*) for 7 d beginning on the eighth day after the surgical protocol. All rats were anesthetized and sacrificed on the fifteenth day of the protocol. Their stomachs were immediately removed and divided into four subsamples that were stored in a freezer at -80 ℃ for analysis of oxidative stress, total glutathione, immunohistochemistry and Western blotting.

Oxidative stress determinations

Gastric oxidative stress was determined by measuring the concentration of aldehydic products (TBARS). Briefly, the frozen tissue was homogenized in a solution containing 140 mmol KCl and 20 mmol phosphate buffer (pH 7.4) and centrifuged at 14000 *g* for 10 min. For TBARS analysis, the amount of aldehydic products generated by lipid peroxidation was measured *via* the thiobarbituric acid reaction using 3 mg of protein per sample. The samples were incubated at 90 ℃ for 30 min following the addition of 500 mL of 0.37% thiobarbituric acid in 15% trichloroacetic acid and then centrifuged at 2000 *g* for 15 min. The spectrophotometric absorbance of the supernatant was determined at 535 nm[23].

Nitric oxide quantification

Nitric oxide production in the gastric tissue was measured indirectly using a quantitative colorimetric assay based on the Griess reaction. This method is sensitive for both nitrite and nitrate ions[24]. Briefly, the samples were deproteinized and subsequently centrifuged for 20 min at 12000 *g*. After incubation of the supernatants with *E. coli* nitrate reductase (37 ℃, for 30 min) to convert nitrates to nitrites, 1 mL of Griess reagent (0.5% naphthylethylenediamine dihydrochloride, 5% sulfonylamide, 25% phosphoric acid) was added. The reaction was allowed to proceed at room temperature for 20 min, after which the absorbance at 546 nm was measured using a sodium nitrate solution as a standard.

Antioxidant enzyme activities

Cytosolic superoxide dismutase (SOD; EC 1.15.1.1) was assayed spectrophotometrically based on the rate of epinephrine autooxidation, which is progressively inhibited by increasing amounts of SOD in the homogenate; the amount of enzyme that results in 50% of the maximum inhibition of autooxidation is defined as 1 unit of SOD activity[25]. For analysis of glutathione (GSH) and GSSG, the livers were homogenized with 5% (w/v) metaphosphoric acid. After centrifugation (16000 *g* for 2 min), the tissue homogenate was assessed spectrophotometrically (415 nm) in a microplate reader employing a modified version of the 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, Sigma)/GSSG reductase (Sigma) recycling method using the 43 *N*-ethylmaleimide (NEM, Fluka) conjugating sample preparation technique for GSSG. Samples (10 L) for both GSH and GSSG determination were assayed in 96-well polystyrene plates (Corning) at 37 ℃ in the presence of 10 mmol DTNB, 0.17 mm β-NADPH (Sigma, dissolved in 0.5% (w/v) $NaHCO_3$ as a Stabilizing agent) and 0.5 U/mL GSSG reductase[26].

Western blotting analysis

The technique used for this measurement was protein expression by Western blotting analysis employing the system described by Laemmli[27] for electrophoresis and the blotting technique described by Towbin *et al*[28]. Proteins (80 g) were separated in a 10%-15% polyacrylamide gel and transferred electrically to polvinylidene difluoro membranes (Millipore, Bedford, MA, United States). Subsequently, the membranes were placed in Tris/saline-tamponade/Tween-20 blocking solution (TBST-5% milk powder in Tris-buffered saline containing 0.05% Tween 20) for 60 min at 37 ℃. The membrane was incubated overnight at 4 ℃ with polyclonal eNOS and VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, United States), P-Akt and PI3K (Cell Signaling Technology, Danvers, MA, United States). Thereafter, the membranes were washed with TBST and incubated for one hour at room temperature with an anti-rabbit immunoglobulin antibody coupled to HRP (SIGMA, Glostrup, Denmark). The proteins were detected by chemiluminescence using a commercial ECL kit (Amersham Pharmacia Biotech, Uppsala, Sweden), and the density of specific bands was quantified using a densitometer image (Image J, United States).

Immunohistochemistry

Tissues sections (4 m) soaked in a formalin fixative and embedded in paraffin were subjected to immunohistochemical analysis[29]. This technique consisted of the following steps: deparaffinization, rehydration, antigen retrieval, inactivation of endogenous peroxidase and blocking of nonspecific reactions. The samples were incubated with the primary antibody for 12 h at 4 ℃ us-

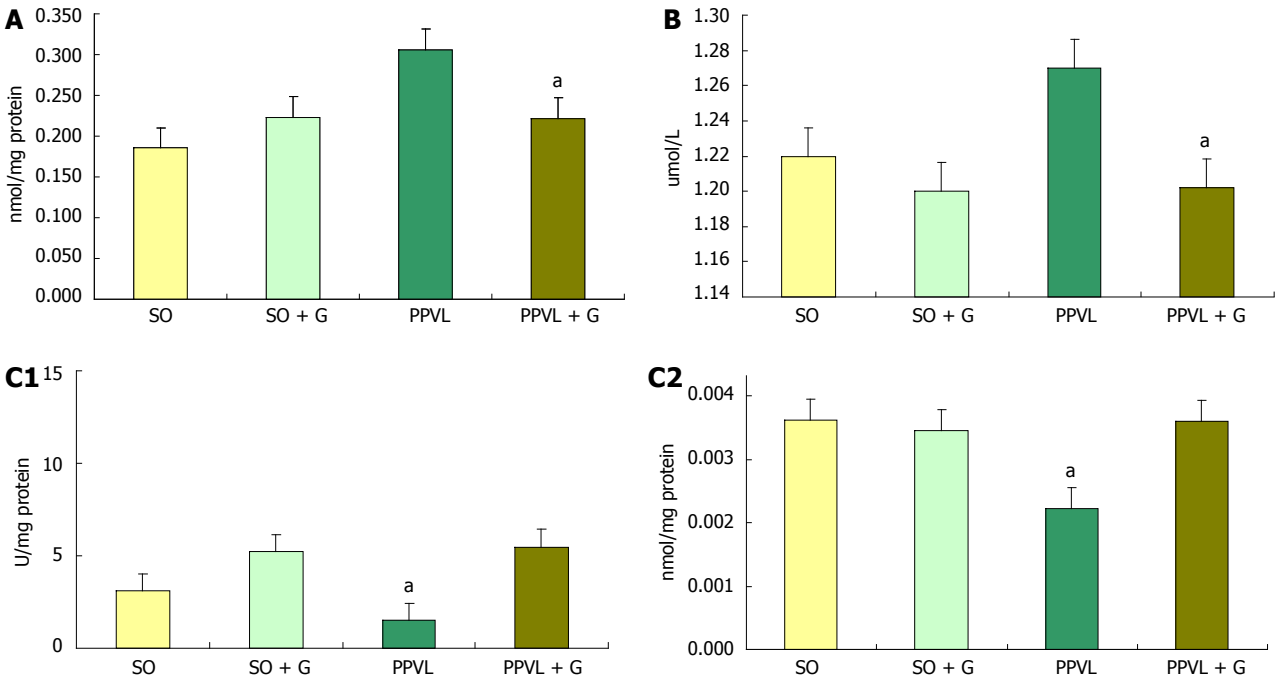


Figure 1 Effect of partial portal vein ligation and glutamine administration on gastric oxidative stress, nitric oxide production, antioxidant enzyme activities. A: Effect of partial portal vein ligation and glutamine administration on gastric oxidative stress; B: Effect of partial portal vein ligation and glutamine administration on gastric nitric oxide production; C: Effect of partial portal vein ligation and glutamine administration on gastric antioxidant enzyme activities: (1) SOD activity; and (2) GSH activity. TBARS concentration. Values are the mean ± SE for 14 rats. $^{a}P < 0.05$ *vs* the sham-operated group. PPVL: Partial portal vein ligation; G: Glutamine; SOD: Superoxide dismutase; GSH: Glutathione; TBARS: Thiobarbituric acid reactive substances; SO: Sham-operated.

ing the specific dilution of each antibody indicated in the instructions. After we applied the streptavidin-biotin complex (LSAB, DAKO) using a diaminobenzidine revelation tetrahydrochloride Kit (DAB, DAKO) and the samples were counterstained with hematoxylin. The antibodies used in the gastric mucosa samples were eNOS and VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, United States), AKT (Cell Signaling Technology, Danvers, MA, United States) and NTT (Sigma, United States).

Statistical analysis

The data were calculated and analyzed using analysis of variance. A *post hoc* multiple comparisons test was performed using the Student Newman-Keuls test. Values were considered significant when $P < 0.05$. All calculations were performed using the statistical program Graphpad Prism, version 14.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Markers of oxidative stress

The cytosolic concentration of TBARS increased in PPVL group, compared to SO. And decrease significantly on PPVL + G group compared to PPVL. Glutamine administration was effective in diminishing TBARS production (Figure 1A).

Nitric oxide levels

The concentrations of nitrites in the gastric tissue (Figure 1B), the values were significantly higher in PPVL group compared to SO. Moreover, nitrite concentrations were similar to PPVL + G and SO groups. This parameter was restored to baseline levels in animals PPVL receiving glutamine.

Antioxidant enzyme activities

Analysis of antioxidant enzyme activities showed that portal vein ligation induced a considerable reduction of gastric SOD in PPVL group compared to SO, SO + G and PPVL + G. Glutamine treatment increased SOD activity and GSH activity (Figure 1C) on PPVL + G group.

Western blotting analysis

Analyses of the protein expression of PI3K (Figure 2B), P-Akt (Figure 2A) and eNOS (Figure 3A) showed that there was reduced expression in the PPVL + G group compared with the PPVL group. This effect was not observed for the expression of VEGF (Figure 3B), for which a significant increase was observed in the PPVL group compared with the SO group, although no reduction was observed when compared with the PPVL + G group.

Immunohistochemistry

Analyses of the reactivity of the eNOS (Figure 4A), Akt (Figure 4C) and NTT (Figure 4D) proteins in the gastric mucosa of rats showed that there was reduced expression in the PPVL + G group compared with the PPVL group. This effect was not observed for the expression of VEGF (Figure 4B), for which a significant increase

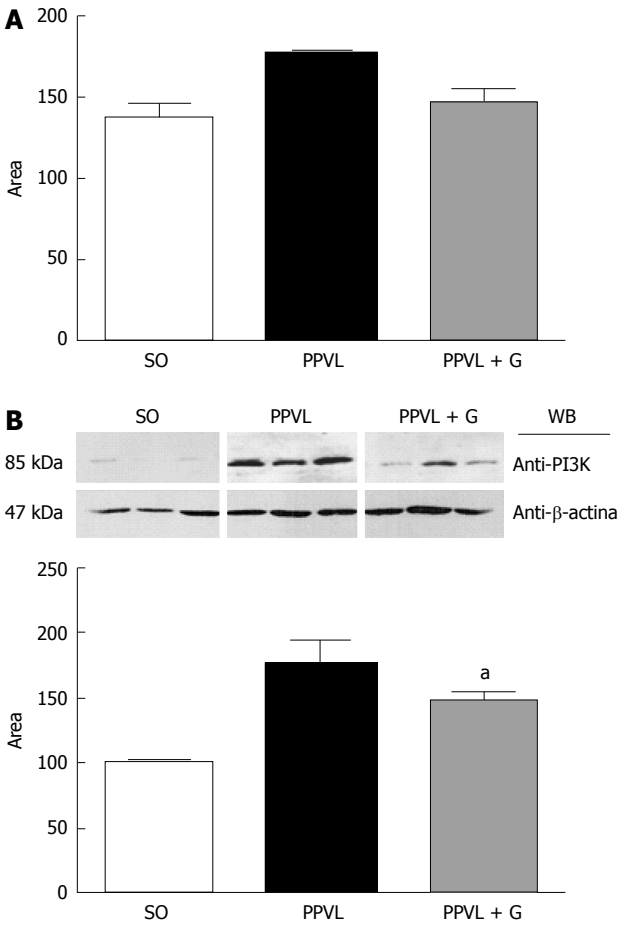


Figure 2 Expression of P-Akt and phosphatidylinositol-3-kinase determined by Western blotting in gastric tissue from rats in the sham-operated group, the partial portal vein ligation group and rats subjected to portal vein ligation and glutamine treatment. A: An increase in P-Akt protein expression was detected in the PPVL group *vs* the SO group; B: An increase in PI3K protein expression was found in the PPVL group *vs* the SO group. The PPVL + G group showed reduced expression of this enzyme *vs* the PPVL group. SO: Sham-operated; PPVL: Partial portal vein ligation; G: Glutamine; PI3K: Phosphatidylinositol-3-kinase; WB: Western blotting. [a]*P* < 0.05 *vs* the SO group.

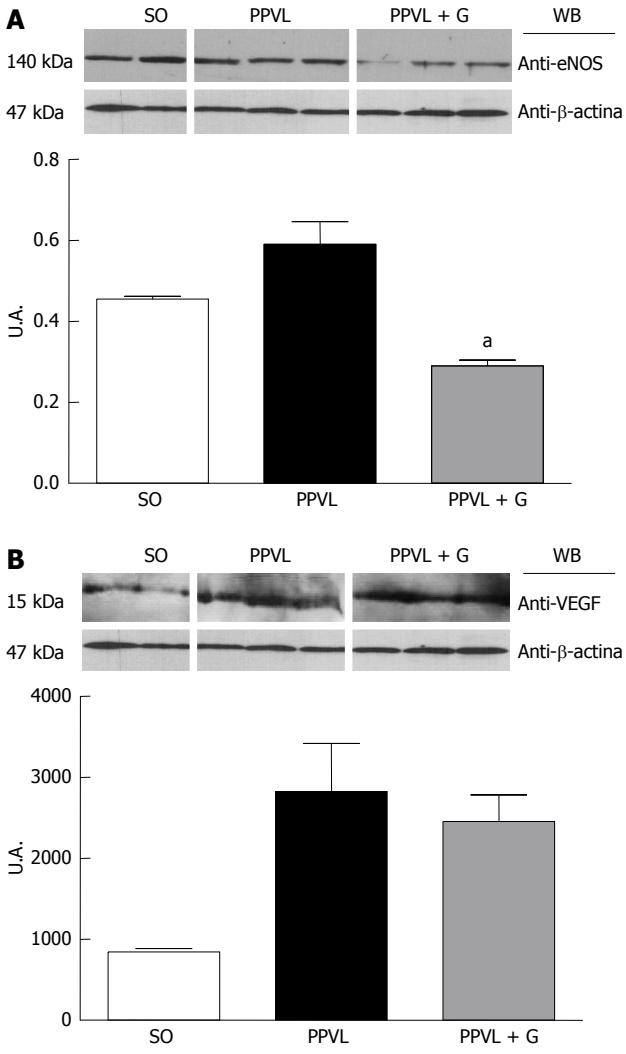


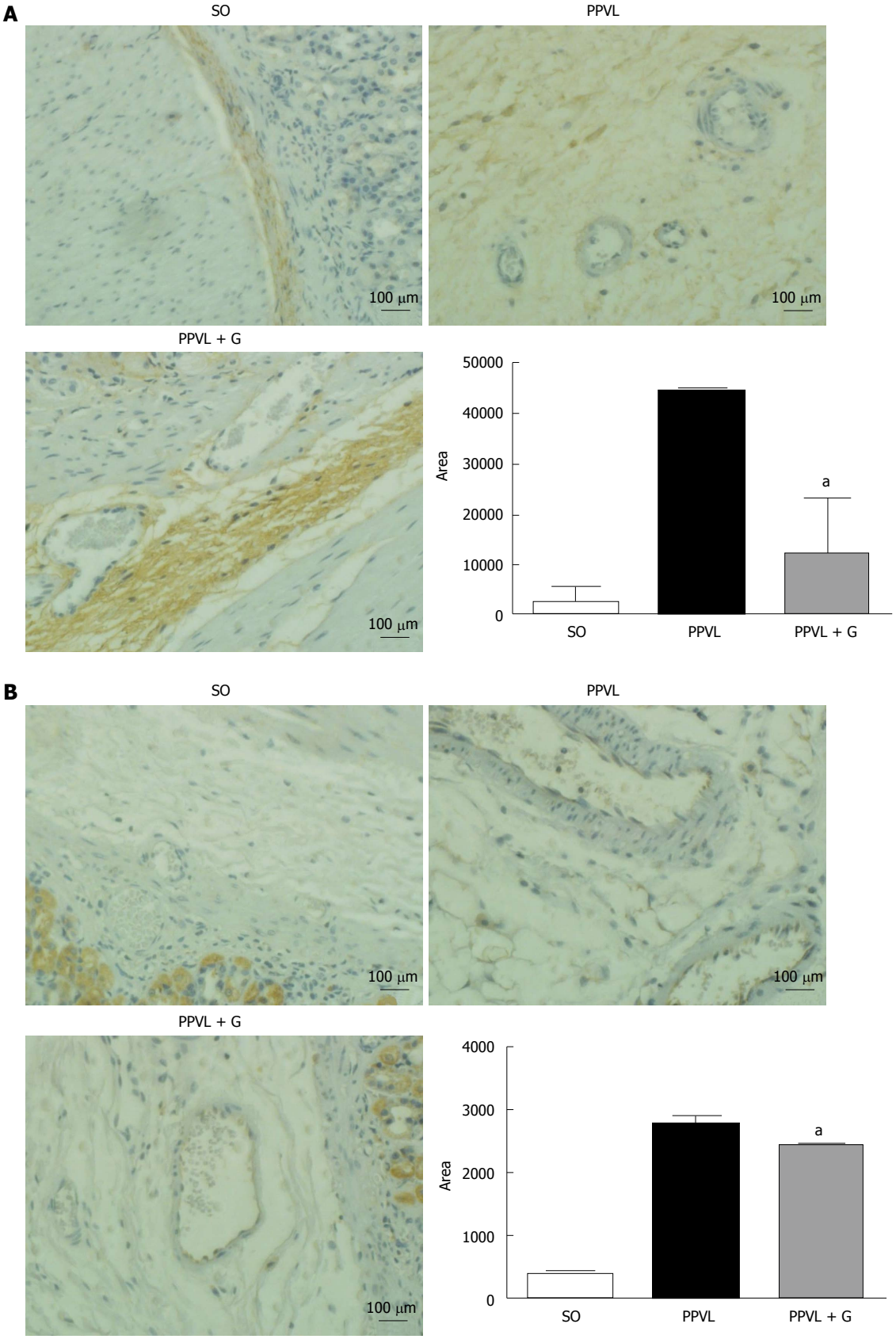
Figure 3 Expression of endothelial nitric oxide synthase and vascular endothelial growth factor determined by Western blotting in gastric tissue from rats in the sham-operated group, the partial portal vein ligation group and rats subjected to partial portal vein ligation and glutamine treatment. A: An increase in eNOS expression was observed in the PPVL group *vs* the SO group. The PPVL + G group showed reduced expression of this enzyme *vs* the PPVL group; B: An increase in VEGF expression was detected in the PPVL group *vs* the SO group. The PPVL + G group did not show a reduction in the expression of VEGF *vs* the PPVL group. SO: Sham-operated; PPVL: Partial portal vein ligation; G: Glutamine; VEGF: Vascular endothelial growth factor; eNOS: Endothelial nitric oxide synthase; WB: Western blotting. [a]*P* < 0.05 *vs* the SO group.

was observed in the PPVL group compared with the SO group, although no reduction was observed when compared with the PPVL + G group.

DISCUSSION

PHG is recognized as a clinical condition in PHI, but the exact pathogenesis of PHG is still unclear. The PPVL model has been extensively studied and found to be a useful tool for understanding the pathophysiology of PHI and PHG. This model has been developed in different animal species, such as rats, mice and rabbits, and it is presently accepted to be suitable for investigating the pathogenesis of PHI and PHG because is highly reproducible and easy to perform, and portal hypertension develops very rapidly. The model used in our study is characterized by prehepatic portal hypertension with maintenance of hepatic structure, hyperdynamic circulation and portal-systemic shunting. Treatment of portal hypertension to prevent complications, particularly gastrointestinal bleeding, is of fundamental importance and occurs under three clinical scenarios: prevention of first bleeding (primary prophylaxis), treatment after an episode of bleeding and prevention of secondary bleeding (secondary prophylaxis). These treatments can be performed using vasoconstrictors, which reduce portal venous flow; vasodilators, which reduce intrahepatic resistance; or a combination of both treatments[30].

Treatment using chemicals or natural products that would prevent complications of PH would represent a significant advance in therapy and could be a way to decrease mortality. Development of experimental models exhibiting pathogenic characteristics similar to those of human disease may help in understanding the mecha-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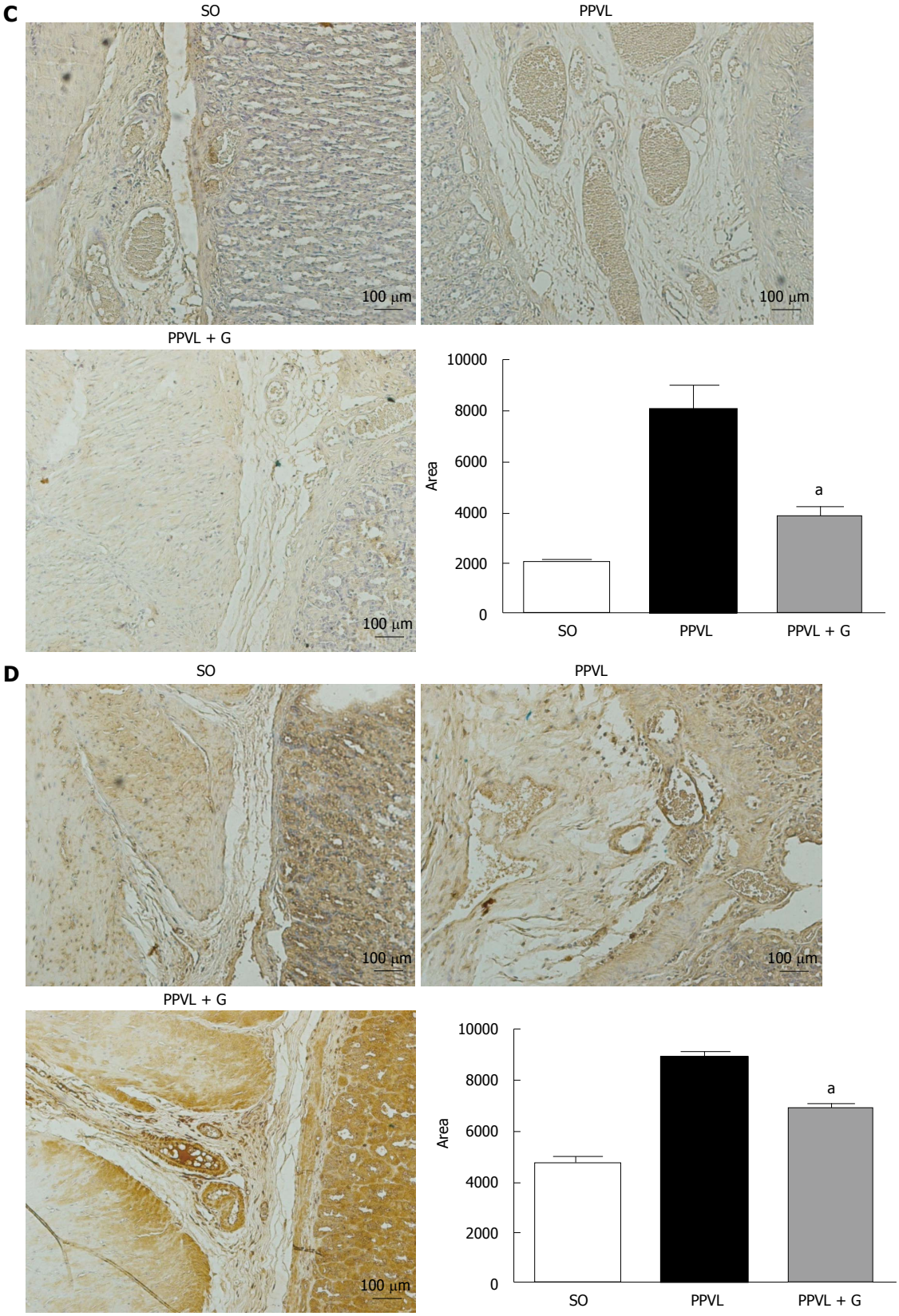

Figure 4 Representative expression of endothelial nitric oxide synthase, vascular endothelial growth factor, Akt and nitrotyrosine determined by immunohistochemical analysis of gastric tissue from rats in the sham-operated group, the partial portal vein ligation group and rats subjected to partial portal vein ligation and glutamine treatment. A: An increase in endothelial nitric oxide synthase (eNOS) expression was observed in the partial portal vein ligation (PPVL) group compared with the sham-operated (SO) group. The PPVL + glutamine (G) group showed reduced expression of this enzyme *vs* the PPVL group; B: An increase in vascular endothelial growth factor (VEGF) expression was observed in the PPVL group *vs* the SO group. The PPVL + G group did not show a reduction in the expression of VEGF *vs* the PPVL group; C: An increase in Akt protein expression was found in the PPVL group *vs* the SO group. The PPVL + G group showed a reduction in the expression of this enzyme *vs* the PPVL group; D: An increase in nitrotyrosine expression was observed in the PPVL group *vs* the SO group. The PPVL + G group showed reduced expression of this enzyme *vs* the PPVL group. Analysis of the field was carried out precisely in the submucosal region, where there is evidence of edema and vasodilatation. eNOS staining; original magnification × 10. $^{a}P < 0.05$ *vs* the SO group.

nisms of portal hypertension and allow testing of new therapeutic modalities. Thus, an experimental model of PH was employed in this study and has contributed to improving our understanding of the pathophysiological conditions presented in humans. PH may be triggered by various agents, leading to cirrhosis.

A study assessing the damage caused by oxidative stress in the gastric mucosa showed that there was an increase of LPO and decreases in the levels of the enzymes SOD, CAT and GPx in injured animals, suggesting that oxidative stress is involved in gastric tissue damage[31]. When we assessed lipid peroxidation in the stomachs of rats, an increase in the level of substances reacting with thiobarbituric acid (TBA-RS) was detected in the PPVL group compared with the SO group, which is suggested to be related to increased oxidative stress. This conclusion was supported by the amounts of TBA-RS observed in the PPVL + G group compared with the PPVL group. Similar results have been shown in other studies that used the flavonoid quercetin and *N*-acetylcysteine, suggesting that the amino acid glutamine exhibits antioxidant potential[32]. In the present study, glutamine reduced submucosal edema and vasodilation as well as reducing lipid peroxidation in homogenized stomachs from animals in the PPVL + G group. Glutamine, as a potential antioxidant, appears to protect the gastric mucosa and decrease oxidative damage to the gastrointestinal tract, which was observed both in this study and in previous studies of colitis employing glutamine[33].

The enzyme SOD, which is responsible for the dismutation of superoxide anion radicals into hydrogen peroxide, is the first line of defense against cellular oxidative stress. The SOD activity determined in the gastric mucosa in a study of patients with liver disease showed that this enzyme is found at reduced levels in these patients compared with control subjects with cirrhosis. However, individuals with chronic liver disease showed no significant changes in the levels of SOD. In liver diseases such as hepatitis, cirrhosis and hepatocellular carcinoma, there are high levels of LPO in the stomach, suggesting involvement of oxidative stress in gastric mucosal lesions in these liver diseases[34]. Studies relate the decrease in SOD enzyme activity to increased oxidative stress[35,36]. In the present study, we observed a decrease in SOD activity in the PPVL group compared with other groups, which could be related to the inactivation of superoxide anions, as this enzyme was acting in the dismutation of EAOS and the formation of H_2O_2. In contrast, animals in the PPVL + G group maintained values of SOD enzyme activity similar to controls. This increased activity of SOD in the PPVL group was due to the increased formation of EAOS, causing oxidative stress. Total GSH has a special physiological importance because glutamine serves as a substrate for glutathione formation. Studies have demonstrated the direct involvement of GSH in colitis[37]. A relationship was shown with glutathione colitis, in which rats with experimental colitis caused by TNBS showed a significant decrease in the level of glutathione compared with the control group[38].

In the evaluation of NO metabolites in stomach homogenates, we observed a reduction in the production of these metabolites in the PPVL + G group compared to the PPVL group. This increase in NO production in the PPVL group can be explained by the process of angiogenesis, which occurs in this model for the purpose of shunting of blood from the obstructed area to the systemic circulation. Moreover, these increased levels of nitric oxide could be reacting with superoxide anions to form peroxynitrite radicals, which are extremely harmful. This NO release becomes more pronounced due to the need for the formation of new blood vessels so that blood can reach the systemic circulation, triggering an increase in RL, thus stimulating lipid peroxidation and oxidative stress[39].

Oxidative stress plays an important role in the pathogenesis of PH because in association with the overproduction of superoxide anions and nitric oxide observed in the model of partial portal vein ligation, peroxynitrite formation occurs. Researchers have noted specific actions of ONOO as a modulator of intracellular signaling pathways regulating inflammatory responses, including induction of angiogenesis and VEGF.

The significant reduction of lipid peroxidation observed in animals in the PPVL + G group demonstrates effective action of the glutamine in the process of lipid peroxidation. This result is consistent with the expected capacity of glutamine in the formation of inactivating EAOS, especially in reducing the formation of peroxynitrite. Peroxynitrite reacts with free tyrosine and tyrosine residues in protein molecules to produce nitrotyrosine. Alternatively, EAOS can activate tyrosine to form tyrosyl, a radical that in turn oxidizes NO to produce nitrotyrosine (NTT)[40,41]. The generation of peroxynitrite was assessed based on the level of expression and immunoreactivity of NTT, with significant reductions being observed for both parameters in animals in the PPVL + G group, demonstrating the effectiveness of glutamine with respect to the oxidative/nitrosative damage that occurs in the gastric mucosa.

Upregulation of eNOS initiates the post-translational level mediated by Akt, which increases its activity at any concentration of cytosolic Ca^{2+}[42]. During early cirrhosis, this pathway is stimulated by different types of stimuli, such as VEGF, inflammatory cytokines and mechanical shear forces[42]. In advanced stages of portal hypertension, bacterial translocation also activates eNOS *via* tumor necrosis factor, increasing tetrahydrobiopterin, which is an essential element that acts as a cofactor of the enzyme. According to several studies, other mechanisms, such as changes in the subcellular localization of eNOS[43], *S*-nitrosylation[44] or degradation of asymmetrical dimethylarginine, may be involved in regulating the activity of eNOS[45]. We observed in this study that glutamine reduced eNOS protein expression and the immunoreactivity of the enzyme in the gastric mucosa of animals in the PPVL + G group. One explanation for

this finding is that glutamine, due to its involvement in NO synthesis, is involved in reduction of the cytosolic levels of Ca^{2+}, which is related to the levels of Akt; in turn, Akt levels are stimulated by mechanical stress shear. Throughout this process, this hemodynamic mechanism would be inhibited due to reduced levels of NO triggered by the action of glutamine.

Angiogenesis is characterized by the formation of new vascular structures and a pathophysiological phenomenon that has been further investigated in recent years due to the critical role it plays in the pathogenesis of disease and its potential as a therapeutic target. Additionally, angiogenesis contributes to a number of physiological processes, such as wound healing and the reproductive cycle[46]. NO released by dilation and increased vascular permeability as well as the migration, proliferation and survival of endothelial cells plays a crucial role in angiogenesis. Many molecules have been implicated as modulators of the angiogenic process, such as tumor necrosis factor, interleukins, angiopoeitins and growth factors, including VEGF. Indeed, VEGF stimulates NO production by NOS, increasing vascular permeability and the proliferation and survival of endothelial cells[47].

Furthermore, overproduction of ONOO, which is formed by the reaction of superoxide anions and nitric oxide, is observed under inflammatory conditions[48], whereas cytotoxic actions of ONOO have been reported *via* the modulation of intracellular signaling pathways that regulate inflammatory responses, including induction of angiogenesis and increased levels of VEGF. Studies in the retina of rats with diabetes induced by streptozotocin demonstrated the formation of nitrotyrosine and increased effects regarding stimulation of VEGF. These conditions have been studied because they induce the generation of reactive oxygen species (ROS). EAOS are involved in triggering the overexpression of VEGF in various cell types[49,50], but the molecular mechanisms underlying this effect remain to be elucidated. Studies in patients, in animals and *in vitro* indicate that formation of nitrotyrosine, a marker of ONOO, is associated with increased VEGF expression during diabetic microvascular disease, atherosclerosis and tumor angiogenesis[51,52]. In addition, studies using a cultured cell line showed that formation of ONOO stimulates an increase in VEGF[53]. In the present study, we observed increased expression and immunoreactivity of VEGF in animals in the PPVL group, but there was no significant reduction of these parameters in animals in the PPVL + G group. We suggest that these results demonstrate direct action of glutamine on NO synthesis to reduce its levels, rather than only affect the action of growth factors and the associated cascade of events.

VEGF also acts by stimulating PI3K, which activates eNOS in an Akt-dependent manner, resulting in activation of eNOS and increased NO production. PI3K phosphorylates Akt, which can rapidly activate eNOS[54]. The Akt pathway has emerged as a signaling pathway in all cells of higher eukaryotes, and Akt has been found to be one of the most important and versatile kinases for understanding human physiology and disease. Recent studies have been performed to elucidate the molecular details of the regulation of Akt and its role in human disease. The NO released due to eNOS stimulation is involved in vasodilation, vascular remodeling and angiogenesis[55]. The Akt signaling pathway also leads to increased production of transcription factors induced by hypoxia (HIF1α and HIF2α), partly through the activation of an mTOR-dependent mechanism[56]. The activation of eNOS through Akt/B kinase leads to increased NO production, vasodilatation and increased splanchnic circulation[57]. Akt activation occurs due to an increase in shear stress-induced endothelial disruption, although other mechanisms may be involved[58].

In this study, we observed a significant increase in the expression and immunoreactivity of Akt in the PPVL group and a reduction in these parameters in the PPVL + G group. These results can be explained by the role that glutamine plays in the production of NO, which in turn, triggers a chain reaction, is overproduced and dilates the splanchnic vessels, gradually increasing shear stress, which is the mechanical stimulus for the activation of Akt. In addition, it was observed that VEGF levels are unchanged in the PPVL + G group compared to the PPVL group, and the VEGF RTK pathway acts as a means for the release of PIK3, eNOS and P-Akt, leading to the production of more NO, which is a feature present in the hyperdynamic circulation associated with PH. Therefore, glutamine acts to mitigate this situation not by reducing the levels of NO *via* VEGF, but by reducing the shear stress triggered by NO synthesis through inhibition of *L*-arginine, thereby reducing the levels of Akt, which is stimulated by mechanical stress in blood vessels. In conclusion, we describe the beneficial effects of glutamine treatment on oxidative stress, including reduced portal pressure, normalization of SOD and reduction of NO production by eNOS, mediated by the PI3K-Akt-eNOS pathway. VEGF levels tended to decrease, although this trend was not found to be statistically significant.

COMMENTS

Background

Portal hypertension (PH) is the main complication of cirrhosis and is through its development is arising other liver diseases. Among them we mention the gastropathy of portal hypertension, which is characterized by the development of hyperdynamic splanchnic circulation. The main modulator of this process is nitric oxide release and their pathways in the pathophysiology of PH is still being studied. As there is no effective treatment for PH, it was decided to study the role of glutamine, an amino acid that has been used in the treatment of colitis, the clinic and evaluate its involvement both in oxidative stress, as in the intracellular stimulus for release nitric oxide.

Research frontiers

Studies are relevant in clinical hypertension, especially in hepatology. In addition, PH may be associated with other diseases such as schistosomiasis and budd-chiari syndrome. Therefore, the study of PH has a great clinical relevance for gastroenterology, since there is still no effective therapeutic treatment.

Innovations and breakthroughs

There are numerous studies using the experimental model of partial portal vein ligation,with which it mimics the complications of PH. Have been evaluated for

treatment of other molecules such as PH, quercetin and *N*-acetylcysteine. The results were similar with glutamine to these molecules, but there is when the comparative results on the role of intracellular pathways.

Applications

These results suggest that the amino acid glutamine is a molecule with therapeutic potential and can be used in the treatment of portal hypertension, reducing damage to the gastric mucosa caused by hypertension portal gastropathy.

Terminology

PH is characterized by increased vascular resistance and/or blood splanchnic flow; Glutamine: Essential amino acid involved in several cellular functions, among them acting as a substrate for the synthesis of antioxidants such as glutathione; Gastropathy of portal hypertension: Clinical syndrome secondary to cirrhosis, which is characterized by the formation of edema and dilation of gastric submucosal tissue.

Peer review

This is a quantitative study, in which the authors analyze the antioxidant effect and the possible involvement of glutamine in the gastric mucosa in an animal model of PH. The results are interesting and show that this molecule may have therapeutic potential in treatment of gastric mucosal lesions triggered by PH.

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ORIGINAL ARTICLE

Resistin mediates the hepatic stellate cell phenotype

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Abstract

AIM: To describe the role of resistin in liver fibrosis.

METHODS: For the *in vivo* animal study, Sprague Dawley rats were subjected to bile duct ligation (BDL) for 4 wk. Rat liver, adipose tissue (epididymal fat) and serum were analyzed for resistin expression. For the *in vitro* experiment, rat primary hepatic stellate cells (HSCs) and Kupffer cells (KCs) were used. HSCs were exposed to recombinant resistin, and collagen Ⅰ, transforming growth factor β1, α smooth muscle actin, tissue inhibitor of metalloproteinase 1 and connective tissue growth factor expression were analyzed. Resistin gene and protein expression was quantified as was the expression of pro-inflammatory cytokines including tumor necrosis factor α (TNFα), interleukin (IL)-1, IL-6, IL-8 and monocyte chemotactic protein-1 (MCP-1). The effects of resistin on HSC proliferation, migration and apoptosis were determined. The effects of resistin on KCs were also investigated.

RESULTS: Following BDL, rat epididymal fat and serum rather than liver showed higher resistin expression compared to control rats. In liver, resistin was expressed in quiescent HSCs and KCs. Resistin treatment resulted in enhancement of *TNFα*, *IL-6*, *IL-8* and *MCP-1* gene expression and increased IL-6 and MCP-1 protein in HSCs. Resistin activated HSC phospho-MAPK/p38, and p38 inhibition diminished IL-6 and MCP-1 expression. Furthermore, resistin facilitated HSC proliferation and migration, but decreased apoptosis which was *via* an IL-6 and MCP-1 mechanism. Finally, resistin-induced transforming growth factor β1 from KCs enhanced HSC collagen Ⅰ expression.

CONCLUSION: Resistin directly and indirectly modulates HSC behavior towards a more pro-fibrogenic phenotype.

Key words: Resistin; Hepatic stellate cell; Kupffer cell; Liver fibrosis; Monocyte chemotactic protein-1

Core tip: Resistin activated hepatic stellate cells (HSCs) phospho-MAPK/p38, and p38 inhibition diminished interleukin 6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) expression. Furthermore, resistin facilitated HSC proliferation and migration, but decreased apoptosis which was *via* an IL-6 and MCP-1 mechanism. Finally, resistin-induced transforming growth factor β1 from Kupffer cells enhanced HSC collagen Ⅰ expression. Resistin directly and indirectly modulates HSC behavior towards a more pro-fibrogenic phenotype.

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INTRODUCTION

Metabolic alterations such as glucose intolerance, increased energy expenditure, and negative nitrogen balance with depletion of fat and skeletal muscle mass are frequently encountered in patients with cirrhosis[1,2]. In particular, glucose intolerance and insulin resistance are almost universal[3,4] and, in part, mediate the progression of fibrosis[5]. However, the mechanisms whereby metabolic alterations mediate disease progression are unclear. Adipose tissue secreted proteins (adipokines) such as leptin and adiponectin modulate metabolic homeostasis and have direct effects on the hepatic fibrogenic cascade. For example, leptin promotes liver fibrosis, while adiponectin is anti-inflammatory and anti-fibrotic[6-9]. Resistin, another adipokine, has been reported to be associated with impaired insulin sensitivity and glucose intolerance[10-13], but its role in hepatic fibrosis has not been adequately delineated[14-17].

Resistin is almost exclusively expressed in the white adipose tissue of rodents, but is expressed in humans predominantly by monocytes/macrophages[18]. Several reports indicate that the serum levels of resistin are elevated in cirrhosis[14-16], increasing progressively with worsening liver function as determined by the Child-Pugh class[17]. Furthermore, in patients with liver disease, resistin levels are correlated with the extent of insulin resistance and with clinical complications and prognosis[16]. In a recent animal study[19], hyperinsulinemia and increased tumor necrosis factor α (TNFα) secretion following bile duct ligation (BDL) were shown to up-regulate adipose tissue resistin gene expression which could subsequently contribute to liver fibrosis. A recent human study noted that resistin expression was low in normal liver, but was increased in severe fibrosis, suggesting that intra-hepatic resistin derived from monocytes/macrophages might contribute to fibrosis[15,20,21].

In the present study, we undertook *in vivo* and *in vitro* studies to elucidate the role of resistin in liver fibrosis. We show that resistin has increased expression in the epididymal fat and serum of cirrhotic rats. Resistin has a pro-inflammatory role in mediating the release of TNFα, interleukin (IL)-6, IL-8 and monocyte chemotactic protein-1 (MCP-1) in hepatic stellate cells (HSCs). Importantly, we demonstrate that resistin directly and indirectly mediates HSC activated phenotype in IL-6/MCP-1 and transforming growth factor (TGF) β1 dependent mechanisms, respectively, indicating that resistin contributes to the pro-inflammatory and pro-fibrotic phenotype of activated HSCs.

MATERIALS AND METHODS

Materials

Recombinant mouse resistin protein, recombinant IL-6, and MCP-1 ELISA kits, IL-6, MCP-1 and TGFβ1 antibodies were purchased from RD Systems (Minneapolis, MN, United States). Nycodenz, α smooth muscle actin (αSMA) mouse antibody was purchased from Sigma-Aldrich (St. Louis, MO, United States). Pronase E, DNase I and collagenase B were purchased from Roche Applied Sciences (Indianapolis, IN, United States). Resistin rabbit polyclonal antibody was purchased from Abbiotec TM (San Diego, CA, United States). p-p38, pERK1/2, pJNK, nuclear factor κB (NF-κB), p-p65 and p-p50 mouse monoclonal antibodies, p-p38 inhibitor (SB203580) and pJNK inhibitor (SP600125) were purchased from Cell Signaling Technology, Inc (Beverly, MA, United States). The BrdU ELISA kit was purchased from Roche Diagnostics (Castle Hills, NSW, Australia). Anti-mouse IgG conjugated to horseradish peroxidase was purchased from GE Healthcare Life Sciences (Piscataway, NJ, United States). DMEM medium was obtained from Invitrogen (Carlsbad, CA, United States).

Animals

Male Sprague Dawley (SD) rats were obtained from the Animal Resources Centre (Perth, Australia). All animals were maintained under 12-h light/dark cycles with food and water *ad libitum*. For the *in vivo* experiment, BDL or a sham surgical procedure was performed on rats. After 4 wk, rat liver, epididymal fat and serum were collected for resistin quantification. All experimental protocols were approved by the Sydney West Area Health Service Animal Research Ethics Committee.

Isolation and culture of rat hepatic stellate cells and Kupffer cells

Rat HSCs were isolated by a two-step (collagenase B and pronase E) perfusion method under ketamine and xylazine anesthesia as reported previously[6]. Briefly, rat liver was perfused through the portal vein using Ca^{2+}- and Mg^{2+}-free Gey's Balanced Salt Solution (GBSS, Sigma, United States) and then sequentially with pronase E followed by collagenase B (Roche Applied Science, Castle Hills, NSW, Australia). The liver was excised, gently dispersed in GBSS containing 0.01% DNase I and the cell suspension filtered through a sterile nylon mesh and subjected to low-speed centrifugation. The resultant cell pellet was mixed with 30% Nycodenz to obtain an 11% final Nycodenz/cell suspension. After centrifugation at 1400 *g* for 20 min, HSCs were collected, resuspended in culture medium, and plated on 6 well plates with 10% FCS/DMEM at a density of 0.8×10^6 cells/well. Cell viability was assessed by trypan blue exclusion and was routinely more than 95%. Purity was 95% as determined by morphology, vitamin A autofluorescence and desmin positivity. HSCs were maintained in 95% air and 5% CO_2 in DMEM (Gibco, United States) with 10% FCS and 1% penicillin/streptomycin. KCs were further obtained and purified by elutriation[6]. KCs were identified by their ability to phagocytose latex beads; viability was > 96% and purity > 98%. KCs were cultured in 10% FCS/DMEM/1% penicillin-streptomycin.

Treatments: For recombinant mouse resistin (RD Systems, Minneapolis, MN, United States), we undertook a dose ranging study based on previous reports[20-23] using 10, 50, 250 and 500 ng/mL. We found that 500 ng/mL

was the optimal dose which was used in all subsequent experiments. Primary rat HSCs and KCs were cultured for the time periods indicated and serum starved (0.2%) for 4 h prior to treatment. Subsequently, control (vehicle) and resistin (500 ng/mL) were added to the culture wells. After 24 h or extended culture as indicated, total RNA and protein were extracted. For the KC-HSC co-culture experiment, control (vehicle) and resistin (500 ng/mL) were added to cultured KCs at day 2 for 24 h, then KCs were washed three times with PBS and fresh medium was added and cultured for another 24 h. Afterwards, KC conditioned medium (KM) was transferred to HSCs at day 4 for 24 h co-culture. In one experiment, lipopolysaccharide (LPS, 50 ng/mL) was used to further activate cultured KCs.

Real-time reverse transcription polymerase chain reaction

Total cellular RNA was prepared from HSCs using TRI@ REAGENT (Molecular Research Center, INC., Cincinnati, OH, United States). Complementary DNA (cDNA) was synthesized from 1 μg RNA using Super-Script Ⅲ reverse transcriptase and 0.5 nmol of random primers (Invitrogen, Carlsbad, CA, United States). Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed using SYBR Green Platinum SYBR Green SuperMix (Invitrogen, United States). The synthesized cDNA was amplified using the following sequence specific primers: resistin 5'-CAAGACTTCAGCTCCCTACTGC-3' (forward) and 5'-GACGGTTGTGCCTTCTGG-3' (reverse); collagenα1 (Ⅰ) 5'-TTCACCTACAGCACGCTTGTG-3' (forward) and 5'-TCTTGGTGGTTTTGTATTC-GATGA-3' (reverse); TGFβ1 5'-TCGACATGGAGCT-GG TGAAA-3' (forward) and 5'-GAGCCT-TAGTTTGGACAGGATCTG-3' (reverse); αSMA5'-CGATAGAACACGGCATCATC-3' (forward) and 5'-CATCAGGCAGTTCGTAGCTC-3' (reverse); tissue inhibitor of metalloproteinase 1 (TIMP1) 5'-AAGGGC-TACCAGAGCGATCA-3' (forward) and 5'-GGTATT-GCCAGGTGCACAAAT-3' (reverse); connective tissue growth factor (CTGF) 5'-CGCCAACCGCAAGATTG-3' (forward) and 5'-ACACGGACCCACCGAAGAC-3' (reverse); IL-6 5'- CCCTTCAGGAACAGCTATGAA-3' (forward) and 5'-ACAACATCAGTCCCAAGAAGG-3' (reverse); IL-1 α 5'-ACATCCGTGGAGCTCTCTT-TACA-3' (forward) and 5'-TTAAATGAAC-GAAGTGAACAGTACAGATT-3' (reverse); IL-1β 5'-TACCTATGTC TTGCCCGTGGAG-3' (forward) and 5'-ATCATCCCACGAGTCACAGAGG-3' (reverse); TNFα 5'-GCCCAGACCCTCACACTC-3' (forward) and 5'-CCACTCCAGCTGCTCCTCT -3'(reverse); IL-8 5'-TCTGCAGCTCTGTGTGAAGG-3' (forward) and 5'-AATTTCTGGTT TGGCGCAGT-3' (reverse); MCP-1 5'-AGCATCCACGTGCTGTCTC-3' (forward) and 5'-GATCATCTTGCCAGTGAATGAG-3' (reverse). The relative amount of mRNA was calculated by reference to a calibration curve. The final result for each sample was normalized to the respective β actin value.

Immunoblotting: Cell culture media were removed and the cells washed with PBS and lysed on ice in a buffer containing 20 mmol/L Tris, 0.5 mmol/L $MgCl_2$, 1 mmol/L Dithiothreitol (DTT), 3 mmol/L NaN_3, and a mixture of protease and phosphatase-inhibitors. Cell lysates were disrupted using a sonicator on ice. After centrifugation at 13000 *g* for 15 min, the supernatant was collected as cytoplasmic protein. Nuclear protein was extracted as described previously[6]. The protein concentration was determined using the Bradford Protein Assay (Bio-Rad, Sydney, Australia). Immunoblotting was performed as previously described with some modifications[6,24]. Total protein (20 μg per lane) was resolved by electrophoresis on 12% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) under reducing conditions. The electrophoresed proteins were electrotransferred onto Polyvinylidene difluoride membranes (Immobilin-P, Millipore, Bedford, MA, United States). The membranes were blocked with 5% skim milk (Resistin, TGFβ1, p-p38, pERK1/2, pJNK, NF-κB p-p65, NF-κB p-p50 and αSMA) for 60 min and then incubated overnight with primary antibody (Resistin 1:200, p-p38 1:1000, pERK1/2 1:1000, pJNK 1:1000, p-p65 1:1000, p-p50 1:1000, TGFβ1 1:500, αSMA 1:2000) at 4 ℃. After 3 washes with 0.05% Tween-20/TBS, anti-mouse IgG (peroxidase conjugate) secondary antibody was applied. Blots were visualized by enhanced chemiluminescence (Pierce Perbio, Rockford, IL, United States). All images were quantified by densitometry.

Sirius red staining and quantification of collagen in HSCs

Sirius red staining and collagen quantification were performed according to a previously published protocol[6,25]. Briefly, Sirius red F3BA solution (0.1% in saturated picric acid) was added to cell layers fixed in Bouin's solution. After 1 h the cell layers were washed in tap water and again in 0.01 mol/L HCl to remove unbound dye. For collagen quantification, the dye was dissolved in 0.1 mol/L NaOH and absorbance determined at 450 nm. The amount of collagen was normalized to the protein concentration using the Bradford Reagent. Assays were performed in duplicate.

Enzyme-linked immunosorbent assay

The media were collected from cultured HSCs following 24 h stimulation with resistin (500 ng/mL). Levels of IL-6 and MCP-1 in the media were determined according to the manufacturer's instructions (RD Systems). Rat serum was collected and resistin concentration detected using a resistin rat ELISA kit (Boivendor) according to manufacturer's instructions.

HSC proliferation: Cell proliferation was analyzed using a BrdU-based enzyme-linked immunosorbent assay (Roche Diagnostics) according to the manufacturer's

instructions. HSCs at day 4 were treated with resistin (500 ng/mL) or other agents as indicated for 24 h. The cells were subsequently labeled with BrdU for 2 h at 37 ℃. Cells were then fixed and incubated with a peroxidase-conjugated anti-BrdU antibody for 90 min at room temperature. After adding the peroxidase substrate, 3,3',5,5'-tetramethylbenzidine, BrdU incorporation was determined by measuring optical densities at 450 nm (background 620 nm).

HSC migration: HSC migration was assessed both with the wound scratch assay and a modified Boyden chamber. For the wound scratch assay, using a sterile 200 μL pipette tip, three separate wounds were generated through the cell monolayer. HSCs (90% confluence) at day 6 cultured in 12-well plates were treated with resistin (500 ng/mL) or other agents as indicated. The scratch area was photographed immediately and 6 h after scratching and cell migration into the scratch area calculated as the area covered by cells in the percentage of the initial scratch area. For the second method, a cell culture insert (12 well, BD) was used and the porous membrane (pore size 8 μm) of the filter was coated with 30 μg/mL collagen I at 37 ℃ for 30-60 min. HSCs at day 6 were trypsinized and placed into the upper chamber (10^5 cells/mL). The lower wells were filled with resistin (500 ng/mL) or other agents as indicated. After 6 h of incubation at 37 ℃, cells adhering to the upper side of the filter were removed with a cotton swab. The filters were then fixed with 100% methanol and stained with HEMA-3. The numbers of HSCs on the lower side of the filter were counted in five randomly chosen microscopic fields at a magnification of × 400 by changing the focus.

HSC apoptosis: Annexin-V/PI labeling was used to detect HSC apoptosis. Briefly, trypsinized HSCs were washed twice in PBS, stained with annexin-V (10 μL) and PI (5 μL) for 10 min, and the apoptotic rate quantified by FACS Calibur flow cytometry (Becton Dickinson Inc.) at 488 nm. More than 1×10^4 cells were detected, and the results were analyzed with FlowJo software (Treestar, United States). The population of apoptotic cells was identified as annexin V+/PI-. The percentage of apoptotic cells was calculated according to total annexin V+/PI- divided by total cells.

Statistical analysis

The results are expressed as mean ± SD. Comparisons between 2 groups were analyzed using the Student *t* test. For the comparison of more than two groups, we used two-way ANOVA. *P* values < 0.05 were considered statistically significant. All calculations were performed using Statistical Program for Social Sciences (SPSS) software 13.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Resistin expression is up-regulated in cirrhotic rats

Resistin expression in liver, epididymal fat and serum in BDL and sham rats was examined. We noted that resistin expression in epididymal fat was considerably higher than that in liver in the BDL or sham rats (Figure 1A and B, all $P < 0.01$). BDL rat epididymal fat mRNA and protein level were further up-regulated compared to sham rats (Figure 1A and B, both $P < 0.05$). Similarly, BDL rat serum resistin level was also elevated (Figure 1C, $P < 0.05$). However, liver resistin mRNA and protein were unchanged in the BDL and sham groups (Figure 1A and B). These findings suggest that increased adipose resistin rather than liver resistin may play a vital role in resistin-mediated liver injury in rodents. Therefore, we undertook detailed *in vitro* experiments in order to explore the impact of exogenous resistin on HSC activated phenotype.

Resistin is expressed in quiescent HSCs and KCs

Resistin mRNA was detected in quiescent rat HSCs (Figure 1D) at day 1 and was reduced by 90% ($P < 0.01$) following activation for 8 d on plastic. Resistin mRNA was expressed in quiescent KCs (day 1) and activated KCs (3 d), without significant changes over time. LPS (50 ng/mL for 24 h) stimulation of KCs at day 3 did not enhance resistin expression (Figure 1D). Consistent with the mRNA data, resistin protein expression declined 6-fold in HSCs at day 8 with no change in KCs at day 3 and after LPS stimulation (Figure 1E). These data indicated that autocrine HSC resistin and paracrine KC resistin are unlikely to be of major importance in mediating any effects on activated HSCs.

Resistin promotes a pro-inflammatory phenotype in HSCs

Pro-inflammatory cytokines and chemokines play a permissive role in liver fibrosis[26-29] and previous reports suggest that resistin increases MCP-1 secretion. We evaluated the expression of TNFα, IL-1α, IL-1β, IL-6, IL-8 and MCP-1 in rat HSCs after stimulation with resistin. As demonstrated, resistin (500 ng/mL) stimulation for 24 h markedly up-regulated the expression of TNFα, IL-6, IL-8 and MCP-1 mRNA (Figure 2A, all $P < 0.05$), but not that of IL-1α and IL-1β. To rule out any potential effects of inadvertent endotoxin contamination, we repeated these studies in the presence of Polymyxin B and noted no difference in the gene expression profile (data not shown). Finally, using trypan blue staining and LDH assays at 24, 48 and 72 h, we excluded the possibility of direct cellular toxicity due to the resistin dose used (data not shown). Since IL-6 and MCP-1 are well documented to play a role in mediating hepatic fibrosis, their protein concentrations were estimated in conditioned medium. As shown in Figure 2B, resistin administration increased IL-6 and MCP-1 concentrations 1.7 and 1.8 fold after 24-h of treatment (both $P < 0.05$).

Resistin enhances HSC proliferation and migration but diminishes HSC apoptosis via an IL-6 and MCP-1 pathway

During the process of chronic liver injury, activated

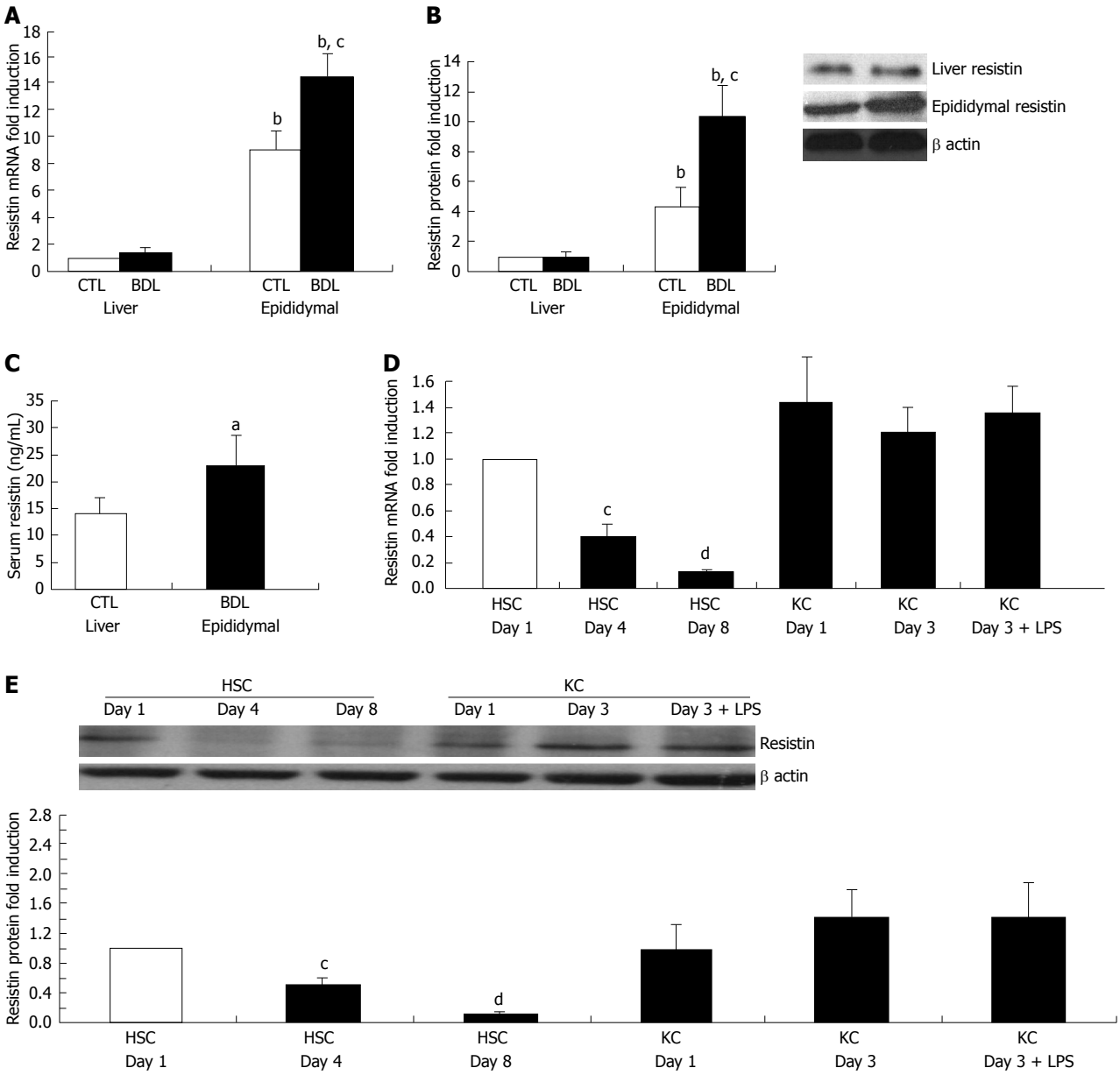

Figure 1 Rat extrahepatic but not intrahepatic resistin is up-regulated in cirrhosis. For the *in vivo* animal study, Sprague Dawley rats were subjected to bile duct ligation (BDL) for 4 wk. Liver, adipose tissue (epididymal fat) and serum were collected to determine resistin expression by quantitative polymerase chain reaction (qPCR), immunoblot and enzyme-linked immunosorbent assay. For the cell culture study, rat hepatic stellate cells (HSCs) and Kupffer cells (KCs) were isolated and cultured on plastic. HSC and KC total RNA/protein were extracted at different culture times (day 1, 4 and 8 for HSCs; day 1 and 3 for KCs). One group of KCs at day 2 were treated with Lipopolysaccharide (LPS) (50 ng/mL) for 24 h. qPCR and Immunoblot were performed for quantification of resistin mRNA and protein. β actin was used as an internal control. A: mRNA expression of resistin in liver and epididymal fat; B: Protein expression of resistin in liver and epididymal fat; C: Serum resistin concentrations; D: mRNA expression of resistin in HSCs and KCs on different culture days; E: Protein expression of resistin in HSCs and KCs on different culture days. Results are mean ± SD of at least three independent experiments performed in triplicate. $^{a}P < 0.05$ and $^{b}P < 0.01$ increased *vs* rat liver, HSC control at day 1 or sham rat serum; $^{c}P < 0.05$ and $^{d}P < 0.01$ decreased *vs* liver of control sham rat or HSC control at day 1.

HSCs proliferate and migrate to sites of inflammation and have reduced apoptosis. This phenotype is part of the expected adaptive wound healing response to injury. Hence, we sought to determine the role of resistin in mediating activated HSC behavior. As demonstrated in Figure 3A, resistin enhanced HSC proliferation by approximately 90% compared to the control ($P < 0.01$). Using the wound scratch assay and a modified Boyden chamber, compared to the control, resistin treatment resulted in an approximately 80% and approximately 220% increase in HSC migration, respectively ($P < 0.05$, Figure 3A and B). We next examined the role of resistin on HSC apoptosis. In contrast, resistin significantly reduced HSC apoptosis (56%, $P < 0.05$, Figure 3A), as shown by annexin V/IP flow cytometry. Finally, we determined whether up-regulation of IL-6 and MCP-1 was responsible for the changed HSC phenotype by resistin. As expected, resistin-mediated HSC proliferation, migration and apoptosis were partially, but significantly reversed (all $P < 0.05$, Figure 3A and B) by IL-6 (5 μg/mL) and

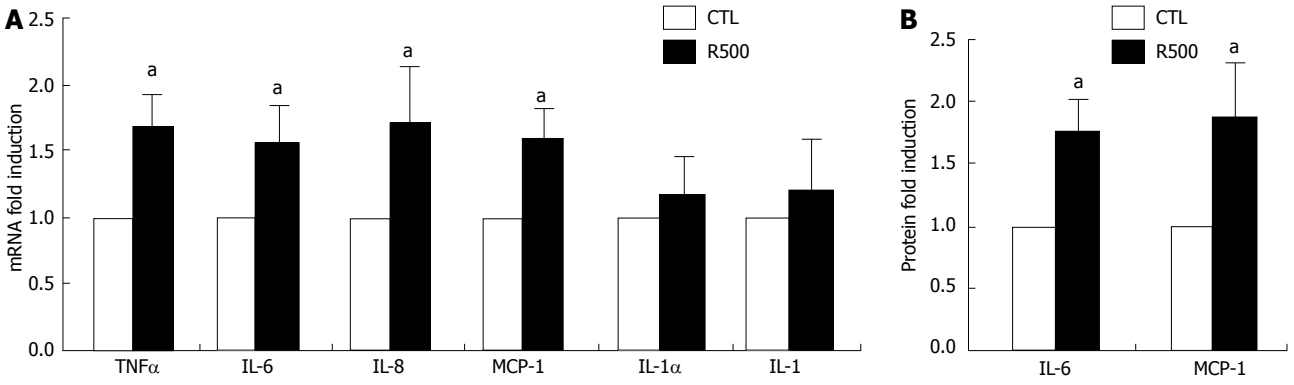

Figure 2 Resistin enhances the expression of tumor necrosis factor α, interleukin 6, interleukin 8 and monocyte chemotactic protein-1 in hepatic stellate cells. Rat hepatic stellate cells (HSCs) at day 4 were cultured with resistin (500 ng/mL) (R500) for 24 h. Total RNA was extracted and quantitative polymerase chain reaction was performed to quantify mRNA expression. Media were collected and enzyme-linked immunosorbent assay conducted to determine interleukin 6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) protein concentrations. A: mRNA expression of tumor necrosis factor α (TNFα), IL-6, IL-8, MCP-1, IL-1α and IL-1; B: IL-6 and MCP-1 protein levels. Data are expressed as mean ± SD. At least three independent experiments were conducted in triplicate for data analysis. [a]*P* < 0.05 *vs* controls (untreated).

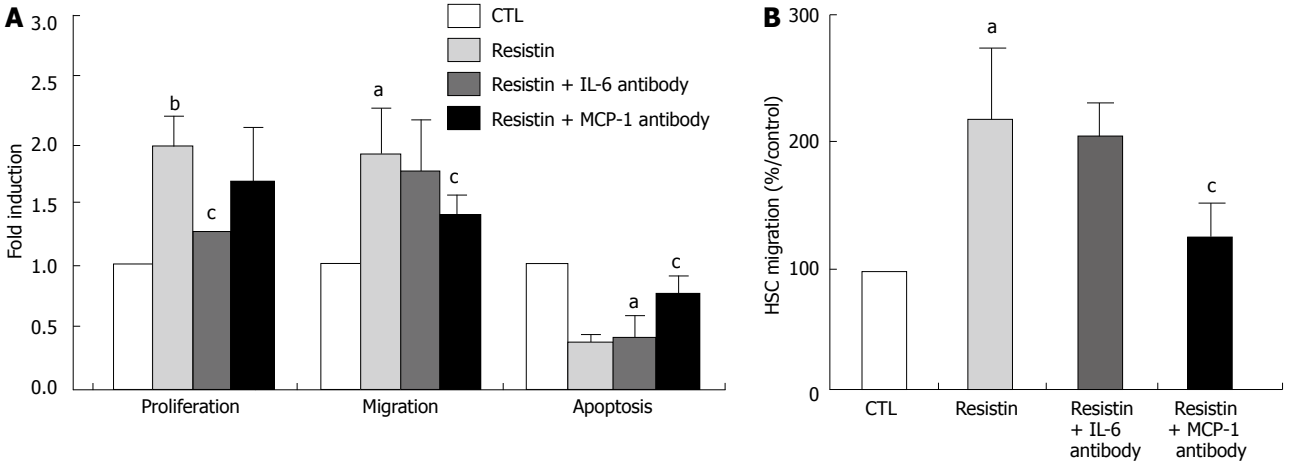

Figure 3 Resistin promotes hepatic stellate cells proliferation and migration but decreases hepatic stellate cells apoptosis in an interleukin 6 and monocyte chemotactic protein-1 dependent mechanism. BrdU, enzyme-linked immunosorbent assay, Wound Scratch Assay (or Boyden chamber) and annexin V/IP flow cytometry were performed to determine hepatic stellate cells (HSCs) proliferation, migration and apoptosis, respectively. For the interleukin 6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) inhibition experiments, IL-6 and MCP-1 neutralizing antibodies (5 μg/mL and 10 μg/mL, respectively) were added to the culture 1 h before resistin (500 ng/mL) administration. Resistin (500 ng/mL) was added to rat HSCs at day 4 for 24 h. Absorbance was measured and apoptosis assessed. For the migration assay, rat HSCs at day 6 were used. After a scratch wound was made, resistin (500 ng/mL) was added and the cells were cultured for 6 h and photographed. For the Boyden chamber assay, the detailed procedure is described in the Materials and Methods section. A: Resistin promoted HSC proliferation and migration, but inhibited HSC apoptosis, while IL-6 and MCP-1 antibodies reversed the resistin-induced HSC phenotype; B: The Boyden chamber assay confirmed that resistin enhanced HSC migration and MCP-1 neutralization reversed this effect. Results are mean ± SD of at least three independent experiments performed in triplicate. [a]*P* < 0.05 and [b]*P* < 0.01 *vs* control (untreated); [c]*P* < 0.05 *vs* resistin treatment alone.

MCP-1 (10 μg/mL) neutralization, respectively. These data suggest that resistin triggered HSC IL-6 and MCP-1 production, thereby modulating HSC phenotype.

Resistin activates HSC MAPK/p38 and nuclear NF-κB p65

Mitogen-activated protein kinases (MAPK) and NF-κB play critical roles in the induction of pro-inflammatory cytokines and chemokines, and regulate cell biological behaviors. Therefore, we determined whether resistin activates HSC MAPK (p38, ERK1/2 and JNK) and NF-κB. Phoshor-p38, ERK1/2 and JNK in the cytoplasm as well as NF-κB p65 and p50 in cytosolic and nuclear extracts were analyzed by immunoblotting. The results showed that cytoplasmic p-p38 and nuclear p-p65 were up-regulated (both $P < 0.05$, Figure 4A and C). Changes in cytosolic pERK1/2, pJNK (data not shown), p65 and cytosolic and nuclear p-p50 (data not shown) were not observed. In the p-p38 inhibition experiment using SB203580, we found that p-p38 activation was responsible for IL-6 and MCP-1 induction in HSCs ($P < 0.05$, Figure 4B). Furthermore, resistin (500 ng/mL) enhanced NF-κB DNA binding ability (luciferase mRNA. $P < 0.05$, Figure 4D). As expected, NF-κB inhibition by pyrrolidine dithiocarbamate (PDTC) (100 μmol/L) attenuated the resistin-induced increase in nuclear p-p65 and NF-κB DNA binding ability ($P < 0.05$, Figure 4C and D). Similarly, PDTC reversed resistin-induced up-regulation of IL-6 and MCP-1 (Figure 4E).

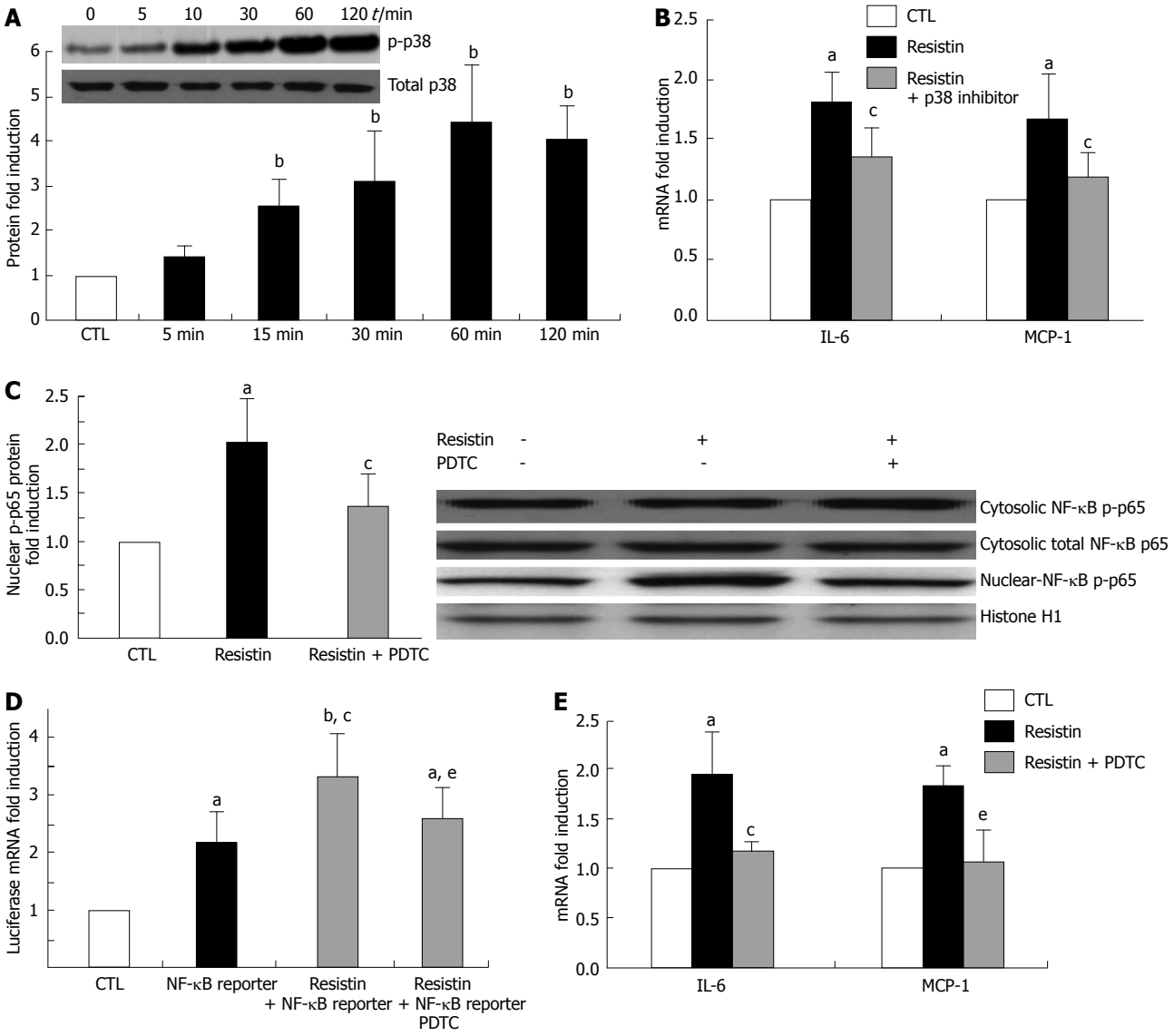

Figure 4 Resistin activates hepatic stellate cells MAPK/p38 and nuclear factor κB p65. Rat hepatic stellate cells (HSCs) at day 4 were cultured with resistin (500 ng/mL) for 120 min. Cytosolic and nuclear proteins were extracted and Immunoblot performed to quantify p-p38 and nuclear factor κB (NF-κB) p-p65. For NF-κB DNA binding capacity, 3 × NF-κB /Luc reporter was added to the culture for 24 h and Luciferase mRNA quantified by quantitative polymerase chain reaction. For the p-p38 and NF-κB inhibition experiments, SB203580 (20 μmol/L, p-p38 inhibitor) or pyrrolidine dithiocarbamate (PDTC) (100 μmol/L, NF-κB inhibitor) was added 1 h before resistin treatment. Resistin (500 ng/mL) was added to rat HSCs for 24 h. A: p-p38 was enhanced by resistin; B: p-p38 inhibition (24 h) diminished resistin-induced interleukin 6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) increase by HSCs; C: Nuclear p-p65 was increased by resistin exposure and decreased by PDTC (120 min); D: Luciferase mRNA was augmented by resistin and diminished by PDTC (24 h); E: PDTC reversed resistin-induced enhancement of IL-1 and MCP-1 (24 h). Data are expressed as mean ± SD. At least three independent experiments were conducted in triplicate for data analysis. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* controls (untreated); $^{c}P < 0.05$ *vs* resistin treatment alone or NF-κB reporter treatment alone; $^{e}P < 0.05$ *vs* combination of resistin and NF-κB reporter.

Resistin indirectly promotes HSC collagen I expression through the actions of KCs

To determine whether resistin affects KCs and whether KCs participate in the process of resistin-mediated HSC phenotype, the appropriate experiments were undertaken. As shown in Figure 5A and B, resistin up-regulated TGFβ1 and CTGF mRNA in KCs and enhanced TGFβ1 protein in KC medium (both $P < 0.05$). Co-culture of HSCs and KC conditioned medium resulted in a significant increase in HSC collagen I and CTGF expression (Figure 5C and D, all $P < 0.05$), however, TGFβ1 (10 μg/mL) neutralization diminished this increase (Figure 5C and D, all $P < 0.05$). Downstream signaling responsible for increased KC TGFβ1 and CTGF expression by resistin were further analyzed. We found that pJNK and p-p38 were activated following exposure to resistin. Furthermore, pJNK and p-p38 inhibition partially, but significantly reversed resistin-induced TGFβ1 and CTGF enhancement (Figure 5E and F, $P < 0.05$ and 0.01). These data suggest that resistin affected HSC activated phenotype by increased TGFβ1 from KCs.

DISCUSSION

Resistin is suggested to play a pathogenic role in insulin resistance and altered glucose metabolism in rodents[3,4].

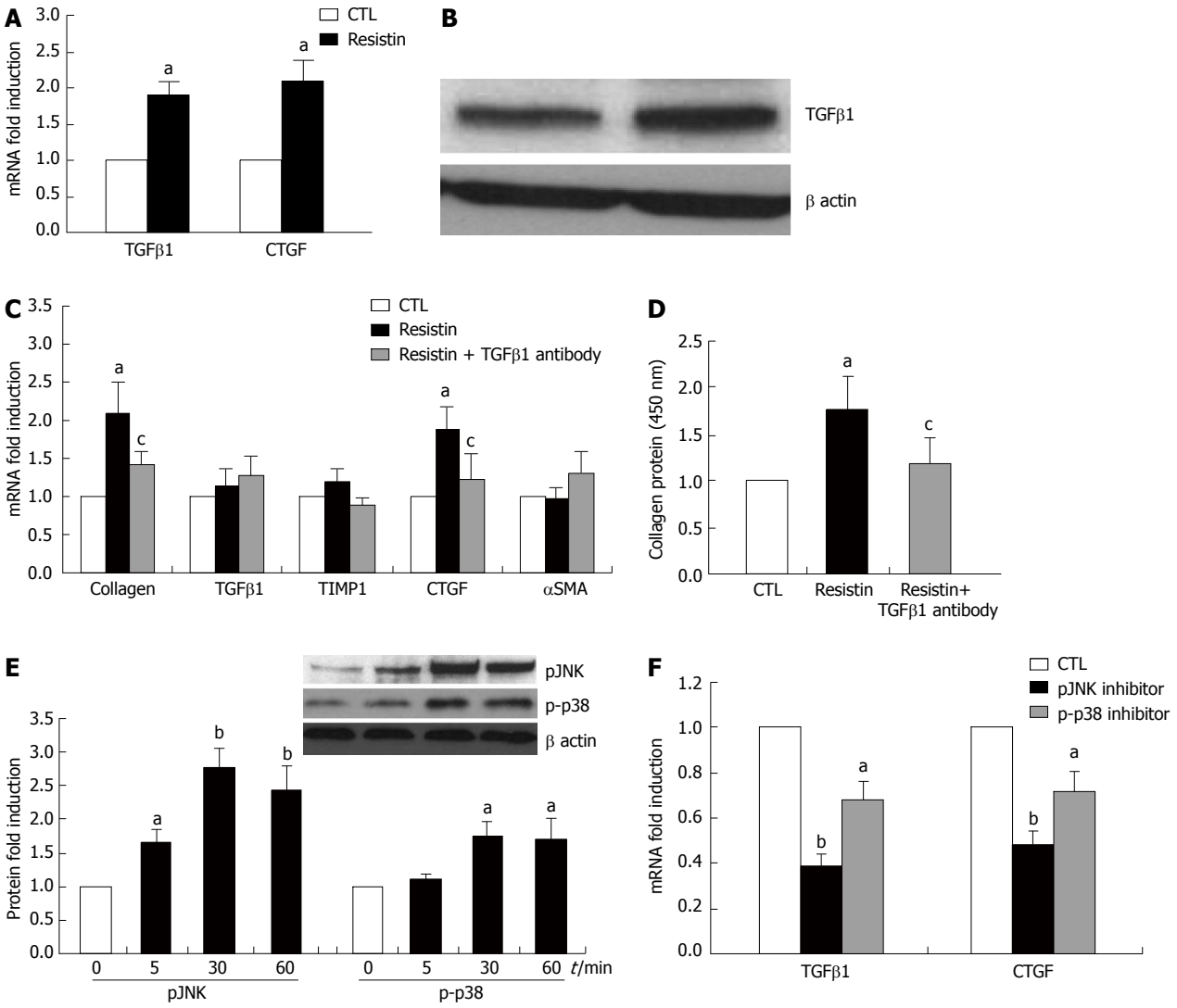

Figure 5 Resistin indirectly enhances hepatic stellate cells collagen I expression through a transforming growth factor β1 dependent mechanism *via* the action of Kupffer cells. Rat Kupffer cells (KCs) at day 2 were cultured with resistin (500 ng/mL) for 24 h. Total RNA was extracted and quantitative polymerase chain reaction was performed. Transforming growth factor β1 (TGFβ1) protein expression in KC conditioned medium (KM) was quantified by immunoblotting. Sirius red was used for collagen I protein quantification in the medium. For the KC-hepatic stellate cell (HSC) co-culture experiment, KCs at day 2 were incubated with resistin for 24 h and then washed three times with phosphate-buffered saline, fresh medium was subsequently added to the culture for another 24 h. KM was then transferred to HSCs at day 4 for 24 h. HSC collagen I , TGFβ1, tissue inhibitor of metalloproteinase 1 (TIMP1), connective tissue growth factor (CTGF) and α smooth muscle actin (αSMA) expression were determined. For inhibition experiments, TGFβ1 monoclonal antibody (10 μg/mL), SP600125 (50 μmol/L) and SB203580 (20 μmol/L) were added to KCs for 24 h. A: Resistin promoted KC T*GFβ1* and *CTGF* gene expression; B: Resistin augmented TGFβ1 expression in KM medium; C: HSC collagen I and CTGF mRNA were augmented by resistin conditioned KM reversed by TGFβ1 neutralization (10 μg/mL); D: HSC collagen protein was increased by resistin conditioned KM but reversed by TGFβ1 neutralization (10 μg/mL); E: Resistin increased KC JNK and p38 phosphor-protein; F: pJNK inhibitor (50 μmol/L, SP600125) and p-p38 inhibitor (20 μmol/L, SB203580) partially, but significantly reversed resistin-induced TGFβ1 and CTGF expression by KCs. Data are expressed as mean ± SD. At least three independent experiments were conducted in triplicate for data analysis. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* controls (untreated); $^{c}P < 0.05$ *vs* resistin treatment alone.

For example, lowering plasma resistin in insulin-resistant mice decreases blood glucose levels and improves insulin sensitivity[10,30,31], while treatment of normal mice with resistin impairs glucose tolerance and insulin actions[10,30]. Resistin may also play a pivotal role in inflammation since it up-regulates IL-6 and TNFα expression in human peripheral blood mononuclear cells *via* NF-κB activation[22]. Furthermore, the addition of resistin protein from both mice and humans to macrophages results in enhanced secretion of pro-inflammatory cytokines including TNFα and IL-12[32]. In human cirrhosis, resistin levels in the liver and plasma are elevated and increase further with the severity of liver disease[14-17,33]. This suggests that the pro-inflammatory activities of resistin may modulate liver inflammation and drive disease progression in cirrhosis.

This study provides evidence that in cirrhotic rats, adipose tissue (epididymal fat) and blood resistin are up-regulated, and adipose tissue may be the main source of resistin secretion. Rat liver, HSCs and KCs express resistin, but are unlikely to be important sources of resistin secretion in cirrhosis. Resistin exerts pro-inflammatory activities on HSCs with enhanced secretion of pro-

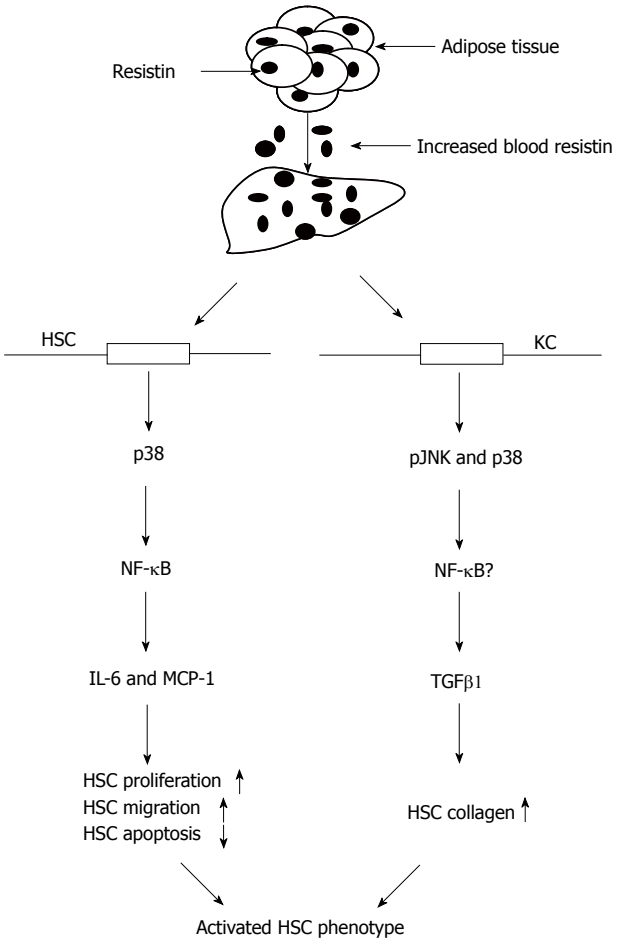

Figure 6 Schematic diagram illustrating a possible mechanism by which resistin potentiates hepatic stellate cells profibrogenic phenotype. Adipose tissue is a predominant source of resistin expression and secretion in rodents. Increased adipose resistin released into the bloodstream and liver stimulates HSCs to produce increased amounts of pro-inflammatory cytokines and chemokines (IL-6 and MCP-1), leading to enhanced HSC proliferation and migration, but attenuation of HSC apoptosis. In addition, resistin promotes KC TGFβ1 which subsequently activates HSC by up-regulation of collagen I. Therefore, resistin is considered one of the pro-fibrogenic adipocytokines. TGFβ1: Transforming growth factor; NF-κB: Nuclear factor κB; IL-6: Interleukin 6; MCP-1: Monocyte chemotactic protein-1; HSCs: Hepatic stellate cells.

inflammatory cytokines (TNFα, IL-6, IL-8 and MCP-1). Most importantly, resistin promotes HSC proliferation and migration, while inhibiting their apoptosis *via* an IL-6 and MCP-1 mechanism. KCs participate in this process by up-regulating HSC collagen I through increased TGFβ1. Taken together, our data suggest that resistin promotes the progression of liver injury.

Resistin is almost exclusively expressed by white adipose tissue in rodents, but is expressed by monocytes and macrophages in humans[18,34,35]. Liver infiltrating CD43 cells and KCs have been suggested as key sources of resistin in the liver of cirrhotic patients[20,21], thus resistin was more abundant in adipose tissue than in human liver[20,21]. In this study, although rat quiescent HSCs expressed resistin, it declined markedly on activation. The relevant mechanism is unclear, however, adipogenic transcriptional regulation may be required for maintenance of the quiescent HSC phenotype[26]. KCs also expressed resistin but no change was found on activation or LPS stimulation. Therefore, it is unlikely that resistin derived from HSCs and KCs contributed to the increase in serum resistin in BDL rats, thus HSCs and KCs are non-critical sources of resistin. However, other liver cell types may not represent a likely source of resistin production as hepatocytes and endothelial cells do not express resistin[15]. Thus, adipose tissue, including epididymal fat, could be the predominant source of resistin in liver injured rodents. It has been demonstrated in *in vivo* and *ex vivo* studies, that increased TNFα and insulin in BDL cirrhotic rats stimulate adipose resistin expression[14,15,19].

Why LPS was unable to trigger resistin secretion by KCs is unknown. KCs belongs to the macrophage family, and many studies have shown that LPS exposure induced resistin production in human and rodent macrophages[36,37]. The mechanisms involved require further clarification.

As expected, HSC expression of TNFα, IL-6, IL-8 and MCP-1 mRNAs was increased on resistin exposure, as was IL-6 and MCP-1 protein. Bertolani *et al*[20] reported similar findings in human HSCs and noted that resistin up-regulated human HSC MCP-1 that was dependent on a Ca^{2+}/NF-κB-dependent pathway[20]. We further demonstrated that resistin directly augmented HSC proliferation and migration, but reduced HSC apoptosis *via* an IL-6 and MCP-1 mechanism. These novel data imply that IL-6 and MCP-1 inhibition may prevent resistin-induced liver fibrogenesis. The pro-fibrogenic effects of IL-6 and MCP-1 are well documented in the literature[38-40]. Moreover, we found that resistin was able to promote KC activation as it stimulated enhancement of KC TGFβ1 expression. Thus, increased TGFβ1 led to up-regulation of HSC collagen I and HSC activation. This is an important finding, as TGFβ1 is a potent profibrogenic cytokine. Interestingly, this phenomenon is similar to our previous report[6]. We observed that the profibrogenic role of leptin could be achieved at least through TGFβ1 from KCs[6]. Collectively, these data indicate that resistin is able to modulate HSC behaviors towards a more profibrogenic phenotype.

Although many functions of resistin in inflammation and inflammation-related diseases have been described, the relevant intracellular signaling pathway of resistin is not yet completely understood. We further demonstrated that resistin mediated HSC IL-6 and MCP-1 *via* p38 and KC TGFβ1 *via* pJNK and p-p38 (Figure 6). These results may provide evidence to prevent resistin-mediated liver injury/fibrosis using relevant signaling inhibitors.

In summary, this study demonstrates that in rodents, resistin production in the context of liver injury is principally non-hepatic in origin. Extrahepatic resistin could contribute to liver fibrosis by its direct and indirect profibrogenic effects on HSCs. Further studies on resistin knockout and transgenic animals are needed.

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COMMENTS

Background

Metabolic abnormalities usually cause the progression of liver fibrosis. To date, the mechanism whereby metabolic alterations mediate disease progression are unclear. Resistin, an adipokine, has been reported to be associated with metabolic alterations, however, its role in hepatic fibrosis has not been clearly investigated.

Research frontiers

Although many functions of resistin in inflammation and inflammation-related diseases have been described, the relevant intracellular signaling pathways of resistin in liver fibrosis are not yet completely understood.

Innovations and breakthroughs

To date, there have been a limited number of studies regarding the impact of resistin on the phenotype of hepatic stellate cells and how it functions in liver fibrosis. In this study, the authors employed a direct analysis to identify the significant correlation between resistin and hepatic stellate cells (HSCs). The authors confirmed that resistin mediated-HSCs move towards a more pro-fibrotic phenotype which is dependent on interleukin 6/monocyte chemotactic protein and/or transforming growth factor β1.

Applications

By understanding the mechanism whereby resistin mediates HSC activation, this study may provide evidence to prevent resistin-mediated liver injury/fibrosis using relevant signaling inhibitors.

Terminology

The serum levels of resistin are elevated in cirrhosis, and the changed phenotype of HSCs play an important role in the pathogenesis of liver fibrosis. Resistin is involved in this process.

Peer review

The paper reported the effects of the adipokine resistin on the biology of hepatic stellate cells and Kupffer cells. It is well presented.

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ORIGINAL ARTICLE

Influence of up-regulation of Notch ligand DLL4 on biological behaviors of human gastric cancer cells

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Abstract

AIM: To investigate the potential roles of Delta-like ligand 4 (DLL4) on the biological behavior of gastric cancer cells and its molecular mechanisms.

METHODS: A recombinant eukaryotic expression vector containing human *DLL4* gene was constructed and transfected into the human gastric cancer cell line SGC7901. Clones with up-regulated DLL4 were selected and amplified. The effect of DLL4 up-regulation on gastric cancer cell growth was assessed using cell growth assay. The migration and invasion were assessed using a transwell migration assay and matrigel invasion assay. Matrix metalloproteinases were detected using the zymogram technique. Cells were implanted subcutaneously into male BALB/c nu/nu mice. Tumor volumes were then calculated and compared. DLL4 staining in the implanted tumor was performed using immunohistochemistry technique.

RESULTS: Growth curves over a six-day time course showed significantly promoted cell proliferation of SGC7901 cells with up-regulated DLL4. DLL4 up-regulation in SGC7901 cells promoted the migration (205.4 ± 15.2 *vs* 22.3 ± 12.1, $P < 0.05$) and invasion (68.8 ± 5.3 *vs* 18.2 ± 6.0, $P < 0.05$) *in vitro* and tumorigenicity *in vivo* (2640.5 ± 923.6 mm^3 *vs* 1115.1 ± 223.8 mm^3, $P < 0.05$). Furthermore, significantly increased mRNA level and increased secretion of matrix metalloproteinase-2 (MMP-2) proenzyme were observed in SGC7901 cells with up-regulated DLL4. However, increased MMP-9 mRNA level but decreased extracellular MMP-9 proenzyme level was observed.

CONCLUSION: Our observations indicated a mechanism by which activation of DLL4-mediated Notch signaling promotes the expression and secretion of MMP-2 proenzyme and influences the progress of gastric cancer.

Key words: Gastric cancer; Delta-like ligand 4/Notch; Matrix metalloproteinase; Migration; Invasion

Core tip: Delta-like ligand 4 (DLL4), one of the five notch signaling ligands in mammals, has been researched mainly with regard to vasculogenesis and tumor angiogenesis. To the best of our knowledge, there is rare study to investigate its role and mechanism in human gastric cancers. We found that DLL4

promotes cellular proliferation, migration, invasion and tumorigenicity in gastric cancer cells. Furthermore, increased mRNA level and increased secretion of matrix metalloproteinase-2 proenzyme, while increased matrix metalloproteinase (MMP)-9 mRNA levels but decreased extracellular MMP-9 proenzyme levels were observed. These results indicated a mechanism by which activation of DLL4-mediated Notch signaling promotes the expression and secretion of MMP-2 proenzyme and influences the progress of gastric cancer.

Li GG, Li L, Li C, Ye LY, Li XW, Liu DR, Bao Q, Zheng YX, Xiang DP, Chen L, Chen J. Influence of up-regulation of Notch ligand DLL4 on biological behaviors of human gastric cancer cells. *World J Gastroenterol* 2013; 19(28): 4486-4494 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i28/4486.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i28.4486

INTRODUCTION

Gastric cancer is one of the most common cancers and lethal malignancies worldwide[1,2]. Approximately 738000 patients with gastric cancer died in 2011[2]. Of these, 80% died within a short period after curative surgery due to locoregional recurrence (87%) and distant metastases (30%)[3,4]. The 5-year survival of gastric cancer is less than 40%, even with adjuvant chemo-radiotherapy[5]. Therefore, there is an urgent need for new therapeutic strategies to improve clinical outcomes of this disease.

Notch signaling, as an evolutionarily conserved signaling pathway, is involved in a variety of cellular processes including cell fate, differentiation, proliferation, survival rate, and apoptosis[1,6]. The Notch signaling in mammals consists of five ligands [Delta-like ligand 41/3/4 (DLL1/3/4) and Jagged 1/2] and four receptors (Notch 1-4). Notch proteins are synthesized as full-length unprocessed proteins and cleaved at the S1 site by furin-like convertase to generate the mature receptor. Notch-ligand binding induces the cleavages of a Notch receptor by metalloprotease and γ-secretase to release the Notch intracellular domain (NICD). The NICD subsequently translocates to the nucleus, where it forms a complex with the members of the CSL (C-promoter binding factor-1, suppressor of hairless in *Drosophila* and lag in *Caenorhabditis elegans*) transcription factor family and regulates the expression of downstream genes such as Hairy/Enhancer of Split (*HES1/5/6/7*) and the HES-related proteins (*HEY1/2/L*)[6-10].

Interestingly, Notch signaling may play distinct biological roles in different tumors. It acts as an oncogene in pancreatic cancer[11], colon cancer[12], breast cancer[13] and most other solid tumors[14,15], whereas it acts as an anti-oncogene in some types of skin cancer[16], lung cancer[17] and prostate cancer[18]. Compared with other solid tumors, less literature relates to the role of DLL4-mediated Notch signaling in gastric cancer.

Although most Notch-related genes are expressed in multiple tissue and cell types, DLL4 is largely restricted to the vascular endothelia and has been researched mainly with regard to vasculogenesis and tumor angiogenesis[7,19-21]. Blockade of DLL4 signaling has been shown to lead to inhibition of tumor angiogenesis by nonproductive angiogenesis in some types of murine tumor models[20,21]. However, the precise function and mechanism of DLL4 in gastric cancer remain unclear. In the present study, we up-regulated the expression of DLL4 in the gastric cancer cell line SGC7901, and assessed its biological function both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell lines and animals

Human gastric cancer cell line SGC7901 was purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Cells were propagated in RPMI-1640 medium (Gibco-Invitrogen, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum (FBS) (Gibco-Invitrogen), penicillin (100 units/mL) and streptomycin (100 μg/mL) in a humidified atmosphere containing 5% CO_2 at 37 ℃.

Animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the protocol was approved by the Animal Research Committee of Zhejiang University, Hangzhou, China. Mouse protocols were conducted in accordance with stringent regulations laid out by Zhejiang University Laboratory Animal Center. Twenty-four 4-wk-old male BALB/c nu/nu mice (Shanghai SLAC Laboratory Animal Co, Shanghai, China) weighing 10-12 g used for subcutaneous tumor implantation were randomly divided into control, SGC7901-vector and SGC7901-DLL4 groups. Animals were housed in a sterile environment, and maintained on daily 12-h light/12-h dark cycle, which was controlled by qualified staff in the Zhejiang University Laboratory Animal Center.

Recombinant eukaryotic expression vector pEGFP-C1-DLL4 construction

A human full-length DLL4 cDNA fragment was amplified using a forward primer 5'-GGAATTCACCATGGCGGCAGCGTCC-3' and a reverse primer 5'-CGGGATCCTTATACCTCCGTGGCAATGACAC-3', verified by sequencing, and then *Eco*RI-*Bam*HI-digested. The 2-kb fragment was purified using a AxyPrep DNA Gel Extraction Kit (Axygen, Union City, CA, United States) and was ligated to a EcoRI-BamHI-digested pEGFP-C1 vector DNA (Clontech, Mountain View, CA, United States). Positive clones were further confirmed by *Eco*RI-*Bam*HI digestion and sequencing.

Transfection and clone selection

SGC7901 cells in logarithmic growth phase were collected and seeded into six-well plates at 4×10^5 cells/well to obtain approximately 80% confluence after overnight

Table 1 Primer sequences for real-time polymerase chain reaction

Genes	Forward sequences	Reverse sequences	Product size (bp)
DLL4	5′-CCCTGGCAATGTACTTGTGAT-3′	5′-TGGTGGGTGCAGTAGTTGAG-3′	73
Notch1	5′-GCCTCAACATCCCCTACAAGA-3′	5′-CCACGAAGAACAGAAGCACAAA-3′	120
HES1	5′-GTCAACACGACACCGGATAA-3′	5′-TTCAGCTGGCTCAGACTTTC-3′	113
HES5	5′-TGGAGAAGGCCGACATCCT-3′	5′-GGCGACGAAGGCTTTGC-3′	65
HEY1	5′-CGCGTTATCTGAGCATCATT-3′	5′-TGGGAAGCGTAGTTGTTGAG-3′	88
MMP-2	5′-GCTGACGGTAAGGACGGACTC-3′	5′-CGTTGCCATTGAACAAGAAGG-3′	158
MMP-9	5′-TTTGACAGCGACAAGAAGTGG-3′	5′-AGGGCGAGGACCATAGAGG-3′	189
E-cadherin	CGTTAGAGGTGGGTGACTACAAA	GAACAGCAAGAGCAGCAGAAT	220
GAPDH	5′-TGCCACTCCTCCACCTTTG-3′	5′-CGAACCACCCTGTTGCTGT-3′	104

DLL: Delta-like ligand 4; MMP: Matrix metalloproteinases; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

incubation. Cultured SGC7901 cells were divided into three groups: (1) transfected cells with recombinant pEGFP-C1-DLL4 vector (SGC7901-DLL4 group); (2) transfected cells with pEGFP-C1 vector (SGC7901-vector group); and (3) nontransfected cells (control group). Transfection was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The cells were then cultured in RPMI-1640 medium containing 10% FBS and G418 (600 μg/mL) for 21 d. G418-resistant clones were selected and amplified in complete medium containing G418 (300 μg/mL). Up-regulation of DLL4 was verified by real-time polymerase chain reaction (PCR) and Western blotting assay.

RNA extraction and real-time PCR

Extraction of total RNA from cultured cells was performed using the TRIzol method (Invitrogen). Total RNA (1 μg) was reverse-transcribed using RevertAid First Strand cDNA Synthesis Kit (Fermentas, ThermoFisher Scientific, Waltham, MA, United States). Real-time PCR was performed in triplicate using SYBR Green PCR Master Mix (TaKaRa, Tokyo, Japan) in an ABI PRISM Stepone Plus Sequence Detection System (Applied Biosystems, Foster City, CA, United States) according to the manufacturer's instructions. Forty cycles were used to amplify DLL4/Notch-related genes (denaturation at 95 ℃ for 5 s, annealing and extension at 60 ℃ for 32 s). Relative quantitation of gene expression was detected using the method described by Pfaffl[22]. The primers for real-time PCR were described in Table 1.

Western blotting analysis

Total protein extracts were prepared and run on 10% polyacrylamide gels. Fractionated proteins were electrotransferred to polyvinylidenefluoride membranes. Antibodies against human DLL4 (1:1000, Abcam, Cambridge, United Kingdom) and β-actin (1:1000, Abcam) were used for detection at 4 ℃ overnight. Horseradish peroxidase-conjugated anti-rabbit antibody was applied as a secondary antibody at 25 ℃ for 1 h. Antigens were identified by luminescent visualization using an ECL Western blotting Detection System (Millipore, Billerica, MA, United States). Signal intensity was measured using a Bio-Rad XRS chemiluminescence detection system (Bio-Rad).

Cell growth assay and growth curve

3-(4,5-dimethyl-2-yl)-5-(3-arboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays using the CellTiter 96 AQueous nonradioactive cell proliferation MTS agent (Promega, Madison, WI, United States) were performed to evaluate cell growth. Approximately 500 cells in 100 μL of medium with 0.5% FBS were plated in 96-well plates and allowed to attach for 24 h. Then 20 μL of the Promega MTS reagent was added to each well, and further incubation was conducted in a humidified incubator for 2 h. Absorbance of each well at 490 nm was determined using a Microplate Reader (Bio-Rad). The cell-growth curve was plotted using the optical densities obtained over 6 consecutive days.

Transwell migration assay

Transwell units with 8.0-μm pore-size polycarbonate filters (Corning Costar, Tewksbury, MS, United States) were used to investigate chemotactic cell migration. Cells were harvested and suspended at approximately 4×10^5 cells/mL in RPMI 1640 medium containing 0.5% FBS. A total volume of 100 μL of suspension was added into the upper compartment of the transwell unit. After 30 min of attachment, the units were transferred to wells containing 600 μL RPMI 1640 medium with 20% FBS as a chemoattractant, and further incubation was conducted for 20 h. After removing the cells on the upper surface of the membrane with a cotton bud and 15-min staining with 0.1% crystal violet, cell numbers on the underside were determined using light microscopy. Five randomly selected fields were counted per insert.

Matrigel invasion assay

Transwell units with 8.0-μm pore-size polycarbonate filters (Corning Costar) were precoated with 50 μL of 1:5 diluted matrigel (Becton Dickinson Biosciences, Franklin Lakes, NJ, United States) and used to investigate cell invasion. A total volume of 100 μL of suspension containing approximately 40000 cells was added to each upper compartment of precoated units. After 30 min of attachment, the units were transferred to wells containing 600 μL RPMI 1640 medium with 20% FBS as a chemoattractant, and incubation was conducted for 20 h. After removing the cells and Matrigel on the upper surface of

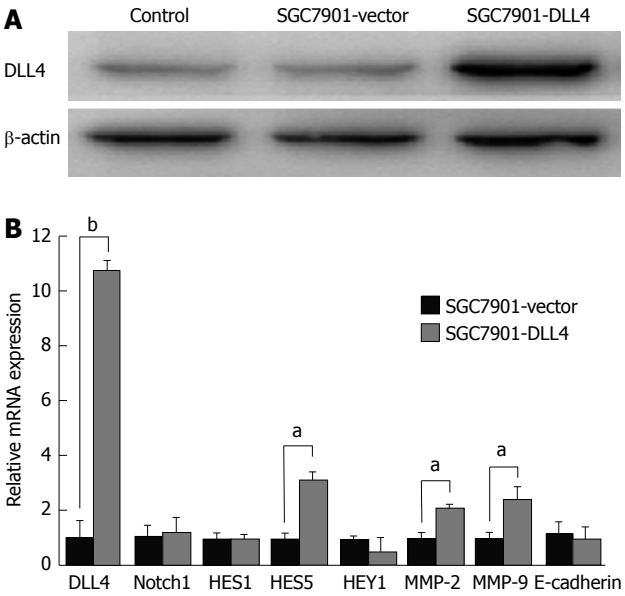

Figure 1 Up-regulation of Delta-like ligand 4 changed downstream gene expression in SGC7901 cells. A: Western blotting confirmed the up-regulation of Delta-like ligand 4 (DLL4) in the SGC7901-DLL4 group at the protein level; B: Real-time polymerase chain reaction was used to assess *Notch1*, *DLL4*, and downstream genes *HES1*, *HES5*, *HEY1*, as well as the matrix metalloproteinases-2 (*MMP-2*), *MMP-9*, and the adhesion protein E-cadherin at the mRNA level. The results showed increased expression of *DLL4*, *HES5*, *MMP-2*, and *MMP-9*, but no significant change of *HES1* and *HEY1* ($^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the SGC7901-vector group). Data show mean ± SD of gene expression compared with the control group. Endogenous references was glyceraldehyde 3-phosphate dehydrogenase; SGC7901-vector: SGC7901 cells transfected with an empty vector; SGC7901-DLL4: SGC7901 cells transfected with a vector encoding human *DLL4* gene; Control: Non-transfected SGC7901 cells.

the membrane with a cotton bud and 15-min staining with 0.1% crystal violet, cell numbers on the underside were determined using light microscopy. Five randomly selected fields were counted per insert.

Gelatin zymography

Matrix metalloproteinases (MMPs) were detected using the zymogram technique according to the standard procedure[23]. Cells were allowed to grow in serum-free medium for 24 h. Then the medium was collected and diluted 1:1 with × 2 sample buffer and run on 10% polyacrylamide gels containing gelatin (1 mg/mL) until the bromophenol blue tracking dye reached the bottom of the gel. After electrophoresis, the gel was washed twice in Triton X-100 solution (2.5%) at room temperature for 15 min, transferred to 100 mL of development buffer containing 2 mL of Triton X-100 solution, and incubation was conducted at 37 ℃ for 72 h. Staining with Coomassie Blue R-250 solution proceeded overnight, then destaining was performed until the bands were clearly visible. Areas of protease activity appeared as clear bands against a dark blue background where the protease has digested the substrate.

Mouse tumor models

Twenty-four 4-wk-old male BALB/c nu/nu mice weighing 10-12 g were purchased and randomly divided into control, SGC7901-vector and SGC7901-DLL4 groups. Cells (1×10^6 cells/animal) in a total volume of 0.1 mL of PBS were implanted subcutaneously into the flank of each mice on day 0. Tumor size was measured on days 7, 14, 28, 35, 42. The tumor volume was calculated using the formula: volume = $D \times d^2 \times$ pi/6, where D and d represent the longer diameter and shorter diameter respectively.

Immunohistochemistry

For DLL4 staining, we used a rabbit monoclonal anti-human DLL4 antibody (1:200, Abcam). Paraffin-embedded tissue blocks were serially sectioned 4 μm in thickness, dewaxed, and rehydrated in serial alcohol washes. Endogenous peroxidase activity was blocked with 0.03% hydrogen peroxide in PBS for 20 min. Immunostaining for DLL4 was done by incubation for 1 h with primary antibody in blocking buffer and visualized using 3,3'-diaminobenzidine chromogen (Invitrogen) with hematoxylin (Invitrogen) counterstaining after treatment with HRP-conjugated Goat anti-rabbit immunoglobulin G (1:100 dilution).

Statistical analysis

Numerical results are shown as mean ± SD. Data were analyzed using SPSS ver. 13.0 statistical software (SPSS, Inc., Chicago, IL, United States). Differences among three groups were examined using one-way analysis of variance analysis. Means between two groups were compared using the Student's t test. Statistical significance was considered a P value of < 0.05. All experiments were performed at least three times.

RESULTS

Up-regulation of DLL4 changed downstream gene expression in SGC7901 cells

SGC7901 cells were transfected with vector encoding human *DLL4* (SGC7901-DLL4 group) or empty vector (SGC7901-vector group) and were then selected by G418 for at least 3 wk. Non-transfected SGC7901 cells were used as a control group. The up-regulating effect of the vector on DLL4 protein levels in the SGC7901-DLL4 group was confirmed by western blot assay (Figure 1A). Real-time PCR was further used to assess the expression of *Notch1*, *DLL4*, and downstream genes including *HES1*, *HES5*, *HEY1*, as well as the mRNA levels of MMP-2, MMP-9 and the adhesion protein E-cadherin. The results show that the mRNA level of DLL4 in the SGC7901-DLL4 group was approximately 10-fold higher than in the SGC7901-vector group. Accordingly, up-regulation of *DLL4* expression resulted in increased expression of *HES5*, *MMP-2* and *MMP-9* (Figure 1B).

Effects of DLL4 up-regulation on gastric cancer cell proliferation

MTS cell proliferation assays (Promega) were used to investigate the effect of DLL4 transfection on gastric

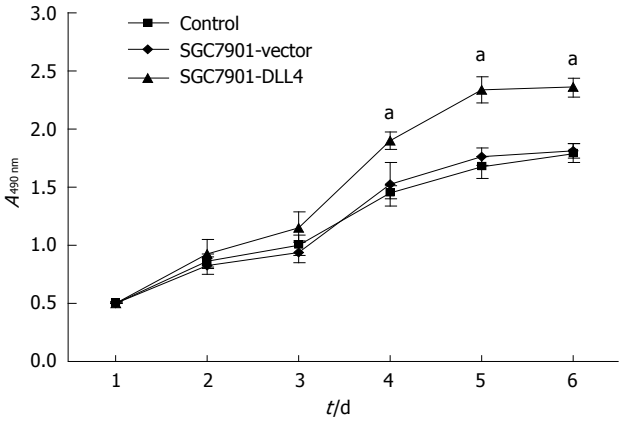

Figure 2 Up-regulation of Delta-like ligand 4 promoted cell proliferation in SGC7901 cells. Growth curve comparing SGC7901-Delta-like ligand 4 (DLL4), SGC7901-vector and SGC7901 cells over a 6-d time course. Up-regulation of DLL4 significantly promoted the proliferation of SGC7901 cells *in vitro*. Data are shown as the mean ± SD of three independent experiments ($^aP < 0.05$ *vs* the SGC7901-vector group); SGC7901-vector: SGC7901 cells transfected with an empty vector; SGC7901-DLL4: SGC7901 cells transfected with a vector encoding human *DLL4* gene; Control: Non-transfected SGC7901 cells.

cancer cells. A growth curve was plotted based on the optical densities obtained during the 6 d after attachment. The results showed that up-regulation of DLL4 resulted in significantly accelerated cell proliferation in the SGC7901-DLL4 group when compared to the SGC7901-vector group ($P < 0.05$, Figure 2).

Up-regulation of DLL4 accelerated migration of SGC7901 cells

The effects of DLL4 up-regulation on SGC7901 cell migration were investigated using 8.0-μm pore-size Corning Costar Transwell units. Approximately 4×10^4 cells from each of the groups were plated on the insert. The results show that the number of SGC7901 cells transfected with DLL4, which migrated across the insert, was 8.3 times higher than those transfected with empty vector (205.4 ± 15.2 *vs* 22.3 ± 12.1, $P < 0.05$) (Figure 3).

Up-regulation of DLL4 accelerated invasion of SGC7901 cells

Matrigel invasion assays were performed using 8.0-μm pore-size Corning Costar transwell units precoated with 50 μL Matrigel (1:5 diluted), which permitted cell migration across the filter. Approximately 4×10^4 cells from each group were plated in the insert. The results show that the number of SGC7901 cells transfected with DLL4, which migrated across both the Matrigel and the insert, was 2.83 times higher than those transfected with an empty vector (68.8 ± 5.3 *vs* 18.2 ± 6.0, $P < 0.05$) (Figure 3).

Effect of DLL4 up-regulation on the secretion and activation of extracellular MMPs

Gelatin zymography was used to analyze the effect of DLL4 up-regulation on the secretion and activation of MMPs, such as MMP-2 and MMP-9. From each group, 20 μL of culture supernatant were diluted 1:1 with × 2 sample buffer, incubated for 30 min at 25℃, and added to a zymogram comprising 10% polyacrylamide, 1 mg/mL gelatin, and electrophoresed at a constant voltage of 110 V until the bromophenol blue tracking dye reached the bottom of the gel. Coomassie Blue R-250 staining of the zymogram revealed significantly increased MMP-2 proenzyme level but decreased MMP-9 proenzyme level in the medium of the SGC7901-DLL4 group compared to the SGC7901-vector group (Figure 4).

Up-regulation of DLL4 accelerated tumor growth in vivo

To examine the effect of DLL4 up-regulation on gastric cancer growth *in vivo*, 24 four-wk-old male BALB/c nu/nu mice were divided randomly and averagely into three groups and implanted subcutaneously with control/SGC7901-vector/SGC7901-DLL4 cells. Six weeks after inoculation, the gastric cancer cells grew as subcutaneous implants in each nude mouse (100%). The size of subcutaneously formed tumor masses of the SGC7901-DLL4 group (2640.5 ± 923.6 mm^3) ($P < 0.05$ relative to the SGC7901-vector group) was significantly larger than the control (1011.1 ± 273.6 mm^3) and SGC7901-vector groups (1115.1 ± 223.8 mm^3) (Figure 5A). Immunohistochemistry staining of DLL4 confirmed the up-regulation of DLL4 expression in the SGC7901-DLL4 group (Figure 5B).

DISCUSSION

The finding that DLL4/Notch signaling participates in vascular development and homeostasis, as well as the evidence that DLL4 is predominantly expressed in the developing endothelium and in some tumor endothelia, suggest that DLL4-mediated Notch signaling activation is involved in tumor angiogenesis[24-26]. However, literature regarding the biological properties of DLL4-mediated Notch signaling in gastric cancer is very limited. The aim of our study is to investigate the potential roles of DLL4 on the biological behavior of gastric cancer cells and its molecular mechanisms.

In this study, we investigated the effect of DLL4 up-regulation on cell growth, migration and invasion *in vitro* and tumor growth *in vivo*. The results showed that up-regulation of DLL4 significantly promotes proliferation, migration, and invasion of SGC7901 gastric cancer cells *in vitro* and tumor growth *in vivo*. Our findings indicate the oncogenic function of DLL4/Notch signaling in gastric cancers, which is in accord with previous studies on other cancers. For example, small interfering RNA (siRNA)-induced knockdown of DLL4 resulted in decreased proliferation, increased apoptosis and retarded growth of tumor *in vitro* and *in vivo*[27-31]. Other Notch-targeting approaches such as chemical inhibitors of gamma-secretase significantly suppressed cell growth in colon cancer cell lines[32,33] and sensitized oxaliplatin- and 5-Fu-induced apoptosis and growth inhibition[34]. These findings indicate that DLL4/Notch signaling is an important molecular pathway involved in oncogenesis and chemoresis-

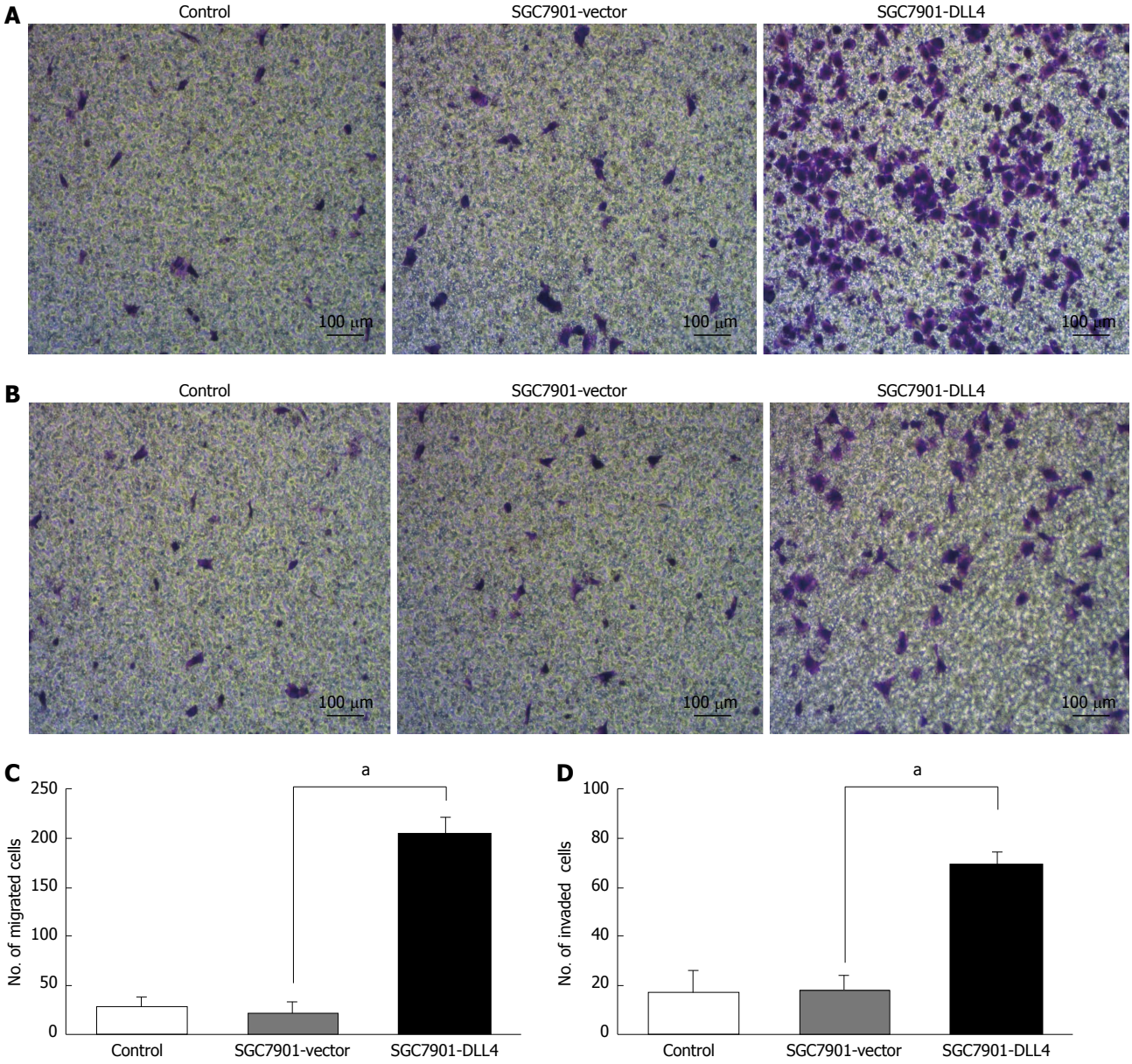

Figure 3 Up-regulation of Delta-like ligand 4 promoted cell migration and invasion in SGC7901 cells. A: Crystal violet staining revealed the migrated cells of each group; B: Crystal violet staining reveals invaded cells from each of the groups; C: The number of cells in the SGC7901-Delta-like ligand 4 (DLL4) group that had migrated was 8.31 times higher than those transfected with an empty vector (205.4 ± 15.2 *vs* 22.3 ± 12.1, $^{a}P < 0.05$); D: The number of cells in the SGC7901-DLL4 group that had migrated across both the Matrigel and the insert was 2.83 times higher than that in the SGC7901-vector group (68.8 ± 5.3 *vs* 18.2 ± 6.0, $^{a}P < 0.05$). SGC7901-vector: SGC7901 cells transfected with an empty vector; SGC7901-DLL4: SGC7901 cells transfected with vector encoding human *DLL4* gene; Control: Non-transfected SGC7901 cells.

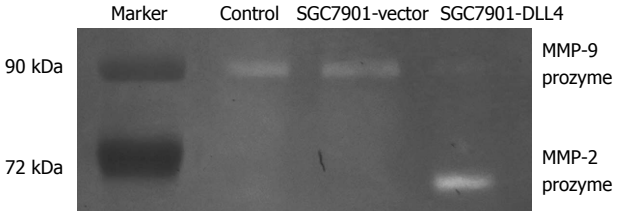

Figure 4 Effect of Delta-like ligand 4 up-regulation on the secretion and activation of extracellular matrix metalloproteinases. Twenty microlitres of culture supernatant from each group was used. Coomassie Blue R-250 staining of the zymogram revealed significantly increased matrix metalloproteinases (MMP)-2 proenzyme but decreased MMP-9 proenzyme in the SGC7901-Delta-like ligand 4 (DLL4) group *vs* the SGC7901-vector group. SGC7901-vector: SGC7901 cells transfected with an empty vector; SGC7901-DLL4: SGC7901 cells transfected with vector encoding human *DLL4* gene; Control: Non-transfected SGC7901 cells.

tance. Targeting DLL4/Notch signaling might constitute a novel molecular therapy for cancers.

Wang *et al*[31] reported that inactivation of Notch signaling in prostate cancer cells leads to decreased expression and activity of MMP-9, which contributes to the inhibition of cell migration and invasion. Our study shows that up-regulation of DLL4 leads to decreased activity of MMP-9 with increased MMP-9 expression at mRNA level. One explanation for this observation could be that Notch signaling targeting genes, as well as MMP-9, have complex post-transcriptional regulations. This explanation might also contribute to diverse roles of Notch signaling in different types of cancers. Another possible explanation is that the activation of MMP-9 is inhibited by

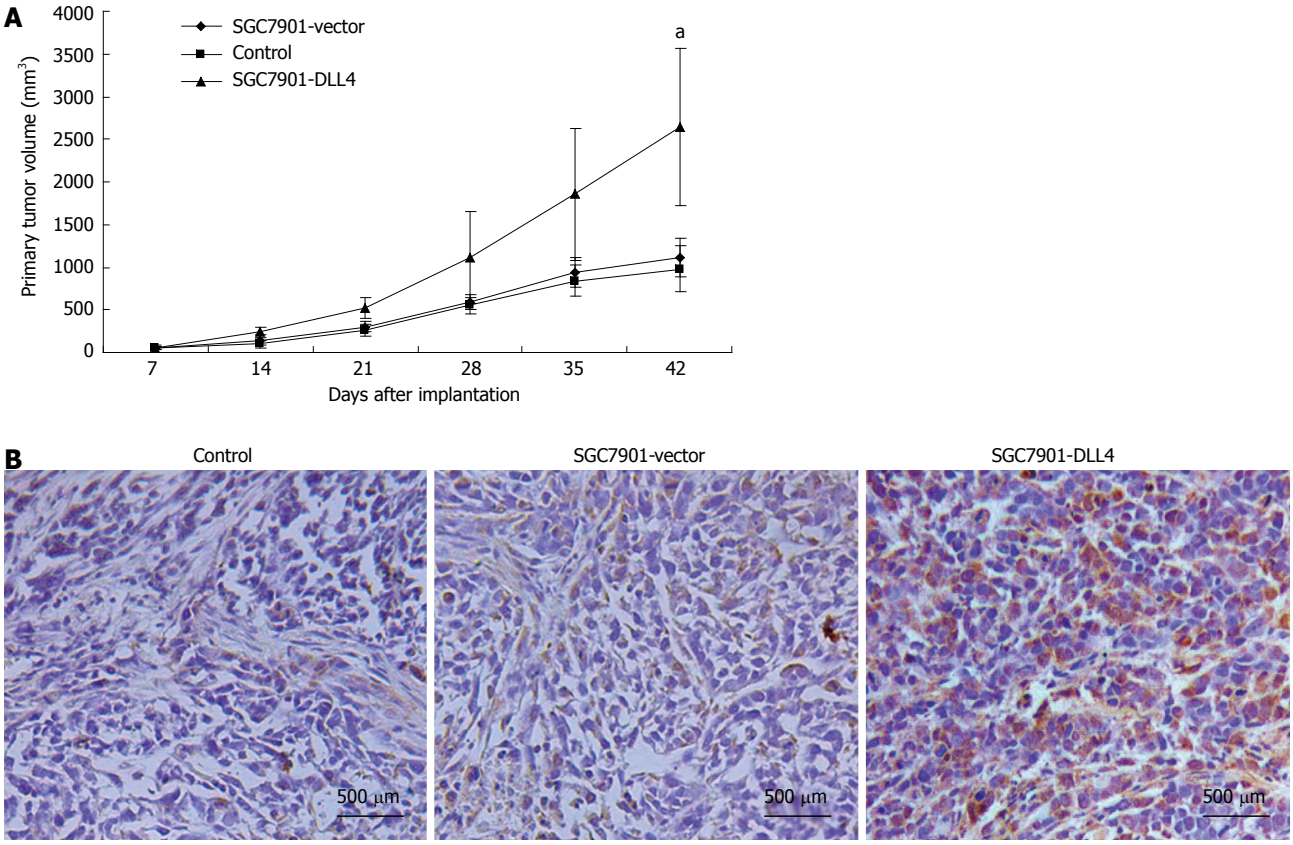

Figure 5 Up-regulation of Delta-like ligand 4 promoted tumorigenesis of SGC7901 cells *in vivo*. Twenty-four 4-wk-old male BALB/c nu/nu mice were divided randomly into three groups and implanted subcutaneously with control/SGC7901-vector/SGC7901-Delta-like ligand 4 (DLL4) cells. A: Six weeks after inoculation, the size of subcutaneous formed tumor masses of the SGC7901-DLL4 group (2640.5 ± 923.6 mm^3) was significantly larger than the control (1011.1 ± 273.6 mm^3) and SGC7901-vector groups (1115.1 ± 223.8 mm^3); B: Immunohistochemistry staining of DLL4 confirmed the up-regulation of DLL4 in the SGC7901-DLL4 group. $^aP < 0.05$ *vs* the SGC7901-vector group.

other signaling pathways or molecules induced by DLL4 up-regulation, such as tissue inhibitor of metalloproteinase-1 (TIMP-1), TIMP-2, or other TIMPs. Furthermore, significantly increased mRNA levels and secretion of MMP-2 proenzymes were observed in gastric cancer cells with up-regulated DLL4. These results suggest that MMP-9 signaling might not be sufficient to exert an effect alone in gastric cancer progression[35], while other molecules such as MMP-2 might play a major role.

In summary, to our knowledge, activation of DLL4-mediated Notch signaling effectively promotes proliferation, migration, and invasion of gastric cancer cells, and desensitizes the cells to chemotherapeutically induced cell senescence. Our data suggest that DLL4-mediated Notch signaling may play an important role in the progression of gastric cancer, and that DLL4-mediated Notch signaling could be a potential target for gastric cancer biotherapy. Meanwhile, we identified MMP-2 as a novel target of DLL4-mediated Notch signaling in gastric cancer cells. However, the precise molecular mechanism of DLL4-mediated Notch signaling in gastric cancer remains unclear. Further studies are required to elucidate possible downstream target genes or potential interacting molecules of DLL4-mediated Notch signaling.

In summary, up-regulation of DLL4 significantly promoted cellular proliferation, migration, and invasion *in vitro* and tumor growth *in vivo*. Significantly increased MMP-2 expression at both the mRNA and the protein level was observed in gastric cancer with up-regulated DLL4. Our observations indicated a mechanism by which activation of DLL4-mediated Notch signaling promotes the expression and secretion of MMP-2 proenzyme and influences the progress of gastric cancer.

COMMENTS

Background

Gastric cancer is one of the most common cancers and lethal malignancies worldwide. Discovering novel biomarkers that correlate with gastric cancer may present opportunities to reduce the severity of this disease. As one of the five Notch signaling ligands in mammals, Delta-like ligand 4 (DLL4) has been researched mainly with regard to vasculogenesis and tumor angiogenesis. However, the precise function and mechanism of DLL4 in gastric cancer remain unclear.

Research frontiers

Notch signaling, as an evolutionarily conserved signaling pathway, is involved in a variety of cellular processes, including cell fate, differentiation, proliferation and apoptosis. Previous data indicates its distinct biological roles in different tumors. It may work as an oncogene or anti-oncogene. Their observations indicated a mechanism by which activation of DLL4-mediated Notch signaling promotes the expression and secretion of matrix metalloproteinase (MMP)-2 proenzyme and influences the progress of gastric cancer.

Innovations and breakthroughs

Previous studies indicated that DLL4 is largely restricted to the vascular endothelia. Recent reports were focused on its roles on vasculogenesis and tumor

angiogenesis. The authors discovered that DLL4 up-regulation promotes cellular proliferation, migration, invasion and tumorigenicity in gastric cancer cells. Increased mRNA level and increased secretion of matrix metalloproteinase-2 proenzyme, while increased MMP-9 mRNA level but decreased extracellular MMP-9 proenzyme level were observed.

Applications

In understanding the role and mechanism of DLL4-mediated Notch signaling in gastric cancer, this study may represent a future strategy as a therapeutic target and/or a way to improve clinical treatment for gastric cancer.

Peer review

The authors found that ectopic expression of DLL4 significantly promoted cellular proliferation, migration, invasion *in vitro* and tumor growth *in vivo*. Significantly increased mRNA levels and secretion of MMP-2 proenzymes were observed, increased MMP-9 mRNA but decreased extracellular MMP-9 proenzyme were observed. The paper is well presented and the results are interesting.

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ORIGINAL ARTICLE

Basic transcription factor 3 is involved in gastric cancer development and progression

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Abstract

AIM: To further analyse cancer involvement of basic transcription factor 3 (BTF3) after detection of its up-regulation in gastric tumor samples.

METHODS: BTF3 transcription rates in human gastric tumor tissue samples (n = 20) and adjacent normal tissue (n = 18) specimens as well as in the gastric cancer cell lines AGS, SGC-7901, MKN-28, MKN-45 and MGC803 were analyzed *via* quantitative real-time polymerase chain reaction. The effect of stable BTF3 silencing *via* infection with a small interfering RNA (siRNA)-BTF3 expressing lentivirus on SGC-7901 cells was measured *via* Western blotting analysis, proliferation assays, cell cycle and apoptosis profiling by flow cytometry as well as colony forming assays with a Cellomic Assay System.

RESULTS: A significant higher expression of BTF3 mRNA was detected in tumors compared to normal gastric tissues (P < 0.01), especially in section tissues from female patients compared to male patients, and all tested gastric cancer cell lines expressed high levels of BTF3. From days 1 to 5, the relative proliferation rates of stable BTF3-siRNA transfected SGC7901 cells were 82%, 70%, 57%, 49% and 44% compared to the control, while the percentage of cells arrested in the G_1 phase was significantly decreased (P = 0.000) and the percentages of cells in the S (P = 0.031) and G_2/M (P = 0.027) phases were significantly increased. In addition, the colony forming tendency was significantly decreased (P = 0.014) and the apoptosis rate increased from 5.73% to 8.59% (P = 0.014) after BTF3 was silenced in SGC7901 cells.

CONCLUSION: BTF3 expression is associated with enhanced cell proliferation, reduced cell cycle regulation and apoptosis and its silencing decreased colony forming and proliferation of gastric cancer cells.

Key words: Basic transcription factor 3; Gastric cancer; Small interfering RNA; Proliferation; Apoptosis; Cell cycle

Core tip: After we found that basic transcription factor 3 (BTF3) transcription rates in human gastric tumor tissue samples were significantly higher than in adjacent normal tissues, we extended our study on gastric cancer cell lines. We silenced BTF3 in SGC7901 cells, which led to 82%, 70%, 57%, 49% and 44% proliferation rates of the control within the first 5 d after infection with a

small interfering RNA-BTF3 containing lentivirus. After BTF3 silencing, the percentage the G_1 phase arrested SGC7901 cells was decreased (P = 0.000), the percentages of cells in the S (P = 0.031) and G_2/M (P = 0.027) phases were increased and the apoptosis rate increased from 5.73% to 8.59% (P = 0.014).

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INTRODUCTION

Basic transcription factor (BTF3) is a 27 kD protein that in humans is encoded by the *BTF3* gene[1,2] and evolutionarily conserved in a variety of cells[3]. BTF3 was initially discovered as a member of the general transcription machinery and functions as a transcriptional initiation factor from proximal promoter elements by forming a stable complex with RNA polymerases[4,5]. There are two ubiquitously expressed isoforms of the *BTF3* gene that encode BTF3a and BTF3b proteins. The BTF3a is the transcriptional active form of BTF3, while the isoform BTF3b, which lacks the first 44 amino acids of the BTF3a N-terminus is transcriptionally inactive, although it is able to bind to the RNA polymerase Ⅱ[6]. However, previous studies indicated that BTF3 was not in fact essential for specific, *in vitro* initiation of transcription, but its biological importance was shown by the fact that mouse embryos, homozygous for a loss of function mutation in the *BTF3* gene, died at the early stage of development indicating its important role during development[7]. In addition, BTF3 was up-regulated strongly in mouse pregnancy, indicating an involvement in alveolar growth[8]. In cancer, it has been reported that the *BTF3* gene has been overexpressed in colorectal cancer, glioblastomas and hepatocellular carcinomas[9-12]. In the pancreatic ductal adenocarcinoma, the median level of the BFT3 and BFT3a mRNA was increased by 1.3 and 4.6 folds compared to the normal tissues, respectively. Down-regulation of the BTF3 expression using small interfering RNA (siRNA) resulted in reduced expression of several cancer-associated genes, including ephrin receptor B2, which is mainly expressed during development and involved in tumor cell survival[13,14] and heparanase 2 an extracellular matrix degrading enzyme involved in cell adhesion. In addition, ataxia-telangectasia mutated gene, which is implicated in cell cycle arrest[15], DNA repair[16] or apoptosis and the oncogene V-abl Abelson murine leukemia viral oncogene homolog 2, a nuclear protein tyrosine kinase for cell differentiation, cell division and cell adhesion were also had reduced expression[17]. By BFT3 silencing, up-regulated genes were *k-ras* oncogene-associated gene, related ras viral oncogene homolog 2, nuclear factor kappa-B, murine retrovirus integration site 1 and mucosal vascular addressing cell adhesion molecule 1, all known to be involved in tumor development[18]. In addition, BTF3 interacts with either 17β-estradiol or epidermal growth factor activated estrogen receptor α (ERα) *via* its AF1 domain in the breast cancer cell line MCF-7 and up-regulates transcriptional responses of ERα reporter genes[19,20]. In this study, we analyzed the expression of BTF3 mRNA and protein in gastric tumors and normal samples and compared BTF3 expressions in different gastric tumor cell lines. Finally we used siRNA-BTF3 to down-regulate BTF3 expressions in gastric tumor cells and measured the changes of proliferation and apoptosis to investigate the relationship between BTF3 and gastric cancer.

MATERIALS AND METHODS

Patients

Human tissue samples of gastric tumor (n = 20) and adjacent normal tissue (n = 18) specimens were obtained from patients with a median age of 63 years (range, 35-83 years) who received gastric resections from November 2011 to March 2012 in the Second Xiangya Hospital of Central South University, Changsha, China. All samples were confirmed histologically. Freshly removed tissues (within 5 min after surgical excision) were: (1) fixed in paraformaldehyde solution for 12-24 h and then paraffin embedded for histological analysis; (2) kept in RNAlater (Ambion Ltd., Huntingdon, Cambridgeshire, United Kingdom) for RNA analysis; or (3) snap-frozen in liquid nitrogen and maintained at -80 ℃ for protein analysis. All studies were approved by The Human Ethics Committee of the First Affiliated Hospital of Hunan Normal University in China, and written informed consent was obtained from all patients.

Cell culture

Gastric cancer cell lines (AGS, SGC-7901, MKN-28, MKN-45 and MGC803) were grown in complete DMEM medium (Gibco, New York, United States), supplemented with 10% fetal calf serum (Gibco, New York, United States) and 5 U/mL penicillin, and incubated at 37 ℃ in 5% CO_2 atmosphere.

Plasmid and vector construction

The selected and optimized siRNA (sense: GCCGAAGAAGCCTGGGAATCA, anti-sense: TGATTCCCAGGCTTCTTCGGC) against human BTF3 and negative control (sense: TTCTCCGAACG TGTCACGT, anti-sense: ACGTGACACGTTCGGAGAA) were used in generating the lentiviral vector. Basically, the BTF3 siRNA transcript template was synthesized, digested with Age I/EcoRI enzymes, and then ligated with pGCSIL-GFP vector. After checking by agarose electrophoresis, successful BTF3-siRNA-pGCIL-GFP recombinants were amplified, purified and sequenced. In order to evaluate the inhibitive effect of BTF3 sequences, the confirmed pGCSIL-GFP recombinants were transfected into 293T cells (data not shown). The expressed BTF3 protein was detected by Western blotting.

Table 1 Primer sequences

Primer	Sequence
GAPDH-F	TGACTTCAACAGCGACACCCA
GAPDH-R	CACCCTGTTGCTGTAGCCAAA
BTF3-F	GCGAACACTTTCACCATTACAG
BTF3-R	AACTTCATCATCATCATCCTCTCC

BTF3: Basic transcription factor 3; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Quantitative real-time polymerase chain reaction

Cells were split during the log-phase growth before plating into the 6-well plate with complete medium, and incubated at 37 ℃ with 5% CO_2. An appropriate amount of lentiviral vectors was added to cells when 30% confluence was reached. Total RNA was extracted 5 d after treatment from cells with GFP expression by Trizol (Invitrogen, San Diego, United States). RNA (2 μg) was reversely transcribed using M-MLV-RTase (Promega, Madison, United States) following manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed using the tested primer set (Table 1) and SYBR Master Mixture (*TAKARA, DRR041B*) following the manufacturer's instructions with a TAKARA's TP800 Real Time PCR Instrument. Following the normalization with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene, relative quantification of BTF3 expression was done by the comparative CT method ($2^{-\Delta\Delta C}$ method).

Total protein extraction, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting

Cells were harvested 36-48 h following the transfection. Pellets were washed twice with cold phosphate-buffered saline (PBS) (Gibco, New York, United States) and then cells were lysed on ice for 10-15 min using proper lysis buffer [1 mol/L Tris-HCl 100 mmol/L, 2% 2-mercaptoethanol, 20% glycerol, 4% sodium dodecyl sulfate (SDS) (Life Technologies, New York, United States)]. After sonication, total protein extraction was performed by centrifugation at 1200 *g* for 15 min at 4 ℃. Protein was separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), with 4% separating and 3%-15% gradient stacking gel. After SDS-PAGE, protein samples were wet transferred to polyvinylidenfluorid (PVDF) (Life Technologies, New York, United States) membranes for immunoblotting. To detect the target protein, the PVDF membrane was incubated with appropriate primary antibodies, either 2 h at the room temperature or overnight in the cold room (Mouse Anti-GFP Santa-Cruz SC-9996, 1:2000, Mouse anti-GAPDH Santa-Cruz SC-32233 1:5000), following 3 times of 10 min TBS-Tween 20 buffer (Life Technologies, New York, United States) washes, membranes were incubated with secondary antibodies for 2 h at room temperature (Goat Anti-Mouse IgG Santa-Cruz SC-2005 1:5000). After further 3 times washing, target proteins were detected using ECL™ Western blotting system (Amersham Cat. No. RPN2135).

Flow cytometric assays

Cells were harvested by Trypsin/EDTA (Life Technologies, New York, United States) solution from the 6-cm dishes at 80% confluence followed by setting up the designed assays. Pellets were then washed with PBS and collected into 5 mL centrifuge tubes in triplicate. After fixation using pre-cooled 4 ℃ 70% ethanol for 1 h, cells were washed again and then stained with propidium iodide (FACSCalibur, BD, New York, United States).

Colony forming assay

Five hundred cells were plated per well in 96-well plates in triplicate. After the colony number reached more than 5 in most of the wells, the colonies were scanned using Cellomics system following the manufacturer's protocol. The colony size and cell numbers within each colony were analyzed by Cellomics system (Thermo, Philadelphia, United States). The data were then statistically analyzed.

Statistical analysis

Results of all calculations were expressed as mean ± SD. Analysis of variance (ANOVA) with multiple comparisons using Bonferroni's test or one-way ANOVA was performed where appropriate. The difference between two groups was analyzed by unpaired *t* test GraphPad Prism (version 3.02-2000). The results of statistical tests were considered significant if the *P* value was < 0.05 and < 0.01.

RESULTS

Level of BTF3 expression in gastric cancer and tumor cell lines

A quantitative real-time PCR was performed to identify the transcription levels of the *BTF3* gene in 20 human gastric tumors and the relative tumor free margins ($n = 18$) as healthy tissue control. A significantly higher transcription of BTF3 mRNA was detected in tumors compared to normal gastric tissues (Figure 1A, $P < 0.01$), especially in female patients' section tissues compared to male patients (Figure 1B) and between the gastric body tumor and adjacent normal tissue, but no significant difference was detectable between gastric antrum or cardia tumor and adjacent normal tissues (Figure 1C). In order to further confirm that the *BTF3* gene was widely expressed in our gastric tumor cell lines, quantitative real-time PCR was performed in five different cell lines AGS, SGC-7901, MKN-28, MKN-45 and MGC803. SGC-7901, MKN-45 and MGC803 which are all poorly differentiated adenocarcinomas, while the MKN-28 and AGS are high and moderately differentiated cell lines. The expected products of BTF3 mRNA were detected in all the above five cell lines (Figure 1D).

Silencing of BTF3 by siRNA

Based on the significant BTF3 up-regulation from the clinical data, we sought that the regulation of BTF3 in gastric tissue might be related to cancer initiation promotion and progression. To investigate this, the gene was

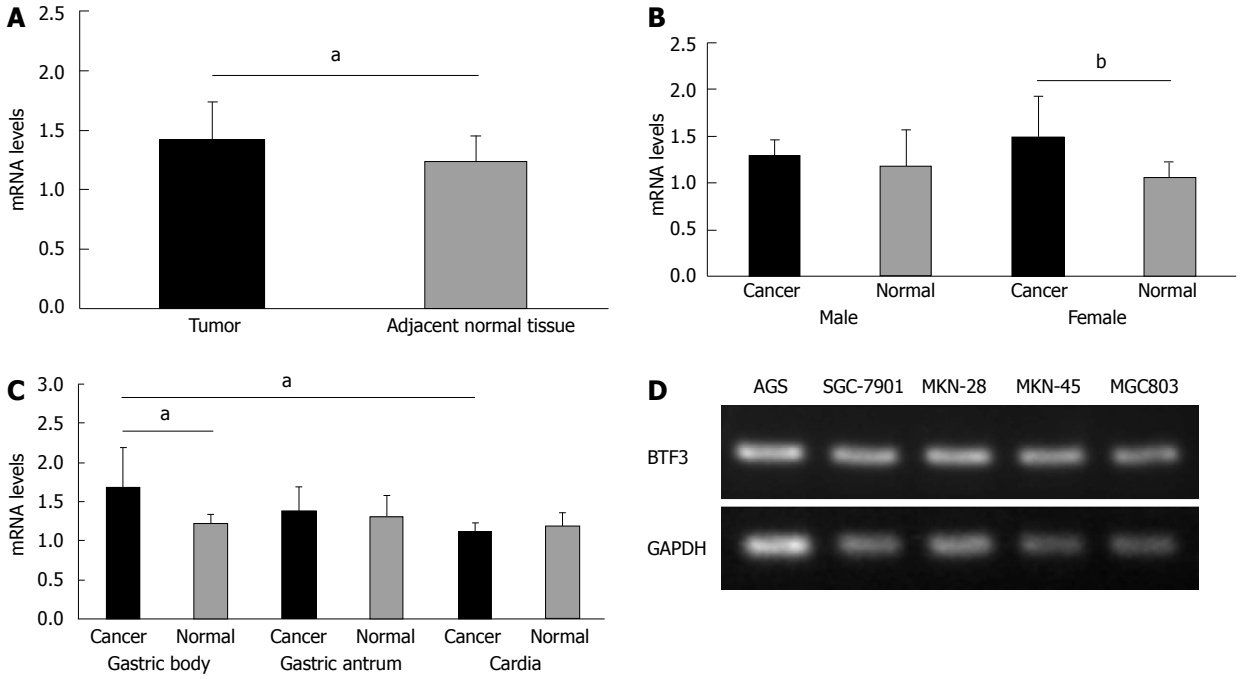

Figure 1 Level of basic transcription factor 3 expression in gastric cancer and tumor cell lines. A: Bar charts represent the mRNA levels of basic transcription factor 3 (BTF3) in gastric tumor and adjacent normal tissue measured by quantitative real-time polymerase chain reaction (PCR); B: BTF3 mRNA expression data from (A) further classified by gender; C: BTF3 expression pattern among gastric body, gastric antrum and cardia tumors; D: The expression of BTF3 mRNA was detected by quantitative real-time PCR among different cell lines. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene. Black bars represent tumors and gray bars normal tissues. The difference in BTF3 expression, $^{a}P < 0.05$, $^{b}P < 0.01$ between groups was assessed by unpaired *t* test and considered significantly different.

silenced using lentiviral vectors expressing the specifically designed synthetic siRNA-BTF3. In pilot experiments, the siRNA-BTF3 was successfully transfected into human SGC7901 and 293T cell lines for mRNA expression profiles and protein analyses *via* GFP expression to estimate the transfection efficiency (Figure 2A). In SGC7901 cells, a significant down-regulation of the *BTF3* gene was measured after siRNA-BTF3 application (Figure 2B). The BTF3 transcription in siRNA transfected cells was 12.4% of the negative control transfected cells ($P = 0.0066$). The BTF3 protein levels in SGC7901 cells, detected by Western blotting 48 h after siRNA-BTF3 transfection, were reduced compared to the control (Figure 2C).

BTF3 silencing effect in SGC7901 cells

In order to identify the effect on gastric tumor cells, proliferation, cell cycle, apoptosis and colony forming assays were performed with siRNA-BTF3 transfected SGC7901 cells. The silencing of BTF3 significantly slowed down the SGC7901 cell growth in the proliferation assay (Figure 3). On day 1, the siRNA transfected cells already started to show an 18% slower proliferation rate compared with the negative controls. The difference became increasingly significant and peaked at the end of the proliferation assay on day 5, on which the proliferation rate in the siRNA transfected cells was only 44% of the negative control. From days 1 to 5, the relative proliferation rates of siRNA transfected SGC7901 were 82%, 70%, 57%, 49% and 44%, respectively. In summary, an obvious effect on proliferation of SGC7901 was induced by silencing BTF3 (Figure 3, BTF3-siRNA). A cell cycle profile of the siRNA-BTF3 transfected cells using PI-FACS and Annexin V showed that the percentage of cells arrested in the G_1 phase was significantly decreased, while the percentages of cells at the S and G_2/M phases were significantly increased (Figure 4). The knockdown of BTF3 in SGC7901 cells also induced an increased level of apoptosis. Cells with good transfection efficiency were harvested on day 5 for flow cytometry (Figure 5). The negative control group which was transfected with empty vectors showed 5.73% and 0.76% apoptosis, while the apoptosis rate of siRNA-BTF3 transfected group was 8.59% and 1.1% (Figure 5B and D). The down-regulation of BTF3 increased the apoptosis rate by nearly 2 folds. To detect the long-term effect on SGC7901 cells following BTF3 silencing, a colony forming assay was performed. Colonies were scanned and analyzed using the Cellomic Assay System. Cells after BTF3 silencing showed a significantly lower colony forming ability compared with the negative controls (Figure 6A). In the silenced group, 14 d following the initiation of the assay in average 10 colonies were detected, whereas a significantly higher number of 22 colonies (mean) was detected in the negative group ($P = 0.014$) (Figure 6B). In addition, the number of cells in each siRNA-BTF3 transfected SGC7901 cell colony was also reduced compared with the control.

DISCUSSION

In cells, multiple protein complexes are required to be

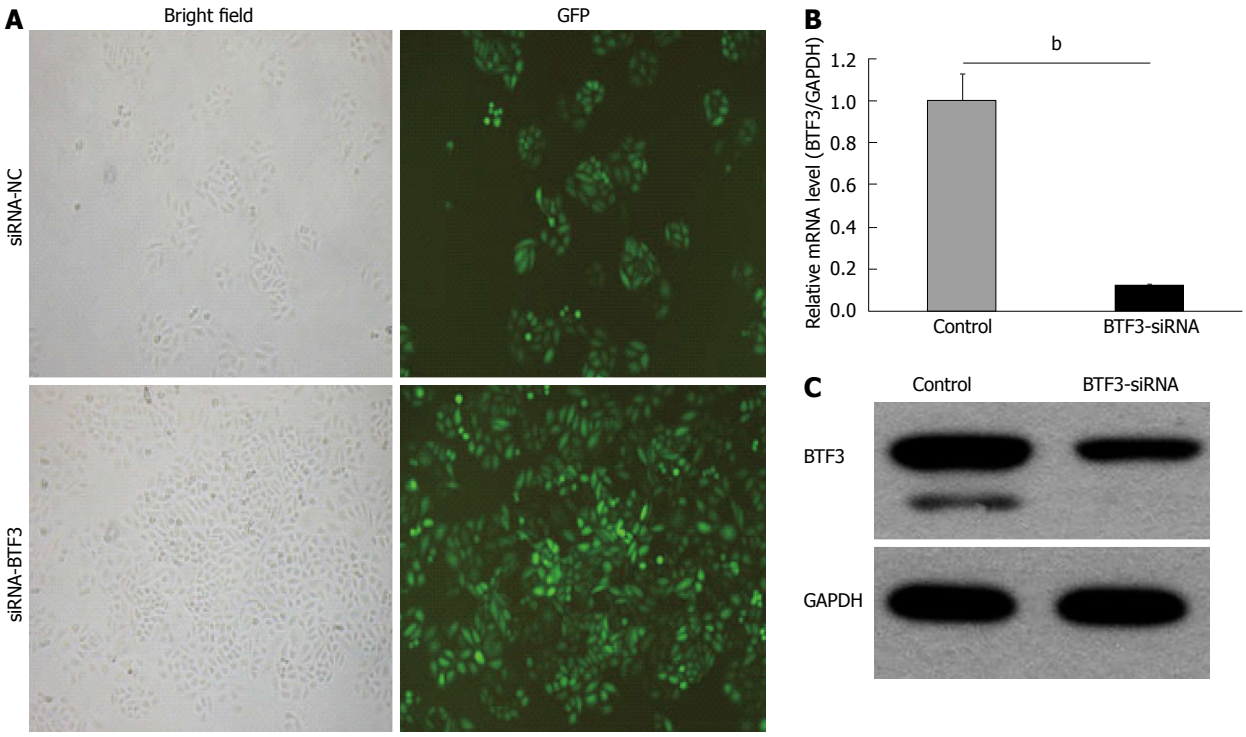

Figure 2 Silencing of basic transcription factor 3 using small interfering RNA. A: Transfection rates of small interfering RNA (siRNA)-normal control (NC)/siRNA-basic transcription factor 3 (BTF3)-green fluorescent protein (GFP) vectors in human SGC7901 cells, estimated by bright field and fluorescence microscope; B: Bar charts represent quantitative real-time polymerase chain reaction data of BTF3 mRNA levels in control and BTF3-siRNA transfected SGC7901 cells ($^{b}P < 0.01$ between groups, unpaired *t* test); C: Immuno-blotting analysis using BTF3-specific antibodies to detect BTF3 protein expressions in control and BTF3-siRNA transfected SGC7901 cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control.

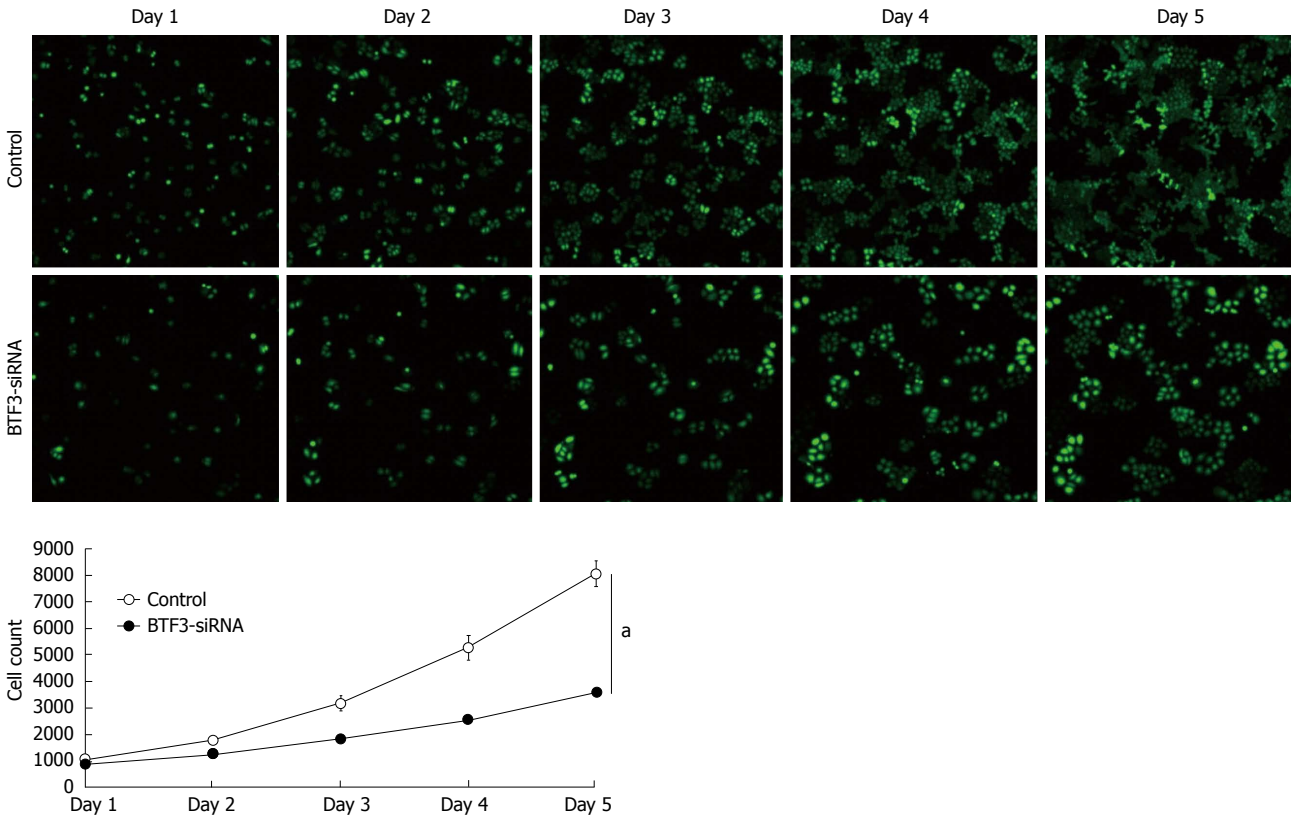

Figure 3 Cell proliferation assay. Fluorescent images of control and small interfering RNA (siRNA)-basic transcription factor 3 (BTF3) transfected cell proliferation assays. Cells were counted based on the green fluorescent protein signals during 5 d. BTF3-siRNA chart of the cell proliferation assay using two-way analysis of variance. $^{a}P < 0.05$ between groups.

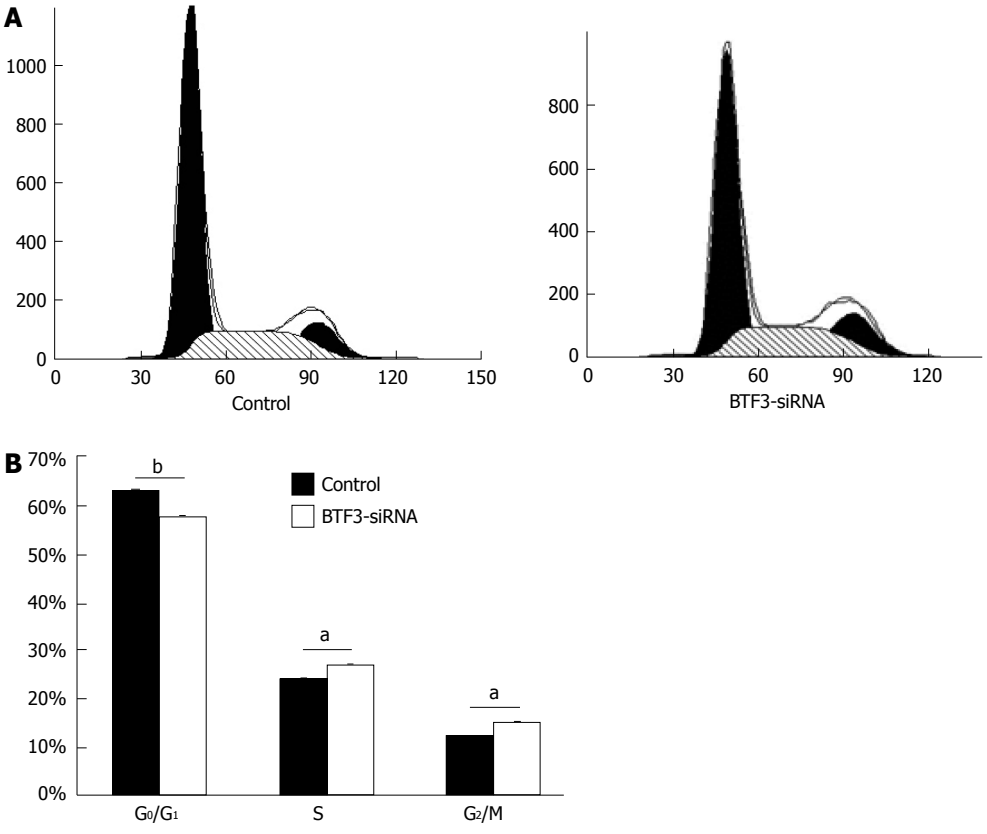

Figure 4 Flow cytometric assays. A: Cell-cycle stages of control and basic transcription factor 3 (BTF3)-small interfering RNA (siRNA) transfected cells were analyzed by flow cytometry. Data are presented as a histogram, with cell number (y-axis) plotted against DNA content (x-axis); B: Cells arrested at different cell-cycle stages were plotted as bar charts. $^{a}P < 0.05$, $^{b}P < 0.01$ between groups was assessed by unpaired *t* test and comparisons were considered significantly different.

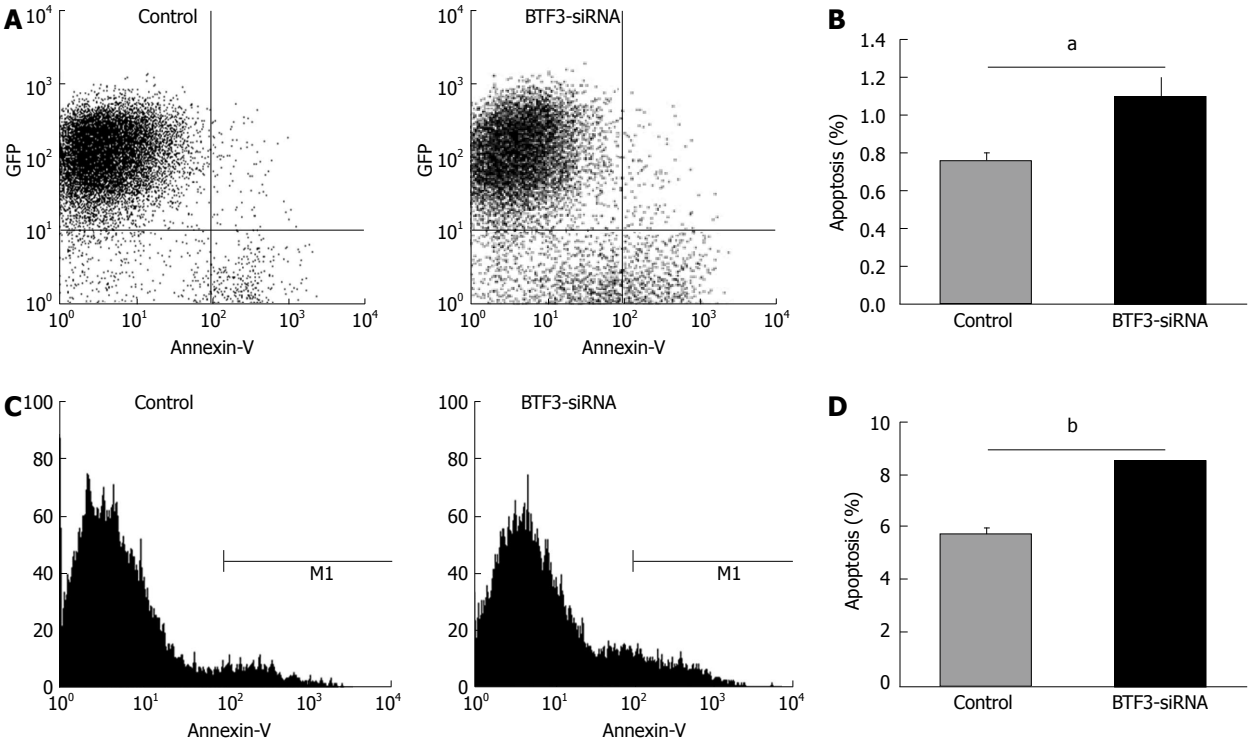

Figure 5 Apoptosis analyses by flow cytometry. A and C: Flow cytometric analysis of control and basic transcription factor 3 (BTF3)-small interfering RNA (siRNA) transfected cells; B and D: Apoptosis rates plotted as bar charts. $^{a}P < 0.05$, $^{b}P < 0.01$ between groups. GFP: Green fluorescent protein.

orderly assembled on proximal promoter elements such as the TATA box and CAAT box sequences in order to initiate the gene transcription. At the very early stage of the transcription initiation, transcription factor class Ⅱ D (TFⅡD), which belongs to the transcription factor class Ⅱ (TFⅡ) family is required to be stably bound to the transcription factor assembling TATA box. The BTF3 protein, which works as an additional TFⅡ re-

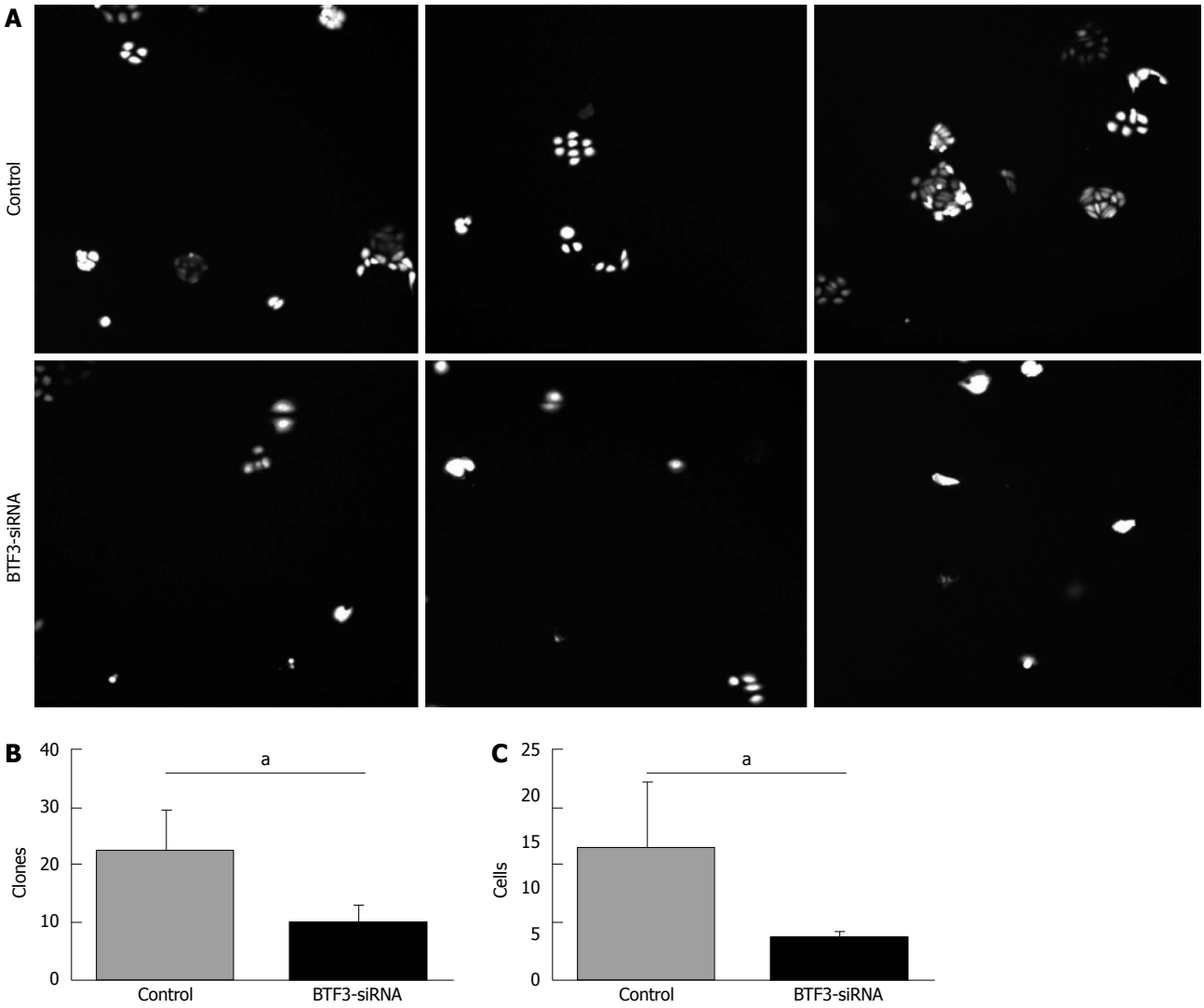

Figure 6 Colony forming abilities identified by Cellomic Assay System assays. A: Colonies formed from control and basic transcription factor 3 (BTF3)-small interfering RNA (siRNA) transfected cells were imaged *via* the Cellomic Assay System; B: Number of clones plotted in a bar chart; C: Colony cell numbers of control and BTF3-siRNA transfected cells plotted in a bar chart. $^{a}P < 0.05$ between groups were analysed with unpaired *t* test.

lated protein, does not directly associate with the proximal promoter, but forms a stable complex with RNA polymerase Ⅱ and is part of the gene transcription initiation complex[4,5]. Several studies have reported that expression pattern of transcription factors is frequently changed in gastric tumors. For example, the transcription factor Sp1 has been reported to be higher in malignant gastric tissues, and might serve as an independent prognostic factor, by influencing the tumor infiltration and progression[21]. Another transcription factor, the E2F transcription factor 1, which plays a critical role in cell cycle regulation and other biological processes of the cells, is also up-regulated in human gastric cancer tissues, but further analysis revealed that its overexpression slowed down the cancer cell growth rate[22]. Other transcription related genes were reported to be differentially expressed in gastric tumors compared to normal tissues. Some genes like the antitumor genes *GATA-4* and *GATA-5* were down-regulated[23,24], whereas others like the oncogenes Forkhead box protein M1[25], and phosphorylated Forkhead box protein O1, which are related to angiogenesis[26] were up-regulated. In addition, the activating transcription factor 4[27], which is cell protective and thereby leading to multidrug resistance, also had enhanced expression. In the present study, we found that the expression of BTF3 is up-regulated in 20 gastric tumor samples compared with normal tissues. We further investigated the expression levels of BTF3 in different malignant gastric tumor cell lines and found uniform expression levels among them (Figure 2). This is in agreement with previous information, because high BTF3 protein levels in gastric cancers have been reported in the Human Protein Atlas[28]. Hence BTF3 is a transcription factor and related to apoptosis[29-32], we thought that the expression level of BTF3 might be important for cell cycle check points, proliferation and further potentially linked with human tumor development and progression. In order to elucidate this, we silenced BTF3 in SGC7901 cells (Figure 3). As a result, the cell cycle arrest shifted from the G_1 to the G_2/M and S phases with a significantly increased apop-

tosis rate, which was also reflected in our colony forming assay with significantly less cell growth after BTF3 silencing (Figure 6). This data indicated that BTF3 silencing might induce G2/M check point failure, which led to the inhibition of cell cycle completion and enhanced apoptotic activity.

In summary, our data indicated that BTF3 has a potential link with gastric cancer development and progression. Low expression or silencing of BTF3 might inhibit tumor growth and be beneficial for cancer treatment. However, the clinical data revealed that the tumors had different BTF3 levels possibly due to the different stages of the gastric tumors examined.

COMMENTS

Background

It has been reported that the basic transcription factor 3 (*BTF3*) gene has been overexpressed in colorectal cancer, glioblastomas as well as pancreatic ductal adenocarcinoma. Down-regulation of the BTF3 expression using small interfering RNA (siRNA) resulted in changed expression of several cancer associated genes involved in tumor cell survival, cell adhesion and cell cycle.

Research frontiers

BTF3 was upregulated in all gastric cancer samples measured in this study. The authors demonstrated that silencing of BTF3 in SGC7901 cells led to a significant decline of proliferation due to enhanced cell cycle arrest and a concomitant higher apoptosis rate. The long-term effect of BTF3 silencing led to a lower amount of colony forming with less cell survival.

Innovations and breakthroughs

In gastric cancer tissues, enhanced expression of BTF3 was reported in the Human Protein Atlas, but the exact role of BTF3 in gastric cancer was not further analyzed. In this study, BTF3 was found to play an essential role in gastric cancer cell proliferation.

Applications

BTF3 was upregulated in all gastric cancer tissues derived from surgical interventions. Silencing of BTF3 led to less proliferation with an enhanced apoptosis rate in SGC7901 cells. The findings may be useful for the diagnosis and treatment of gastric cancer in the future.

Terminology

BTF3 was initially discovered as a member of the general transcription machinery and functions as a transcriptional initiation factor from proximal promoter elements by forming a stable complex with RNA polymerases, and mouse embryos, homozygous for a loss of function mutation in the *BTF3* gene, died at the early stage of development. In later developmental stages, the role of BTF3 has been described as transcriptional regulator and modulator of apoptosis.

Peer review

The authors analyzed the BTF3 expression in human gastric cancer tissues and compared the rates with expression in adjacent non-tumor tissues. As a result, all cancer tissues showed enhanced BTF3 expression. Further analysis *via* stable silencing of BTF3 in gastric cancer SGC7901 cells revealed that inhibiting BTF3 activity led to lower proliferation and enhanced apoptosis rates. The results are interesting and BTF3 might be a target gene for gastric cancer treatment.

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BRIEF ARTICLE

Neurotensin receptor 1 overexpression in inflammatory bowel diseases and colitis-associated neoplasia

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Author contributions: Gui X is the guarantor of the work as well as the main/lead researcher who was instrumental in the design, planning, selecting tissue samples, conducting the study, data review and analysis of the results, and writing the manuscript; Liu S was the major contributor to the laboratory work; Yan Y assisted with the laboratory work; Gao Z contributed partly to the results review/analysis, comments, and review of the manuscript.

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Abstract

AIM: To explore the association of neurotensin receptor 1 (NTSR1) with inflammatory bowel diseases (IBD) and colitis-associated neoplasia.

METHODS: NTSR1 was detected by immunohistochemistry in clinical samples of colonic mucosa with IBD colitis, colitis-associated raised low-grade dysplasia (LGD) including dysplasia-associated lesions or masses (DALMs, *n* = 18) and adenoma-like dysplastic polyps (ALDPs, *n* = 4), colitis-associated high-grade dysplasia (HGD, *n* = 11) and colitis-associated colorectal carcinoma (CACRC, *n* = 13), sporadic colorectal adenomatous polyp (SAP, *n* = 17), and sporadic colorectal carcinoma (SCRC, *n* = 12). The immunoreactivity of NTSR1 was semiquantitated (as negative, 1+, 2+, and 3+) and compared among different conditions.

RESULTS: NTSR1 was not detected in normal mucosa but was expressed similarly in both active and inactive colitis. LGD showed a significantly stronger expression as compared with non-dysplastic colitic mucosa, with significantly more cases showing > 2+ intensity (68.75% in LGD *vs* 32.26% in nondysplastic mucosa, *P* = 0.001). However, no significant difference existed between DALMs and ALDPs. CACRC and HGD showed a further stronger expression, with significantly more cases showing 3+ intensity than that in LGD (61.54% *vs* 12.50% for CACRC *vs* LGD, *P* = 0.022; 58.33% *vs* 12.50% for CACRC/HGD *vs* LGD, *P* = 0.015). No significant difference existed between colitis-associated and non-colitic sporadic neoplasia.

CONCLUSION: NTSR1 in colonic epithelial cells is overexpressed in IBD, in a stepwise fashion with sequential progress from inflammation to dysplasia and carcinoma.

Key words: Neurotensin; Neurotensin receptor; Inflammatory bowel diseases; Dysplasia; Colitis-associated neoplasia; Dysplasia-associated lesion or mass; Sporadic adenoma; Colorectal carcinoma

Core tip: Neurotensin receptor 1 (NTSR1) in colonic epithelial cells is overexpressed in inflammatory bowel diseases, in a stepwise fashion with the sequential progress from inflammation to low-grade dysplasia, high-grade dysplasia, and carcinoma. Both colitis-associated and sporadic dysplasia/carcinoma showed a similar pattern of NTSR1 overexpression. NTSR1 could be a potential pharmacological target in the treatment of inflammatory bowel diseases and prevention of colitis-associated neoplasia.

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plasia. *World J Gastroenterol* 2013; 19(28): 4504-4510 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i28/4504.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i28.4504

INTRODUCTION

Neurotensin (NTS) is a 13-amino-acid peptide secreted by neurons and specialized endocrine cells (N-cells) in the small intestine, which acts as a paracrine and endocrine modulator of various gut functions. The biological activities of NTS are mediated mainly through the high-affinity neurotensin receptor 1 (NTSR1), a member of the G-protein-coupled receptor family[1].

Many studies have suggested a possible role of NTS/NTSR-1 in the pathogenesis of inflammatory bowel disease (IBD) and colitis-associated neoplasia. The NTS/NTSR-1 signaling pathway has a complex dual effect (both proinflammatory and proregenerative) in the regulation of intestinal mucosal inflammation. NTS/NTSR-1 enhances the progression of acute colonic inflammation. NTS enhances mast cell degranulation and neutrophil recruitment[2-4]. NTSR-1 expression is increased in the human colonic mucosa with active ulcerative colitis (UC)[5,6] as well as in rodent colitis induced by *Clostridium difficile* toxin A[4] or by dextran sulfate sodium (DSS)[6], whereas pretreatment of NTSR-1 antagonist SR48692 inhibits the inflammatory changes[4]. Both NTS and NTSR1 expression are also increased in the mesenteric fat of mice during trinitrobenzenesulfonic-acid-induced colitis[7]. Moreover, NTS/NTSR1 activation in colonocytes stimulates interleukin (IL)-8 secretion from colonocytes through activating GTPase-mediated nuclear factor κ-light-chain-enhancer of activated B cells, mitogen-activated protein kinase, and protein kinase C[8-11]. NTS/NTSR-1 augments mucosal healing and regeneration following chronic colitis[5,6]. Pretreatment with NTSR1 antagonist worsens the severity of experimentally induced colitis and delays mucosal healing, whereas coadministration of exogenous NTS exerts the opposite effect[5,6,12,13]. NTS also stimulates colonic epithelial cell migration and proliferation through *COX-2* gene expression[5,6].

As a part of their diverse bioactivities, NTS/NTSR1 signaling is also involved in the early carcinogenesis and progression of colonic carcinoma. First, NTSR-1 is overexpressed in colonic neoplasms. A previous investigation of one of the present authors (Gui X) showed a stepwise increase in NTSR-1 mRNA with progression of colonic adenoma to adenocarcinoma[14]. Second, NTS is an epidermal growth factor (EGF)-like factor in a number of tumors[15-18]; it transactivates EGF receptor, hence it works synergistically with EGF[19,20]. Third, *NTSR-1* gene activation is linked with the *Wnt/APC/Tcf/β-catenin* pathway in colonic neoplasia. Upregulation of NTSR-1 in colorectal adenocarcinoma is the result of *Wnt/APC* pathway activation, and the increased NTSR-1 expression correlates with β-catenin cytosolic or nuclear accumulation. Additionally, *NTSR-1* gene can be activated by agents that cause β-catenin cytosolic accumulation[21]. Recently, it was also found that NTSR1 activation stimulates the expression of miRNAs 21 and 155 in colonocytes, in the experimentally induced colonic cancer (HCT-116 xenograft tumors) in mice as well as in human colonic carcinoma tissues[22].

It is well known that long-standing colitis (IBD) predisposes to the development of colorectal carcinoma (CRC). The relative risk of the development of CRC in IBD patients is 10-40-fold higher compared to that in the general population[23]. This so-called colitis-associated colorectal cancer (CACRC) is the most serious complication and the major cause of death of IBD patients. The carcinogenesis of CACRC is believed to be initiated and/or promoted by persistent active inflammation of colorectal mucosa[24,25] in the inflammation-dysplasia-carcinoma sequence, which differs from the adenoma-carcinoma sequence in sporadic CRC.

The bidirectional effect of NTS/NTSR1 on colonic mucosal inflammation-particularly the stimulation of cytokines/chemokines production and promotion of epithelial cell growth-makes NTS/NTSR1 signaling a possible unique link between chronic mucosal inflammation and carcinogenesis. For example, IL-8 [or chemokine CXC ligand (CXCL)8], an inflammatory component as a chemotactic factor for leukocytes, affects cancer (including colon cancer) progression through mitogenic, angiogenic, and motogenic effects[26]. The secretion of IL-8 can be stimulated by NTSR1 activation in colonic epithelial cells under either inflammatory or neoplastic conditions[27].

Taken together, it seems reasonable to postulate that NTSR1 in colonic epithelial cells is upregulated in IBD and that NTSR1 overexpression may play a role in the development of colitis-associated dysplasia/neoplasia. In this study, we analyzed the expression of NTSR1 in human colonic mucosa with various pathological changes characteristic of IBD and IBD-associated dysplasia and carcinoma. In order to demonstrate whether the change in NTSR1 was specific to IBD-associated neoplasia, we compared it to the sporadic colorectal neoplasia that developed in non-colitic patients.

MATERIALS AND METHODS

Study subjects

All cases were retrieved retrospectively from the surgical pathology files of the Calgary Laboratory Services in 2009 and 2010. The study was approved by the Research Committee of Calgary Laboratory Services, and ethical approval was granted by the University of Calgary Conjoint Health Research Ethics Board. The classification and grading of IBD, dysplasia and carcinoma were carried out on hematoxylin and eosin (HE)-stained slides, based on the morphological features and according to the standardized histological consensus criteria used widely

in clinical pathology practice. An attempt was made to distinguish, based on the strict morphological criterion currently available, between dysplasia-associated lesions or masses (DALMs) and adenoma-like dysplastic polyps (ALDPs) for the raised dysplastic lesions developed in a background of IBD. DALMs were defined as lesions that met all of the following criteria: (1) location within areas of chronic colitis; (2) irregularly elevated and broad-based with indistinct boundaries; and (3) surrounding mucosa also dysplastic, and in a full-thickness or "bottom-up" pattern of dysplasia. ALDPs were defined by the presence of well-circumscribed typical adenoma-looking polyps seen in the colitic regions; mostly in a "top-down" pattern of dysplasia. ALDPs are considered most likely to be sporadic adenoma.

Four separate groups of cases were included in the study.

Group 1: Eighteen colectomy cases of long-standing IBD complicated by colorectal neoplasia (14 males, 4 females, aged 26-84 years), including 13 UC, three Crohn's disease, and two indeterminate colitis. In this group, we looked for sequential histological changes of inflammation/dysplasia/carcinoma. In each case, the tissue blocks were selected from those with proven histology of normal/unremarkable colonic mucosa (n = 16), active colitis (n = 16), inactive colitis (n = 14), raised low-grade dysplasia (LGD, n = 16), high-grade dysplasia (HGD, n = 11), and adenocarcinoma (CACRC, n = 13). For the LGD lesions, 12 DALMs and four ALDPs were subgrouped.

Group 2: Eighteen colonoscopic biopsies of DALMs detected in longstanding IBD patients (10 males, 8 females, aged 23-68 years) for neoplasia surveillance.

Group 3: Seventeen randomly selected biopsies of sporadic colorectal adenomatous polyps detected in non-IBD patients (10 males, 7 females, aged 35-79 years) for colon cancer screening.

Group 4: Twelve randomly selected cases of colectomy for sporadic colorectal adenocarcinoma (SCRC) detected in non-IBD patients (7 males, 5 females, aged 42-85 years).

Detection of NTR-1 in colonic epithelial cells

The expression of NTSR1 was detected by immunohistochemistry of deparaffinized sections using the avidin-biotin-peroxidase complex method. The formalin-fixed paraffin-embedded tissue sections were pretreated in CINTec Epitope Retrieval Solution (10 mmol/L Tris/1 mmol/L EDTA, pH 9.0) for 20 min at 95-100 ℃, and then cooled down slowly to room temperature. The NTSR1 antibody was a rabbit polyclonal antibody (Imgenex, San Diego, CA, United States) against the third cytoplasmic domain of human NTSR1. All slides were stained with Ventana Nexes IHC autostainer at 1:40 dilution using UltraView Universal DAB Detection (Ventana 760-500). Immunoreactivity of NTSR1 appeared in a cytoplasmic pattern. Only the surface and cryptal epithelial cells of colonic mucosa were analyzed (in order to eliminate the variability of mononuclear cells in lamina propria). The positivity and intensity of the immunoreactivity of NTSR1 were semiquantitated independently by two pathologists as absent (negative), weak (1+), moderate (2+), and strong (3+). If the signal intensity was heterogeneous, the level assigned was based on the intensity in at least 50% of tumor cells or epithelial cells. A comparison of NTSR1 expression was analyzed between the different groups (active *vs* inactive colitis, dysplastic *vs* non-dysplastic lesions, DALMs *vs* ALDPs, colitis-associated dysplasia *vs* sporadic non-colitic dysplasia, and colitis-associated CRC *vs* sporadic non-colitic CRC).

Table 1 Neurotensin receptor 1 expression in 18 cases of inflammatory bowel diseases with colectomy *n* (%)

Histology	Case				*P* value
	Negative	1+	2+	3+	
Normal	12 (100.00)	0 (0.00)	0 (0.00)	0 (0.00)	
Colitis	1 (3.23)	20 (64.52)	10 (32.36)	0 (0.00)	
Active	0 (0.00)	12 (75.00)	4 (25.00)	0 (0.00)	
Inactive	1 (6.67)	8 (53.33)	6 (40.00)	0 (0.00)	0.338[1]
LGD	0 (0.00)	3 (18.75)	11 (68.75)	2 (12.50)	0.007[2]
DALM	0 (0.00)	3 (25.00)	7 (58.33)	2 (16.67)	
ALDP	0 (0.00)	0 (0.00)	4 (100.00)	0 (0.00)	0.298[1]
HGD	0 (0.00)	1 (9.09)	4 (36.36)	6 (54.55)	0.063[3]
CACRC	0 (0.00)	1 (7.69)	4 (30.77)	8 (61.54)	0.022[3]

[1]Within two subgroups (active *vs* inactive); [2]As compared with colitis; [3]As compared with low-grade dysplasia (LGD). HGD: High-grade dysplasia; DALM: Dysplasia-associated lesions or masses; ALDP: Adenoma-like dysplastic polyps; CACRC: Colitis-associated colorectal carcinoma.

Statistical analysis

Analysis of variance test was used to determine the statistical significance of the NTSR1 expression intensity between different groups. Differences were considered significant if P was < 0.05.

RESULTS

NTSR1 expression in colonic mucosa is associated with stepwise progression from colitis to LGD, HGD and carcinoma

Within the 18 colectomy specimens, relatively normal (uninvolved/colitis-spared) mucosa was identified in 12 cases. NTSR1 was not detected in any of the samples of normal mucosa. In the colitic mucosa, however, NTSR1 was detected in 30 out of 31 representative tissue samples and expressed similarly in both active and inactive colitis, as shown in Table 1 and Figure 1.

LGD lesions were identified in 16 of the 18 cases. The epithelium with LGD showed a significantly stronger expression of NTSR1 as compared to the non-dysplastic colitic mucosa, with most cases showing a $\geq$ 2+ intensity (68.75% in LGD *vs* 32.26% in non-dysplastic mucosa, P = 0.001) but fewer cases showing a 1+ intensity (18.75%

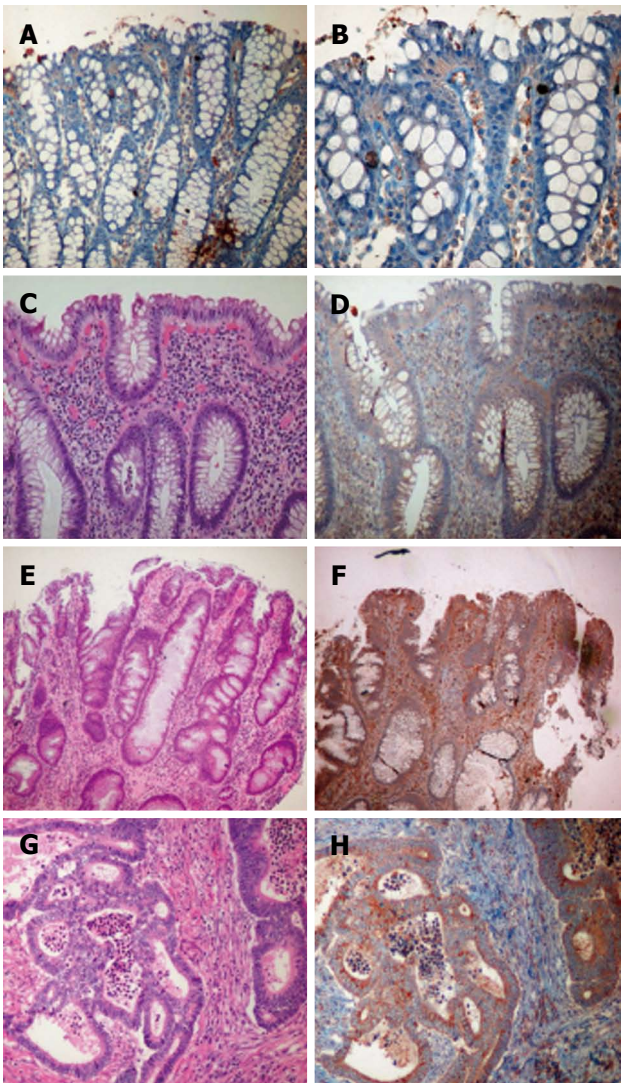

Figure 1 Neurotensin receptor 1 expression in colonic mucosa under different conditions. A and B: Normal colonic mucosa; C and D: Active chronic colitis; E and F: Dysplasia-associated lesions or masses with low-grade dysplasia; G and H: Invasive adenocarcinoma (hematoxylin and eosin histology and neurotensin receptor 1 expression immunohistochemistry).

in LGD *vs* 64.52% in non-dysplastic mucosa, $P = 0.007$). However, no significant difference existed between DALMs and ALDPs, as shown in Table 1 and Figures 1 and 2.

HGD was identified in 11 cases, and CACRC was identified in 13. As shown in Table 1 and Figures 1 and 2, expression of NTSR1 in the CACRC and HGD samples was stronger, with significantly more cases showing a 3+ intensity than in LGD of both DALMs and ALDPs (61.54% *vs* 12.50% for CACRC *vs* LGD, $P = 0.022$; 58.33% *vs* 12.50% for CACRC/HGD *vs* LGD, $P = 0.015$). However, no significant difference existed between CACRC and HGD ($P = 0.942$).

NTSR1 expressed similarly between DALMs and non-colitic sporadic adenoma

In the cases of colitis-associated DALMs ($n = 18$) and non-colitis sporadic adenomas ($n = 17$), the increased expression of NTSR1 showed a similar pattern, as shown in Table 2 and Figure 2.

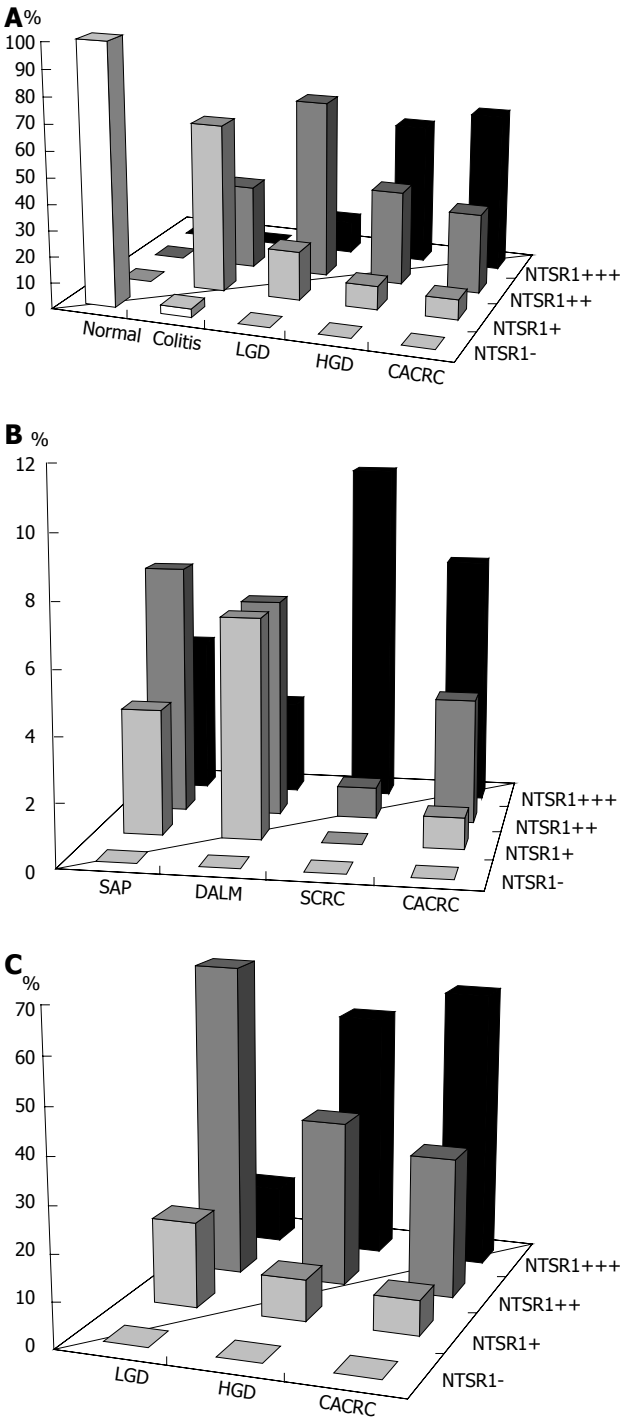

Figure 2 Neurotensin receptor 1 expression. A: Neurotensin receptor 1 (NTSR1) expression in colonic mucosa under different conditions in cases of colectomy for inflammatory bowel diseases (IBD) (percentage of cases in each subgroup); B: NTSR1 expression in colonic mucosa with dysplasia and carcinoma in cases of colectomy for IBD (percentage of cases in each subgroup); C: Comparison of colonic mucosal NTSR1 expression in sporadic neoplasia and colitis-associated neoplasia (percentage of cases in each subgroup). LGD: Low-grade dysplasia; HGD: High-grade dysplasia; DALM: Dysplasia-associated lesions or masses; CACRC: Colitis-associated colorectal carcinoma; SAP: Sporadic colorectal carcinoma; SCRC: Sporadic colorectal adenomatous polyp.

Table 2 Neurotensin receptor 1 expression *n* (%)

	Negative	1+	2+	3+
SAP	0	4 (23.53)	8 (47.06)	5 (29.41)
DALM	0	7 (41.42)	7 (41.42)	3 (17.65)
SCRC	0	0	1 (8.33)	11 (91.67)
CACRC	0	1 (7.69)	4 (30.77)	8 (61.54)

Comparison between colitis-associated dysplasia/carcinoma and sporadic dysplasia/carcinoma. Sporadic colorectal adenomatous polyp (SCRC) *vs* colitis-associated colorectal carcinoma (CACRC), $P = 0.198$; Dysplasia-associated lesions or masses (DALMs) *vs* CACRC, $P = 0.028$; DALMs *vs* sporadic colorectal carcinomas (SAPs), $P = 0.50$.

NTSR1 expressed similarly between colitis-associated and sporadic CRC

The increased expression of NTSR1 also showed a similar pattern to that in CACRC and in conventional sporadic colorectal carcinoma (SCRC, $n = 12$), as shown in Table 2 and Figure 2.

DISCUSSION

Through the detection of NTSR1 expression directly in human colonic mucosa with various IBD-related pathologies and those with sporadic colonic neoplasia in non-IBD patients, the present study demonstrated that both active and inactive IBD colitis upregulated NTSR1 in colonic epithelial cells; colitis-associated LGD to HGD and carcinoma was associated with stepwise higher expression of NTSR1; and the overexpression of NTSR1 showed a similar pattern in colitis-associated and non-colitic sporadic dysplasia/neoplasia, which suggests that NTSR1 is commonly unregulated in colonic neoplasia with or without a background of colitis.

The first two findings support the hypothesis that the upregulation of NTSR1 is involved in IBD inflammation and colitis-associated neoplasia. The findings corroborate various studies carried out in the past in animal models and *ex vivo* systems. In a similar study reported by Bossard *et al*[28], identical findings were shown with a slightly different methodology. Their study also demonstrated that coexpression of NTS/NTSR1 is present in a majority of the inflammatory and neoplastic/dysplastic lesions, suggestive of a self-activation of NTSR1 secondary to increased production of ligands; and β-catenin nuclear translocation is seen in a minority of the dysplastic and carcinomatous lesions in which no NTS was detected, suggestive of a different pathway.

To the best of our knowledge, the finding that NTSR1 is similarly overexpressed in colitis-associated dysplasia/neoplasia and sporadic dysplasia/neoplasia in non-IBD patients has not been reported previously. This finding indicates that NTSR1 is a neoplastic marker irrespective of its underlying etiology. In other words, the NT/NTSR1 signaling pathway is intrinsic and common to the tumorigenesis of all colorectal carcinomas, regardless of the tumor-promoting factors or the predisposing/initiation processes. This finding and interpretation are supported by an animal study reported recently by Bugni *et al*[29]. They developed a chemical-carcinogen-induced colonic adenoma by administration of azoxymethane to mice with or without DSS-induced colitis. NTSR1-deficient (gene knockout) mice had a < 50% chance, compared to wild-type mice, of developing colonic adenoma in the absence of colitis (*i.e.*, a model of sporadic colonic neoplasia). The difference, however, disappeared in the mice that had colitis (*i.e.*, a model of colitis-associated colonic neoplasia), even though significantly higher levels of IL-6 and CXCL2 (the mouse homolog of IL-8, both known as tumor-promoting cytokines) were seen in the latter. Our study, as well as that of Bugni *et al*[29], suggests that NTS/NTSR1 are not particularly responsible for the link between chronic inflammation and neoplasia in IBD, although it is commonly involved in the entire multistep process as an intrinsic regulator. The tumorigenetic process in colitis-associated dysplasia/neoplasia appears far more complex. However, it is still possible that increased NTSR1 expression associated with pre-existent or coexistent chronic colitis may further enhance the carcinogenesis.

It was noted in a minority of cases that NTSR1 expression was less upregulated, which occurred nonspecifically for each of the conditions and in different cases. We have no solid explanation for these relative negative cases. It is possible that NTSR1 expression in the epithelium is regulated by multiple factors, including a variety of cytokines and other gut peptides in the local mucosa or circulation that are not always the same in different patients.

Overall, our findings further provide a rationale for exploring the anti-NTSR1 approach in the treatment of IBD and in the chemoprevention of IBD-associated colorectal neoplasia as well as the treatment of sporadic colonic neoplasia. Hopefully, the development of clinically useful NTSR1 blockers will become a reality in the near future with the recent better understanding of the chemical structure of NTSR1[30].

ACKNOWLEDGMENTS

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COMMENTS

Background

Inflammatory bowel diseases (IBD) are chronic and debilitating inflammatory conditions of the colon, which also increase the relative risk of colorectal cancer. Identification of the factors associated with both mucosal inflammation and colitis-associated carcinogenesis could lead to novel treatments.

Research frontiers

Neurotensin (NTS) is a 13-amino-acid peptide that acts as a paracrine and endocrine modulator of various gut functions. Its bioactivities are mediated mainly through the high-affinity neurotensin receptor 1 (NTSR1). The NTS/NTSR1 signaling pathway has dual effects (both proinflammatory and proregenerative) in the regulation of intestinal mucosal inflammation. NTS/NTSR1 signaling is also involved in the carcinogenesis and progression of colonic carcinoma. It is suggested that NTSR1 in colonic epithelial cells is upregulated in IBD and NTSR1 overexpression may play a role in the development of colitis-associated dysplasia/neoplasia.

Innovations and breakthroughs

The present study used colonic tissue samples to detect the expression of NTSR1 in the context of various pathological conditions of IBD, with a focus on NTSR1 expression in the progressive changes from active inflammation to low-grade dysplasia, high-grade dysplasia, and carcinoma. A stepwise increase in NTSR1 expression was identified in the sequential progression. Moreover, a similar pattern of NTSR1 overexpression in colitis-associated and sporadic dysplasia/neoplasia was also determined for the first time.

Applications

The findings provide a rationale for exploring the anti-NTSR1 approach in the treatment of IBD and in the chemoprevention of IBD-associated colorectal neoplasia, a clinically useable anti-NTSR1 agent becomes available in the near future.

Terminology

Colitis-associated dysplasia and carcinoma develop in association with the longstanding chronic colitis in IBD patients. It is now well recognized that this type of colorectal carcinogenesis is clearly initiated and/or promoted by chronic, persistent and repetitively active mucosal inflammation. This type of colorectal carcinoma (CRC) develops and progresses in an inflammation-dysplasia-carcinoma sequence and therefore differs from the adenoma-carcinoma sequence in the sporadic CRC.

Peer review

The study investigates the relationship between NTSR1 expression in IBD and the possibility of its association with mucosal inflammation and colitis-associated neoplasia. They found a strong correlation with the progression from normal mucosa to colitis, degree of dysplasia, and carcinoma. The methodological approach was correct and the findings are interesting.

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BRIEF ARTICLE

First-line erlotinib and fixed dose-rate gemcitabine for advanced pancreatic cancer

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Abstract

AIM: To investigate activity, toxicity, and prognostic factors for survival of erlotinib and fixed dose-rate gemcitabine (FDR-Gem) in advanced pancreatic cancer.

METHODS: We designed a single-arm prospective, multicentre, open-label phase Ⅱ study to evaluate the combination of erlotinib (100 mg/d, orally) and weekly FDR-Gem (1000 mg/m^2, infused at 10 mg/m^2 per minute) in a population of previously untreated patients with locally advanced, inoperable, or metastatic pancreatic cancer. Primary endpoint was the rate of progression-free survival at 6 mo (PFS-6); secondary endpoints were overall response rate (ORR), response duration, tolerability, overall survival (OS), and clinical benefit. Treatment was not considered to be of further interest if the PFS-6 was < 20% (p0 = 20%), while a PFS-6 > 40% would be of considerable interest (p1 = 40%); with a 5% rejection error (α = 5%) and a power of 80%, 35 fully evaluable patients with metastatic disease were required to be enrolled in order to complete the study. Analysis of prognostic factors for survival was also carried out.

RESULTS: From May 2007 to September 2009, 46 patients were enrolled (male/female: 25/21; median age: 64 years; median baseline carbohydrate antigen 19-9 (CA 19-9): 897 U/mL; locally advanced/metastatic disease: 5/41). PFS-6 and median PFS were 30.4% and 14 wk (95%CI: 10-19), respectively; 1-year and median OS were 20.2% and 26 wk (95%CI: 8-43). Five patients achieved an objective response (ORR: 10.9%, 95%CI: 1.9-19.9); disease control rate was 56.5% (95%CI: 42.2-70.8); clinical benefit rate was 43.5% (95%CI: 29.1-57.8). CA 19-9 serum levels were decreased by > 25% as compared to baseline in 14/23 evaluable patients (63.6%). Treatment was well-tolerated, with skin rash being the most powerful predictor of both longer PFS (P < 0.0001) and OS (P = 0.01) at multivariate analysis (median OS for patients with or without rash: 42 wk *vs* 15 wk, respectively, Log-rank P = 0.03). Additional predictors of better outcome were: CA 19-9 reduction, female sex (for PFS), and good performance status (for OS).

CONCLUSION: Primary study endpoint was not met. However, skin rash strongly predicted erlotinib efficacy, suggesting that a pharmacodynamic-based strategy for patient selection deserves further investigation.

Key words: Pancreatic cancer; Gemcitabine; Fixed dose-rate; Erlotinib; Prognostic factors; Cutaneous rash; Phase Ⅱ trial

Core tip: The most important finding reported in this study is the strong predictive value of the appearance of skin rash, related to epidermal growth factor receptor (EGFR)-pathway inhibition. Our data suggest that patients developing any grade of skin rash during the treatment, can achieve disease control and survival comparable to those obtained with more intensive and more toxic chemotherapy. These findings underline the relevance of further investigation of the biological mechanisms related to the occurrence of skin rash upon EGFR blockade in order to identify clinical/molecular biomarkers predicting toxicity and efficacy and to prospectively select a subset of patients who could potentially benefit from Gem/erlotinib.

Vaccaro V, Bria E, Sperduti I, Gelibter A, Moscetti L, Mansueto G, Ruggeri EM, Gamucci T, Cognetti F, Milella M. First-line erlotinib and fixed dose-rate gemcitabine for advanced pancreatic cancer. *World J Gastroenterol* 2013; 19(28): 4511-4519 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i28/4511.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i28.4511

INTRODUCTION

Pancreatic adenocarcinoma (PDAC) is arguably the most aggressive solid malignancy, with nearly as many deaths as the number of newly diagnosed cases each year. In 2012 an estimated 43920 new cases and an estimated 37390 deaths are expected to occur, making pancreatic carcinoma the fourth leading cause of cancer-related death in the United States. The prognosis of pancreatic cancer is extremely poor due to difficulties in early detection and early metastatic dissemination, with a 5-year survival rate of only 6%[1].

The majority of PDAC patients present with metastatic or inoperable disease. In this setting, systemic chemotherapy remains the treatment of choice, with a palliative objective and a disappointing, marginal, survival advantage. Single-agent gemcitabine (Gem), administered as weekly 30-min *iv* infusions, has become the standard care for advanced PDAC based on a small but statistically significant advantage over bolus 5-fluorouracil (5-FU), in terms of both clinical benefit (CB) and survival[2].

Until recently, efforts to improve on single-agent Gem efficacy[3], by combining Gem with either a second cytotoxic drug or a molecularly targeted agent, have failed[4,5]. The addition of erlotinib, an oral epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, to Gem has produced a minimal, albeit statistically significant, improvement in overall survival (OS), leading to FDA approval of the Gem/erlotinib combination in the setting of advanced, inoperable PDAC[6]. On the other hand, pharmacokinetic Gem modulation, achieved by prolonging its infusion time, is justified by the observation that deoxycytidine kinase, the enzyme converting Gem into its active triphosphate metabolite, is rapidly saturated at plasma concentrations achieved with the standard 30-min infusion. Consequently, the infusion of Gem over a prolonged period at the constant dose rate of 10 mg/m^2 per min (FDR-Gem) avoids enzyme saturation and permits greater intracellular accumulation, possibly increasing Gem antitumor activity. This strategy has proven promising in a randomized phase Ⅱ trial, in which FDR-Gem significantly improved time to treatment failure as compared with the standard 30-min infusion[7]. Although formally negative, a phase Ⅲ trial comparing standard Gem with either FDR-Gem or the GEMOX combination, produced a clear signal in favor of FDR-Gem, which was as effective as the GEMOX combination[8].

Recently, a four-drug combination including 5-FU, folinic acid, oxaliplatin and irinotecan (FOLFIRINOX regimen) has demonstrated to improve objective response rate (ORR), progression-free survival (PFS) and OS over single-agent Gem administered by standard 30-min infusion in metastatic PDAC patients[9]. However, such improved efficacy comes at the price of significantly higher toxicity (both hematological and non-hematological), which restricts the use of such regimen to accurately selected, young and fit patients.

Based on our previous experience with a modified FDR-Gem regimen, which utilizes a lower Gem dose (1000 mg/m^2) as compared with the original FDR-Gem described by Tempero *et al*[7] (1500 mg/m^2) resulting in reduced hematological toxicity[10,11], we prospectively investigated the activity and tolerability of FDR-Gem in combination with erlotinib in advanced, inoperable PDAC patients.

MATERIALS AND METHODS

Patient population

Patients with cytologically or histologically proven, treatment-naïve, unresectable or metastatic PDAC and measurable disease were eligible for the study. Prior radiation for the management of local disease was allowed, provided that disease progression had been documented, all toxicities had resolved and treatment was completed at least 4 wk before study enrollment. Prior chemotherapy was not permitted, except for fluorouracil or Gem given concurrently with RT for radiosensitization purposes. Other inclusion criteria included: age > 18 years; Eastern Cooperative Oncology Group (ECOG) performance status (PS) ≤ 3; life expectancy > 12 wk; adequate hematological, renal, hepatic function; absence of other relevant medical conditions, potentially precluding the delivery of the planned treatment. The study was

Table 1 Patients' characteristics *n* (%)

Characteristics	Categories	Data
Age (yr)	Median (range)	64 (35-81)
Gender	Male	25 (54)
	Female	21 (46)
Stage	Locally advanced	5 (11)
	Metastatic	41 (89)
ECOG PS	0	10 (22)
	1	26 (56)
	2	9 (20)
	3	1 (2)
Basal CA 19-9 (U/mL)	Median (range)	897 (1-49, 483)
Interval between symptoms and treatment (wk)	Median (range)	12 (2-179)
Clinical benefit	Evaluable	33 (75)
	Not evaluable	13 (25)
Follow-up (wk)	Median (range)	21.5 (2-91)
Number of administrations	Median (range)	9 (1-29)

PS: Performance status; ECOG: Eastern Cooperative Oncology Group; CA 19-9: Carbohydrate antigen 19-9.

Table 2 Objective response, clinical benefit response and carbohydrate antigen 19-9 reduction in the overall population *n* (%)

Parameter	Patients (*n* = 46)
CR/PR	5 (10.9)
SD	21 (45.7)
PD	20 (43.4)
CB	
Pos	15 (42.9)
Neg	20 (57.1)
CA 19-9 reduction > 25%	14 (63.6)[1]

[1]In 23 evaluable patients. CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; CB: Clinical benefit; Pos: Positive; Neg: Negative; CA 19-9: Carbohydrate antigen 19-9.

reviewed and approved by the institutional review board of the Regina Elena National Cancer Institute (Rome, Italy), and written informed consent, according to Institutional requirements, was obtained from all patients before entering the study.

Treatment and study design

This was a single-arm, open-label, multicenter phase II study, evaluating the activity and tolerability of the combination of FDR-Gem and erlotinib in patients with advanced PDAC. Study patients received Gem at the dose of 1000 mg/m^2, administered as a 10 mg/m^2 per min FDR *iv* infusion (100 min total infusion time)[10,11], weekly for 7 consecutive weeks and on days 1, 8, and 15 of a 4-wk cycle thereafter for a maximum of 6 cycles in the absence of progressive disease (PD) or unacceptable toxicity; erlotinib was administered as a daily oral dose of 100 mg from day 1 until PD or unacceptable toxicity. Toxicities were recorded according to the National Cancer Institute-Common Toxicity Criteria Version 3.0. Appropriate dose reductions of each study agent were planned in case of severe toxicities. Tumor assessments were performed

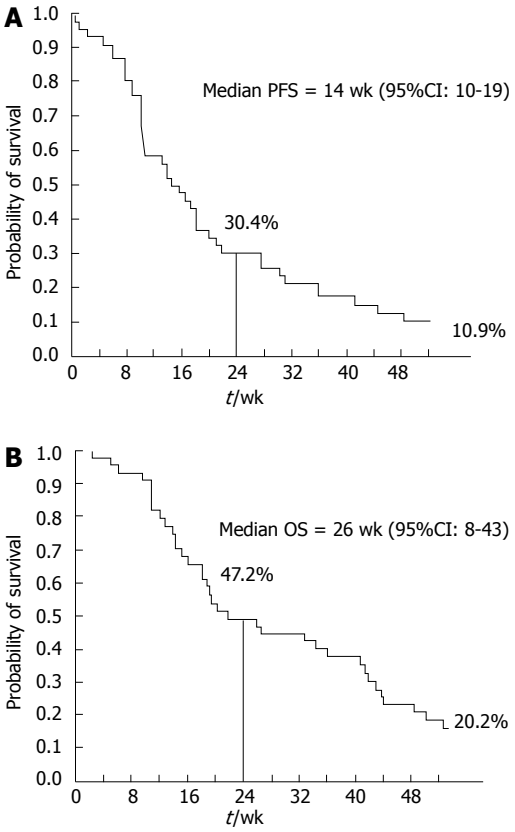

Figure 1 Kaplan-Meier analysis of progression-free survival and overall survival in the intent to treat population. A: Progression-free survival (PFS); B: Overall survival (OS).

at the end of cycle 1 and every 2 cycles thereafter.

Response and progression were evaluated using the Response Evaluation Criteria in Solid Tumours (RECIST 1.0)[12]. All patients who had measurable lesions and who had at least one objective tumour assessment after baseline were considered evaluable for response. The composite end point of CB was evaluated according to the criteria established by Burris *et al*[2] and included the assessment of pain (pain intensity and analgesic consumption) and functional impairment (assessed by Karnofsky PS) as primary measures and weight change (assessed by body weight) as a secondary measure. Each patient was classified as positive, stable, or negative for each of the primary CB measures (pain intensity or PS)[2]. For all patients, positive indicated a sustained (≥ 4 wk) improvement over baseline. If the patient was stable on both primary measures of clinical benefit, the patient was then classified as either positive or non-positive on the basis of the secondary clinical benefit measure of weight. For patients to achieve an overall rating of a positive CB, they had to be positive for at least one parameter without being negative for any of the others.

Statistical analysis

PFS rate at 6 mo (PFS-6) was selected as the primary study endpoint. Secondary endpoints were ORR, response duration, tolerability, OS, and CB. Sample size

Table 3 Toxicity (maximum toxicity per patient) *n* (%)

Variables	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Haemoglobin	15 (32.6)	17 (37.0)	10 (21.7)	4 (8.7)	-
Leucopenia	29 (63.0)	3 (6.5)	9 (19.6)	5 (10.9)	-
Neutropenia	25 (54.3)	2 (4.3)	9 (19.6)	8 (17.4)	2 (4.3)
Febrile neutropenia	44 (95.7)	-	2 (4.3)	-	-
Platelet	32 (69.6)	6 (13.0)	2 (4.3)	6 (13.0)	-
Fever	35 (76.1)	9 (19.6)	2 (4.3)	-	-
Bleeding	45 (97.8)	-	-	1 (2.2)	-
Alopecia	41 (89.1)	4 (8.7)	1 (2.2)	-	-
Anorexia	38 (82.6)	7 (15.2)	1 (2.2)	-	-
Asthenia	22 (47.8)	12 (26.1)	11 (23.9)	1 (2.2)	-
Cardiac	45 (97.8)	-	1 (2.2)	-	-
Skin	24 (52.2)	15 (32.6)	5 (10.9)	2 (4.3)	-
Diarrhoea	18 (39.1)	16 (34.8)	11 (23.9)	1 (2.2)	-
Constipation	46 (100.0)	-	-	-	-
Stomatitis	42 (91.3)	1 (2.2)	3 (6.5)	-	-
ALT	24 (52.2)	13 (28.3)	6 (13.0)	3 (6.5)	-
AST	19 (41.3)	12 (26.1)	10 (21.7)	4 (8.7)	1 (2.2)
Bilirubine	42 (91.3)	2 (4.3)	2 (4.3)	-	-
Renal	43 (93.5)	3 (6.5)	-	-	-
Neurological	45 (97.8)	1 (2.2)	-	-	-
Nausea	39 (84.8)	3 (6.5)	4 (8.7)	-	-
Vomiting	40 (87.0)	3 (6.5)	3 (6.5)	-	-

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

Table 4 Multivariate analysis for progression-free survival and overall survival

Variables	PFS		OS	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
PS	NA		8.78 (1.60-48.2)	0.01
Gender	2.99 (1.13-7.90)	0.03	2.66 (0.85-8.35)	0.09
CB	NA		3.07 (0.87-10.8)	0.08
Skin rash[1]	8.66 (2.65-28.32)	< 0.0001	5.10 (1.41-18.4)	0.01
CA 19-9 decrease[2]	2.64 (0.93-7.45)	0.07	3.36 (1.05-10.6)	0.04

[1]None *vs* any grade; [2]Carbohydrate antigen 19-9 (CA 19-9) decrease of ⩾ 25%. PFS: Progression-free survival; OS: Overall survival; PS: Performance status according to Eastern Cooperative Oncology Group (PS 0-1 *vs* PS 2-3); CB: Clinical benefit; NA: Not applicable.

was computed according to the exact single-stage Phase II design described by A'Hern[13]. The treatment was not considered to be of further interest if the PFS rate at 6 mo was < 20% (p0 = 20%). The alternate hypothesis assumed that a PFS rate at 6 mo of > 40% would be of considerable interest (p1 = 40%). With a 5% rejection error (α = 5%) and a power of 80%, a total of 35 fully evaluable patients were needed to complete the study. In order to have enough power to also analyze the 'pure' metastatic sub-population separately, 46 patients were planned to enter the study, taking into account a dropout rate of approximately 15%. The Kaplan-Meier method was used to estimate PFS and OS[14]. PFS was defined as the time from the first day of treatment to the first observation of disease progression or death due to any cause and OS was defined as the time from the first day of treatment to death from any cause. ORR was estimated as the proportion of patients evaluable for response who met RECIST criteria for complete or partial response (CR or PR). Response duration was calculated for all patients achieving a PR or CR as the time from first objective status assessment of CR/PR to the first time documented PD or death. Cox proportional hazards models were used to compare survival among different patient/disease characteristics and treatment response groups[15]; hazard ratios were appropriately derived from these models. The SPSS statistical software package version 20.0 (SPSS, Inc, Chicago, IL, United States) was used for all statistical analyses.

RESULTS

Between May 2007 and September 2009, 46 patients with advanced-stage PDAC were enrolled in the study from 3 institutions. Patient characteristics are shown in Table 1.

Treatment outcome

All 46 patients were evaluable for response according to RECIST criteria. PR and stable disease (SD) were observed in 5/46 (10.9%) and 21/46 (45.7%) patients, respectively, for an overall disease control rate (DCR), defined as the percentage of patients who had CR, PR or SD as their best response, of 56.5% (95%CI: 42.2-70.8); PD was documented at the first response assessment in 20 patients (43.5%). Median response duration was 27.4 wk (range 11-45 wk); median duration of stable disease was 27.6 wk (range 10-85 wk). Fifteen out of 35 evaluable patients (42.9%) experienced a positive CB. CA 19-9 serum levels were decreased by > 25% as compared to baseline in 14/23 evaluable patients (63.6%). Similar results were obtained in the pure metastatic population

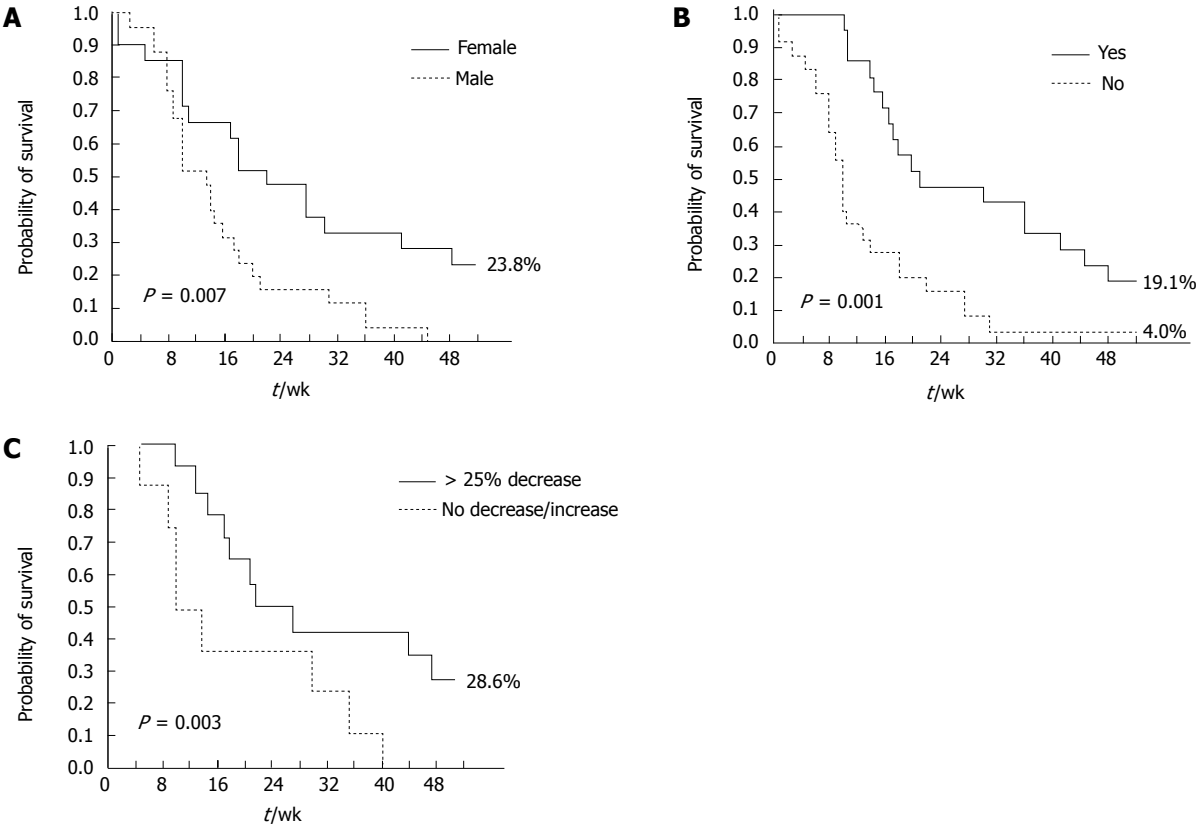

Figure 2 Kaplan-Meier analysis of independent progression-free survival predictors. A: Progression-free survival (PFS) by sex; B: PFS by skin rash; C: PFS by carbohydrate antigen 19-9 decrease.

(data not shown). At a median follow-up of 23.6 wk (range 2-139 wk), the median PFS and 1-year PFS rate were 14 wk (95%CI: 10-19) and 10.9%, respectively; PFS-6 (primary study endpoint) was 30.4% (Figure 1A). The median OS and 1-year OS rate were 26 wk (95%CI: 8-43 wk) and 20.2%, respectively (Figure 1B). In the pure metastatic population the corresponding figures were: median PFS: 14 wk, PFS-6: 24.4 %, median OS: 20 wk, 1-year OS: 12.7% (Table 2, data not shown).

Toxicity

All patients were evaluable for toxicity. Main hematological and non-hematological toxicities are summarized in Table 3. Treatment protocol was well tolerated, with only 3 serious adverse events that required hospitalization: 2 episodes of GI bleeding and 1 duodenal perforation. Three patients (7%) reported grade 4 toxicities (neutropenia in 2 patients and asymptomatic transaminase elevation in 1 patient). Grade 3 hematological toxicity was also rare: anemia in 4 patients (9%), neutropenia in 8 patients (18%), thrombocytopenia in 5 patients (11%); only 1 patient (2%) experienced febrile neutropenia. The main non-hematological toxicity were: asymptomatic serum transaminase elevation and hyperbilirubinemia (grade 3 in 9% of patients); grade 3 diarrhea in 1 patient (2%); grade 2 and 3, erlotinib-related skin rash in 11% and 4% of patients, respectively. Median time to rash was 7 d. Gem and erlotinib doses were reduced in 14 and 3 patients, respectively. No toxic deaths were recorded.

Clinical predictors of response and survival

A positive CB and skin rash (any grade) were significant, independent predictors of DCR at multivariate analysis, in both the overall and pure metastatic populations. Female gender ($P = 0.03$) and skin rash (any grade, $P < 0.0001$) were significant, independent predictors of longer PFS (Table 4). ECOG PS (0-1, $P = 0.01$), skin rash (any grade, $P = 0.03$), and carbohydrate antigen (CA 19-9) decrease (> 25% relative to baseline, $P = 0.04$) were significantly associated with longer OS at multivariate analysis (Table 4). Conversely, the occurrence of other Gem- or erlotinib-related toxicities, such as hematological toxicity and diarrhea, did not significantly impact on survival outcomes. The impact of these factors on PFS and OS was further confirmed by Kaplan-Meier analysis (Figures 2 and 3): in particular, median PFS and median OS were both significantly longer in patients experiencing any grade of skin rash (21 wk *vs* 10 wk, Log-rank $P = 0.001$, and 42 wk *vs* 15 wk, Log-rank $P = 0.03$, respectively) (Figures 2A and 3A). In the "pure metastatic" population, gender and skin rash ($P = 0.007$ and $P = 0.002$), and gender, PS, CB and skin rash ($P = 0.01$, $P = 0.06$, $P = 0.02$ and $P = 0.02$) were significant, independent predictors of PFS and OS, respectively, at multivariate analysis (data not shown).

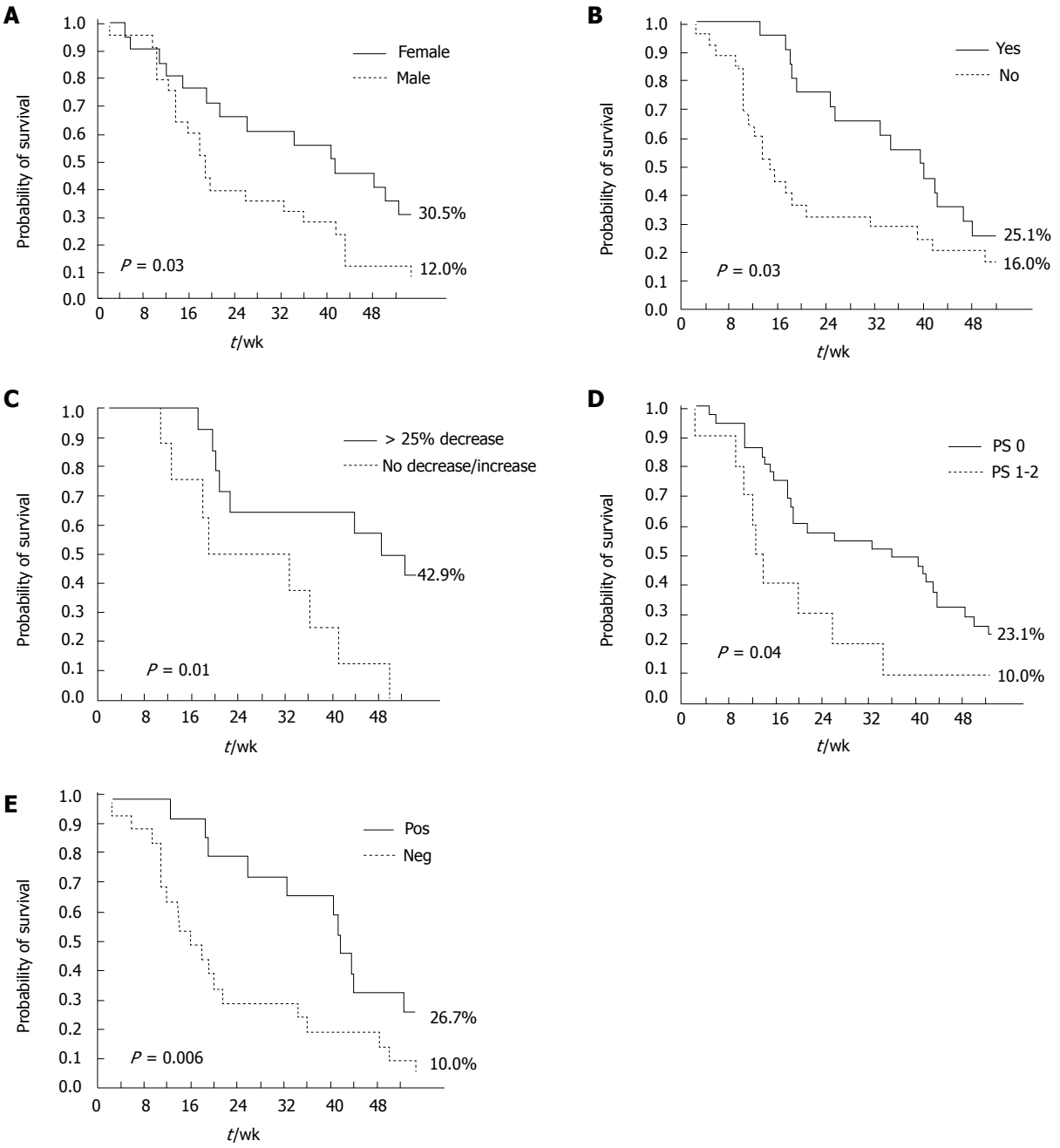

Figure 3 Kaplan-Meier analysis of independent overall survival predictors. A: Overall survival (OS) by sex; B: OS by skin rash; C: OS by carbohydrate antigen 19-9 decrease; D: OS by Eastern Cooperative Oncology Group performance status (PS); E: OS by clinical benefit. Pos: Positive; Neg: Negative.

DISCUSSION

In this study, performed in an unselected patient population, the administration of FDR-Gem in combination with erlotinib proved to be feasible, well tolerated, and moderately active. However, the planned goal to obtain a PFS-6 > 40% was not reached (PFS-6: 31.8%). Thus, the addition of erlotinib to an FDR-Gem backbone in unselected patients is unlikely to improve on historical result; indeed 1-year OS (21.6%), median OS (26 wk, 95%CI: 9-43), and activity in terms of responses, with a DCR of 59% are within the ranges reported with single-agent Gem, administered either as a 30-min *iv* infusion or as FDR, or with the combination of Gem and erlotinib[2,6-8,10].

The safety profile of the tested FDR-Gem/erlotinib combination is extremely manageable, an important issue in advanced PDAC patients, who are often frail and at a high risk of an adverse impact of treatment on quality of life. In particular, we confirm here that administering FDR-Gem at 1000 mg/m^2, as in previous experiences from our group[10,11], decreases hematological toxicity in comparison with the original FDR-Gem schedule developed by Tempero *et al*[7], where FDR-Gem was administered at the 1500 mg/m^2 dose level (grade 3-4 neutropenia 23% in the present trial *vs* 48.8% in Tempero's trial).

Other experiences with a different EGFR-TKI (gefitinib) have also confirmed an extremely safe and manageable toxicity profile of Gem-FDR at a lower dose (1200 mg/m^2), thus suggesting these combinations as feasible platforms for associations with additional chemotherapeutics or different targeted agents[16].

Though the combination under study proved feasible and well tolerated, the question remains as to whether such a strategy (*i.e.*, adding an EGFR kinase inhibitor to a FDR-Gem backbone in unselected patients) is worthy pursuing if it does not improve efficacy. As the results of the trial are technically negative (primary endpoint was not met), the easiest answer would be that this combination does not merit further investigation, particularly in a scenario, such as that of advanced PDAC treatment, where novel polychemotherapy strategies (FOLFIRINOX and Gem/nab-paclitaxel combinations) are moving the field forward and, for the first time in almost 20 years, show increased efficacy and improved survival as compared with single-agent Gem. However, survival analysis of the present trial and of two other recently reported experiences[17,18] clearly show that, at least in some patients, the addition of erlotinib to Gem has both biological and clinical activity: indeed, the most relevant finding reported herein is the strong predictive value of the appearance of skin rash. Patients developing erlotinib-related skin toxicity experience a more than doubled median OS (42 wk *vs* 15 wk, $P = 0.03$), and PFS (21 wk *vs* 10 wk, $P = 0.001$); conversely, the occurrence of other toxicities, such as hematologic toxicity or diarrhea, has no impact on treatment activity and/or survival outcomes. A similar predictive effect had already been described in the registration trial of erlotinib in PDAC, where patients experiencing grade 2 skin rash had a 1-year survival of 43%[6] and is shared by other agents targeting the EGFR pathway, either small molecules or monoclonal antibodies, regardless of the disease setting[19-25]. The trial exploring the addition of bevacizumab to Gem and erlotinib, also showed a significantly better outcome for patients developing skin rash, regardless of the treatment arm[26]. A more recent randomized trial showed that skin rash is able to dichotomize patients receiving erlotinib between good and poor prognosis[27].

In addition to skin rash, survival analysis of the current study also underlines the importance of two other treatment-modified factors to guide the management of advanced PDAC patients: clinical benefit and decline in CA 19-9 levels. Though chosen as the primary end-point in the Gemcitabine registration trial by Burris *et al*[2], the relationship between CB and OS has never been validated. Interestingly, in the present trial the occurrence of CB was a significant independent predictor of longer OS, while objective response, as assessed by RECIST criteria, was not, a finding of great clinical relevance in the context of a disease with dismal prognosis, where symptom control represents a real issue for clinical practice. A reduction in CA 19-9 levels > 25% from baseline was also an independent prognostic factor for survival, thus adding to the numerous evidence supporting the prognostic role (and clinical utility) of a CA 19-9 reduction, regardless of the chosen cut-off point[28-31].

In conclusion, although the study reported herein failed to meet its primary endpoint of prolonging PFS with the addition of erlotinib to FDR-Gem, intriguing data on skin rash do suggest that a subset of advanced PDAC patients could actually achieve disease control and survival comparable to those obtained with more intensive (and more toxic) polychemotherapy approaches, such as FOLFIRINOX and Gem/nab-paclitaxel combinations, with a well tolerated and easily manageable regimen, potentially also suitable for elderly and unfit patients. However, in order for this strategy to become a concrete treatment option, an in-depth investigation of the biological mechanisms underlying the occurrence of skin rash upon EGFR blockade is required to identify clinical/molecular biomarkers predicting toxicity and efficacy and to prospectively select patients who could potentially benefit from Gem/erlotinib combinations.

COMMENTS

Background

Pancreatic adenocarcinoma has a dismal prognosis. Although the disappointing survival advantage obtained in many studies, chemotherapy is the only treatment option for the majority of patients, and single agent gemcitabine (Gem) remains standard care for many of them. Recently, the polychemotherapy regimen named FOLFIRINOX has produced an improvement in survival over single agent Gem but require an accurate selection of young and fit patients to limit treatment-related toxicities. In order to improve Gem efficacy, pharmacokinetic Gem modulation and combination with other chemotherapeutic agent has been proposed. To this regard, the prolonged infusion at constant dose rate has shown promising results in phase Ⅱ and Ⅲ trials and the addition of erlotinib to Gem has provided a minimal, albeit statistically significant, improvement in survival.

Research frontiers

In the field of advanced pancreatic adenocarcinoma (PDAC), the research hotspot is to find active regimen, for patients not suitable for aggressive combination chemotherapy, able to improve survival over single agent Gem, without worsening tolerability. In the context of targeted therapies, applied to PDAC, but also any other malignancy, the opportunity of prospectively select patients who could benefit from targeted therapy plays a fundamental role.

Innovations and breakthroughs

The combination of fixed dose-rate (FDR)-Gem at 1000 mg/m^2 and erlotinib appears feasible, well tolerated and extremely manageable. In comparison to other FDR-Gem schedules with different doses (1500 or 1200 mg/m^2), the regimen shows a reduced hematological toxicity profile. This suggests that the schedule is a feasible platform for combining targeted therapies. In the study, a strong predictive value of the appearance of skin rash is demonstrated. Patients developing erlotinib-related skin toxicity experienced a more than doubled median overall survival, comparable to that obtained with more intensive polychemotherapy approaches. This relation has not been reported for other toxicities (hematologic toxicities or diarrhea). Moreover, occurrence of clinical benefit and reduction in carbohydrate antigen levels > 25% from baseline also proved to be an independent prognostic factor for survival. All these data confirm these factors as an important guide for the management of advanced PDAC patients.

Applications

The study results suggest the importance of investigating the biological mechanisms underlying the occurrence of skin rash upon epidermal growth factor receptor blockade. The identification of clinical/molecular biomarkers is strongly required to predict toxicity and efficacy and to prospectively select patients who

could potentially benefit from Gem/erlotinib combinations.

Peer review

This study investigated activity, toxicity, and prognostic factors for survival of erlotinib and FDR-Gem in advanced pancreatic cancer. They highlighted the correlation between the rash and efficacy. The similar studies were published in the past and they had the similar results, furthermore there were randomized controlled trials among them. This study is the confirmation of result of those studies, but it has reference to clinical practice.

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BRIEF ARTICLE

Negative capsule endoscopy in patients with obscure gastrointestinal bleeding reliable: Recurrence of bleeding on long-term follow-up

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Abstract

AIM: To assess the rate of recurrent bleeding of the small bowel in patients with obscure bleeding already undergone capsule endoscopy (CE) with negative results.

METHODS: We reviewed the medical records related to 696 consecutive CE performed from December 2002 to January 2011, focusing our attention on patients with recurrence of obscure bleeding and negative CE. Evaluating the patient follow-up, we analyzed the recurrence rate of obscure bleeding in patient with a negative CE. Actuarial rates of rebleeding during follow-up were calculated, and factors associated with rebleeding were assessed through an univariate and multivariate analysis. A *P* value of less than 0.05 was regarded as statistically significant. The sensitivity, specificity, and positive and negative predictive values (PPV and NPV) of negative CE were calculated.

RESULTS: Two hundred and seven out of 696 (29.7%) CE studies resulted negative in patient with obscure/overt gastrointestinal bleeding. Overall, 489 CE (70.2%) were positive studies. The median follow-up was 24 mo (range 12-36 mo). During follow-up, recurrence of obscure bleeding was observed only in 34 out of 207 negative CE patients (16.4%); 26 out of 34 with obscure overt bleeding and 8 out of 34 with obscure occult bleeding. The younger age (< 65 years) and the onset of bleeding such as melena are independent risk factors of rebleeding after a negative CE (OR = 2.6703, 95%CI: 1.1651-6.1202, *P* = 0.0203; OR 4.7718, 95%CI: 1.9739-11.5350, *P* = 0.0005). The rebleeding rate (CE+ *vs* CE-) was 16.4% *vs* 45.1% (χ^2 test, *P* = 0.00001). The sensitivity, specificity, and PPV and NPV were 93.8%, 100%, 100%, 80.1%, respectively.

CONCLUSION: Patients with obscure gastrointestinal bleeding and negative CE had a significantly lower rebleeding rate, and further invasive investigations can be deferred.

Key words: Capsule endoscopy; Enteroscopy; Anemia; Obscure gastrointestinal bleeding; Rebleeding

Core tip: Although capsule endoscopy (CE) is widely used as a first-line diagnostic modality for obscure gastrointestinal bleeding after the execution of a work-out negative for gastrointestinal bleeding properly done by following the guidelines proposed by American Gas

Association, the rebleeding rate after negative CE varies according to different studies. We tried to elucidate the outcomes after a negative CE for obscure gastrointestinal bleeding (OGIB) and to determine the risk factors associated with rebleeding. Based on the results of our study patients with OGIB and negative CE had a significantly lower rebleeding rate, and further invasive investigations can be deferred.

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INTRODUCTION

Obscure gastrointestinal bleeding (OGIB) remains a major clinical challenge. Many instances of OGIB originate from the small bowel, which is beyond the reach of an ordinary endoscope, including an esophagogastroduodenoscope (EGD) and colonoscope. The scene was recently revolutionized by the availability of the capsule endoscopy (CE), which is noninvasive and well tolerated by patients.

CE is currently indicated as part of the workup for OGIB (obscure-overt or obscure-occult), undiagnosed iron deficiency anemia, Crohn's disease, polyposis syndromes and cancer, celiac disease, for monitoring after small bowel transplant and, occasionally, for undiagnosed abdominal pain or diarrhea[1-5]. Since its development, several studies have compared the diagnostic yield of CE with other modalities commonly used to investigate the small bowel. Many studies have shown CE to be more sensitive and more effective compared with either push enteroscopy or small bowel follow-through[6-20]. The sensitivity and specificity of CE have been cited to be as high as 89% and 95%, respectively[20]. Although many papers have attempted to estimate the effectiveness of CE based on its diagnostic yield, few studies have considered the utility of a negative CE. In fact, only one of them[21-23] has considered a negative CE as a failure.

A great amount of data about a positive CE and its therapeutic and prognostic implications are available in literature. However, few data on the outcomes of patients with a negative CE are available. It is also somewhat more difficult to ascertain the value of a negative test. Previous studies have considered objective measures such as whether a negative test leads to other tests or therapeutic interventions. The impact on patients' overall outcome remains poorly defined, particularly in patients with OGIB. It remains uncertain that CE findings predict rebleeding.

A negative CE, though it does not confirm a specific diagnosis, may still be useful, because it allows the physician to quit a certain line of investigation, thereby impacting patient care.

MATERIALS AND METHODS

We reviewed the medical records of all patients referred to the Digestive Endoscopy Unit of the Catholic University in Rome to undergo a CE analysis for the investigation of OGIB between December 1st, 2002 and January 30th, 2011. All of them presented an overt or occult gastrointestinal bleeding as clinical presentation according to the guidelines of the American Gastroenterological Association (AGA)[24]. All patients had undergone both EGD and ileo-colonoscopy resulted negative before to referral for CE.

All patients, opportunely consented, underwent a CE with the PillCam capsule endoscopy system (Given Imaging, Yoqneam, Israel), according to the standard protocols endorsed by the American Society for Gastrointestinal Endoscopy[25]. All the procedures were performed in an out patient setting, after fasting for 8 h without any bowel preparation. The PillCam small bowel (Given Imaging) was then administered. The patients had a light breakfast 2 h after and a light meal 4 h after the administration of the PillCam as recommended in the standard protocol. After 8 h, they returned to the Endoscopy Unit, data recorder was removed and images were downloaded on the computer. The recordings of CE were reviewed by 2 experienced endoscopists/gastroenterologists independently (Riccioni ME, Urgesi R) at 8-10 frames per second using the Rapid® Reader (version 5.0). When possible, the stomach and the colon were also observed. The interobserver difference in interpretation about any findings was less than 5% and if and when it existed, it was resolved by reexamination.

A positive CE was defined as the presence of CE findings that may account for the clinical bleeding (angiodysplasia, ulcers or erosions, tumor, Crohn's disease, and active bleeding with no identifiable source), whereas a negative CE was defined as the absence of abnormalities on CE as reason of the bleeding.

In all cases in which CE did not reach the valve or with inadequate small bowel cleansing the examination was repeated. The analysis was considered negative when the second procedure rule out any GI abnormalities[26].

The median follow-up for all patients, strictly monitored for rebleeding, was 24 mo (range 12-36 mo). Patients' records, including blood tests, hospital admissions (especially for anemia and/or recurrent gastrointestinal bleeding), blood transfusions, need of iron supplementation, additional endoscopies (including push endoscopies), and surgery were considered from the date of the CE. Overt clinical rebleeding was defined as passing melena or fresh blood per rectum with a drop in hemoglobin of 2 g/dL or more. Occult rebleeding was defined as an unexplained hemoglobin drop of more than 2 g/dL in the absence of melena or hematochezia.

We defined patients with no recurrent obscure gastrointestinal bleeding or anemia during follow-up as "negative for rebleeding" and those with a confirmed bleeding source identified by an invasive interventions, clinical rebleeding, or recurrent unexplained anemia (using standardized and published criteria: blood haemoglobin level

of < 13.8 g/dL for men, < 11.5 g/dL for postmenopausal women, and < 11 g/dL for pre-menopausal women, with a plasma ferritin level of < 30 μg/L and a mean corpuscular volume of < 80 fL[26]) as "positive for rebleeding".

Statistical analysis

The Statistical Package for Social Science (version 13.0; SPSS Inc., Chicago, IL, United States) was used for all statistical computation. Actuarial rates of rebleeding during follow-up were calculated, and factors associated with rebleeding were assessed through an univariate and multivariate analysis. A *P* value of less than 0.05 was regarded as statistically significant.

The sensitivity, specificity, and positive and negative predictive values (PPV and NPV) were calculated[27] using as "gold standard" the patients negative for obscure bleeding.

RESULTS

CE indications included obscure overt bleeding (532), obscure occult bleeding (164) and several other indications (282). CE studies resulted negative in 207 out of 696 (29.7%) with obscure/overt gastrointestinal bleeding: 110 male (53.1%) and 97 female (46.8%) with a median age of 61.4 years (range 8-92 years). Overall, 489 patients (70.2%) were positive patients. The flowchart of the selection process of patients involved in the study and characteristics of the patients with negative CE are showed in Figure 1 and Table 1 respectively.

Follow-up

The median follow-up for all patients closely monitored for rebleeding was 24 mo (range 12-36 mo). Clinical recurrence of bleeding was observed during follow-up in 34 (16.4%) out of 207 patients with CE "negative for bleeding". In details, 2 out of 34 patients had a new episode of melena, otherwise 32 patients had a recurrence of obscure occult bleeding with anemia and positive fecal occult blood test.

A Meckel diverticulum and a gastrointestinal stromal tumor were respectively diagnosed in the two patients presenting a new episode of obscure overt bleeding. In the group of 32 patients affected by a recurrence of obscure occult bleeding, the cause was undiagnosed in 13 cases (40.6%), whereas an extra-intestinal disease was diagnosed in 11/32 (34.3%) patients, a parasitic infestation in 3/32 (9.37%) and the chronic use of NSAIDs was indicated as the probable cause of the recurrence of bleeding in 5/32 (15.62%) patients. Moreover, we note that 2 out of 34 (5.8%) patients with negative CE and episodes of early rebleeding were receiving chronic therapy with oral anticoagulation, at 10 mo of follow-up.

Data analysis

The results of univariate and multivariate analysis about factors associated with rebleeding in patients with negative CE and reccurence re-bleeding are summarized in Table 2. Patients with OGIB and negative CE have a low percentage of probability of rebleeding (34/207). The statistical analysis of our data shows that the age of onset of the first episode of bleeding < 65 years and the type of bleeding (melena) are the only factors that statistically significantly influence the risk of re-bleeding (OR = 2.6703, 95%CI: 1.1651-6.1202, *P* = 0.0203; OR = 4.7718, 95%CI: 1.9739-11.5350, *P* = 0.0005) (Table 3). Other parameters considerated during our survey such as gender, number of blood transfusions, hemoglobin level, intake of anticoagulants, number of hospitalizations didn't show a statistically significant correlation with rebleeding episodes. The final diagnosis and treatment of these patients are summarized in Table 3.

The patients "negative for rebleeding" with CE negative didn't need a hospital admission at the time of rebleeding and no blood transfusions were required.

Regarding CE "positive" patients, clinical rebleeding was observed in 221 out of 489 (45.1%) patients. Rebleeding rate was 16.4% *vs* 45.1% (χ^2 test, *P* = 0.00001). The sensitivity, specificity, and PPV and NPV were 93.8%, 100%, 100%, 80.1%, respectively. Most rebleedings occurred within the first 12 mo after the CE examinations (18/34; 52.9%).

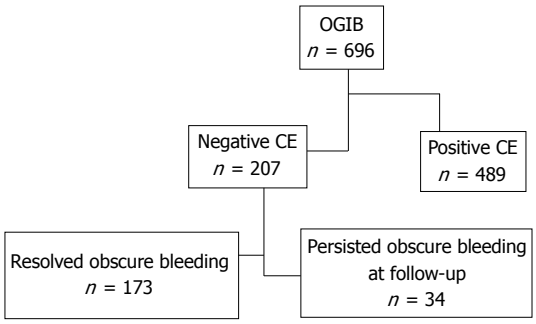

Figure 1 Flow chart of the selection process of patients involved in the study. CE: Capsule endoscopy; OGIB: Obscure gastrointestinal bleeding.

Table 1 Characteristics of patients with negative capsule endoscopy

Variables	Total patients	Negative for rebleeding	Rebleeding patients
n	207	173	34
Age (yr)	56.8	56.8	57.1
Sex (M/F)	110/97	70/59	22/12
Obscure-overt bleeding	157	121	2
Obscure-occult bleeding	50	52	32
Median Hb level	8.2	8.8	8.2
OAT/LMWH	36	32	4
Rebleeding time			
At 10 mo of follow-up			28
At 24 mo of follow-up			34

OAT: Oral anticoagulant therapy; LMWH: Low molecular weight heparin. M/F: Male/female.

DISCUSSION

Despite the wide diagnostic yield of CE compared to

Table 2 Results univariate analysis *n* (%)

Variables	No rebleeding	Rebleeding	*P* value	Odds ratio	95%CI	*P* value
Patients	173	34	-			
Sex (M/F)	88/85	22/12	0.1403	1.8447[1]	0.8175-4.1625[1]	0.1403[1]
Age < 65 yr	23 (67.6)	89 (51.4)	0.0838	2.6703	1.1651-6.1202	0.0203
CE indications (melena)	77 (44.5)	26 (76.5)	0.0006	4.7718	1.9739-11.5358	0.0005
Blood transfusion	64 (36.9)	14 (41.2)	0.6463			
Hb < 8 g/dL	48 (27.7)	14 (41.2)	0.1189	2.0064	0.8891-4.5274	0.0935
Use of FANS	30 (17.3)	5 (14.7)	0.7085			
Oral anticoagulant therapy	13 (7.5)	4 (11.9)	0.4104			
Hospitalizations (*n*)	62 (35.8)	14 (41.2)	0.5559			

[1]Variables with $P < 0.05$ are significant. Variables with $P < 0.125$ entered in the multivariate analysis (logistic regression): (χ^2 test). Likelihood ratio: 22.1037; $P = 0.0002$. The younger age (< 65 yr) and the onset of bleeding such as melena are an independent risk factor of rebleeding after negative capsule endoscopy (CE). M/F: Male/female.

Table 3 The final diagnosis and treatment of the 34 negative capsule endoscopy patients with rebleeding

Type of rebleeding	*n*	Final diagnosis	Treatment
Obscure overt bleeding	2		Surgery
		Extraluminal GIST	
		Meckel's diverticulum	
Obscure occult bleeding	32		
	13	Causes not found	SR
	5	Myelodisplastyc syndrome	MT
	3	Uterine fibroma	Surgery
	1	Metastatic breast cancer	Surgery + MT
	3	Giardia lamblia infection	MT
	5	Chronic use of NSAID	
	2	Erosive gastritis	MT

GIST: Gastrointestinal stromal tumor; SR: Spontaneous resolution; MT: Medical therapy.

conventional diagnostic techniques, the impact of this relatively new investigation on patient outcome remains poorly defined. In particular, few CE studies used long-term rebleeding as the primary outcome. In this study, we determined the long-term clinical outcome and characteristics of patients with OGIB after negative CE. As reported previously[22,23,28,29], CE could not identify all bleeding lesions in patients with OGIB. Up to 36.7% of patients in this study had a negative CE despite overt clinical bleeding at presentation. Notably, this group of patients with negative CE had a low (19.8%) rebleeding rate in a more than 1 year follow-up. The rebleeding rate was significantly lower in patients with negative CE than in cases with positive CE. Moreover, considering the group of patients with negative CE, chronic therapy with oral anticoagulation and NSAIDs seems to be related to a higher risk of rebleeding. In accordance with our findings, Neu *et al*[30] found that rebleeding occured in 20% of patients with negative CE after a median follow-up of 13 mo. In a more recent study Lorenceau-Savale *et al*[31] showed as in 35 patients with a history of OGIB and negative CE and a minimum follow-up duration of one year (median: 15.9 mo) eight patients presented a recurrence of bleeding, with an overall rebleeding rate of 23%. Four women with recurrence before new investigations. In the four remaining patients, repeat endoscopy work-ups after negative CE were performed and revealed previously missed lesions with bleeding potential, mainly in the stomach. Overall, 13 patients, with or without rebleeding, had repeated endoscopy work-ups after a negative CE, leading to a definitive diagnosis in nine patients, with lesions located in the stomach and colon in eight of them.

Since the patients with OGIB and a negative CE had a low rate of rebleeding, further interventions or investigations could be deferred until clinical rebleeding occurs. In these cases, after ruling out a gastrointestinal lesions as causes of the recurrence of bleeding after the execution of a work-out negative for gastrointestinal bleeding properly done by following the guidelines proposed by AGA, the search for causes of obscure bleeding outside of the digestive system should be "necessarily" done.

Otherwise, patients with positive CE had a significantly higher rebleeding rate on long-term follow-up. Recently, Kim *et al*[32] performed CE in 125 patients with OGIB. The complete visualization of the small bowel was achieved in 93 patients (74.4%). Of the 63 patients (50.4%) with negative CE results, 60 patients did not receive any further specific treatment for OGIB. Rebleeding episodes were observed in 16 out of 60 patients (26.7%). Substantial rebleeding events were observed with similar frequency both after negative CE without subsequent treatment (26.7%) and after positive CE without specific treatment (21.2%) ($P = 0.496$). The Authors conclude that in some cases despite a negative CE, approach such as double balloon endoscopy (DBE) should be considered as complementary procedures for further evaluation.

Our study confirms, also in accordance with Lai *et al*[33] that patients with positive CE have a high rebleeding rate, a longer hospital stay and require more units of blood transfused than those with negative CE; in accordance with Kim *et al*[32], the device-assisted enteroscopy (DBE or single-balloon enteroscopy) could be helpful in patients with a high index of suspicion for small bowel pathologies and with a high risk of rebleeding and negative CE.

The limits of the present study are discussed below. First, the lack of a gold standard for small bowel diagnosis limits the accuracy in the determination of CE performance. It is highly possible that some lesions may be

missed despite an extensive investigation. However, unlike many published studies, we used long-term clinical rebleeding instead of small bowel lesions as the primary end point for the determination of CE performance. It may be interesting to determine, in future studies, whether the use of the recently available methods of device-assisted enteroscopy[34,35] and their future technical developments could overcome this problem. Secondly, all our "negative for rebleeding" patients refused to undergo further endoscopic examinations for various reasons. Third, we only recruited patients with "genuine" OGIB, meaning that these patients had undergone multiple upper and lower gastrointestinal endoscopies by experienced endoscopists to rule out other possible sources of bleeding. Consequently, these results could not necessarily be generalized to all patients with suspected small bowel bleeding.

In conclusion, even if additional studies are warranted to confirm these results, we found that in a follow-up of a mean 24 mo, patients with OGIB and negative CE had a significative low long-term rebleeding rate, suggesting that further invasive investigations could be deferred and may not be necessary in this group of patients. Only an accurate and careful clinical observation can help us to identify false negative patients at CE.

COMMENTS

Background

Obscure gastrointestinal bleeding (OGIB) remains a major clinical challenge to gastroenterologists. Many instances of OGIB originate from the small bowel, which is beyond the reach of an ordinary endoscope, including an esophagogastroduodenoscope and colonoscope.

Research frontiers

The scene was recently revolutionized by the availability of capsule endoscopy (CE), which is noninvasive and well tolerated by patients. In this study the authors assess the rate of recurrent bleeding of the small bowel in patients with obscure bleeding already undergone CE with negative results.

Innovations and breakthroughs

Recent reports have highlighted the importance of CE in the clinical assessment of patients with presumed small bowel diseases. For OGIB, CE is recommended as an investigation modality for the detection of a bleeding source after traditional endoscopy. However, even after full evaluation of the small bowel, CE is not able to highlight the bleeding focus. In the present study, they sought to reveal the outcomes after negative CE for OGIB and the risk factors associobscure bleeding already undergone CE with negative results.

Applications

By understanding the value of negative CE and the value of long-term of follow-up in these patients. Patients with OGIB and negative CE had a significantly lower rebleeding rate, and further invasive investigations can be deferred. However, when a confirmatory diagnosis is made by CE study, specific treatments can be applied according to the diagnosis.

Terminology

CE is the most innovative and less invasive resource for the study of the small bowel playing an essential role in the diagnosis of small bowel diseases until now disregarded and the setting of therapeutic decisions.

Peer review

The authors retrospectively present information on 696 patients who underwent CE for OGIB with negative standard tests. They excluded for detailed analysis 489 patients an instead concentrated on outcome in 207 patients in whom the CE proved negative. They found a statistically lower rebleed rate over a median of 24 mo in these CE negative patients compared to the CE positive group. The CE-patients had various other explanations found later in 60%. No explanation was found in the rest. In multivariate analysis age < 65 years and melena on presentation were found to be predictors of rebleed in CE-patients.

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BRIEF ARTICLE

Gallbladder polyps: Factors affecting surgical decision

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Abstract

AIM: To determine the factors affecting the decision to perform surgery, and the efficiency of ultrasonography (USG) in detecting gallbladder polyps (GP).

METHODS: Data for 138 patients who underwent cholecystectomy between 1996 and 2012 in our clinic with a diagnosis of GP were retrospectively analyzed. Demographic data, clinical presentation, principal symptoms, ultrasonographic and histopathological findings were evaluated. Patients were evaluated in individual groups according to the age of the patients (older or younger than 50 years old) and polyp size (bigger or smaller than 10 mm) and characteristics of the polyps (pseudopolyp or real polyps). χ^2 tests were used for the statistical evaluation of the data.

RESULTS: The median age was 50 (26-85) years and 91 of patients were female. Of 138 patients who underwent cholecystectomy with GP diagnosis, only 99 had a histopathologically defined polyp; 77 of them had pseudopolyps and 22 had true polyps. Twenty-one patients had adenocarcinoma. Of these 21 patients, 11 were male, their median age was 61 (40-85) years and all malignant polyps had diameters > 10 mm (P < 0.0001). Of 138 patients in whom surgery were performed, 112 had ultrasonographic polyps with diameters < 10 mm. Of the other 26 patients who also had polyps with diameters > 10 mm, 22 had true polyps. The sensitivity of USG was 84.6% for polyps with diameters > 10 mm (P < 0.0001); however it was only 66% in polyps with diameters < 10 mm.

CONCLUSION: The risk of malignancy was high in the patients over 50 years old who had single polyps with diameters > 10 mm.

Key words: Gallbladder; Polyps; Ultrasound; Cholecystectomy; Malignancy

Core tip: Early stage gallbladder cancers can often be detected as polyps in imaging studies. The aim of this study was to determine the factors affecting surgery by analyzing the incidence of malignancy of gallbladder polyps (GP) and the efficiency of ultrasonography in detecting GP. Of 138 patients with GP on imaging, 99 had polyps and 21 had histopathologically confirmed adenocarcinoma. Of these 21 patients, all malignant polyps were solitary and had a diameter > 10 mm. In our study, the risk of malignancy correlated with age over 50 years old, solitary polyp and polyp diameter > 10 mm.

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INTRODUCTION

Gallbladder polyps (GP) present as masses protruding from the gallbladder mucosa. They are found in 0.3% to 12% of healthy individuals[1]. The actual prevalence is

unknown; however, at present, GPs are diagnosed more frequently because of the widespread use of abdominal imaging techniques. GPs are usually asymptomatic and are diagnosed incidentally during radiological examinations done for other reasons. GPs are classified as pseudopolyps or true polyps. Pseudopolyps consist of cholesterol polyps/cholesterolosis, adenomatous polyps, adenomyoma, inflammatory polyps and hyperplastic polyps; these are all benign lesions. True polyps are grouped into benign (adenoma), premalignant (dysplastic polyps) and malignant (adenocarcinoma)[2]. Cholesterol polyps are the most frequently observed GPs. Therefore, most GPs are benign lesions. Occasionally, early stage gallbladder cancers can be detected as a polyp in imaging studies.The prevalence of malignant polyps of GPs can reach 27%[3]. In patients older than 50 years old, the presence of polyps larger than 10 mm has been reported as a risk factor for malignancy[3-8]. The most commonly used imaging modality for diagnosis is ultrasonography (USG). However, USG is poor at differentiating benign and malignant polyps. Additional diagnostic tools comprise computed tomography and endoscopic USG.

In this study, patients in our clinic diagnosed with-GPs who had surgery were examined; and indication for surgery, frequency of polyp types, malignancy rates of polyps and reliability of USG in identification and differentiation of polyps were investigated.

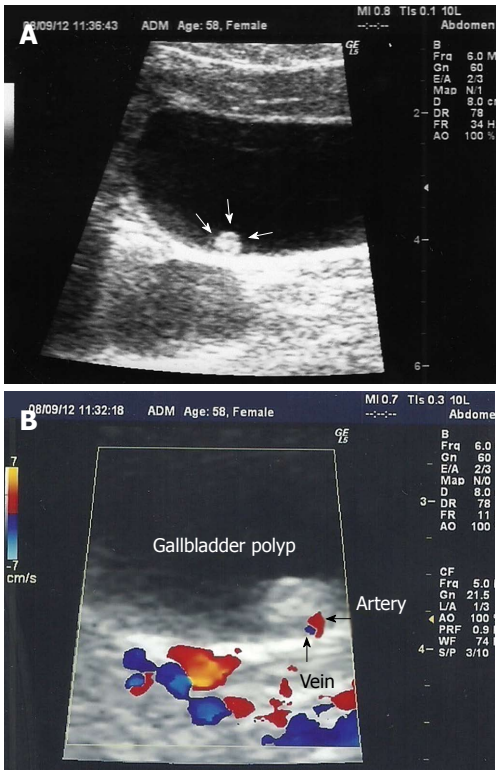

Figure 1 The ultrasonographic image of a 6-mm gallbladder polyp (A) and the same polyp with a feeding artery in Doppler ultrasonography (B).

MATERIALS AND METHODS

Patients

Age, sex, clinical signs and symptoms, preoperative ultrasound and histopathological diagnoses of patients were analyzed retrospectively in patients admitted to our clinic with GP and underwent cholecystectomy from 1996-2012. All the patients were evaluated with USG before surgery in the Radiology Department of Uludag University Faculty of Medicine. Hyperechoic lesions that had no acoustic shadowing and didnot move with position change represented a confirmed diagnosis of GP[8]. Detection of polyps > 10 mm, suspicious findings in USG (such as a vascularization pattern, Figure 1), growth during follow-up, and personal request of the patient were indications for surgery.

Polyp size, number and presence of stones were evaluated in preoperative USG reports, and compatibility of these findings with histopathological data was analyzed.

According to histopathological diagnoses, cholesterol polyps/cholesterolosis, hyperplastic and adenomatous polyps were assembled under the title of "pseudopolyp"; adenoma and adenocarcinoma were assembled under the title of "real polyps". In addition, patients were evaluated in individual groups according to the age of the patients (older or younger than 50 years old) and polyp size (bigger or smaller than 10 mm).

Statistical analysis

The χ^2 test was used, when appropriate, to calculate the statistical significance of the different demographic and clinical variables. *P* values of < 0.05 were deemed significant.

RESULTS

Demographic and clinical characteristics of patients with PLG

Cholecystectomy was performed in 5832 patients between 1996-2012 and surgical indication of 138 patients (2.3%) was GPs. Ninety-one of the patients were female and 47 of them were male, with a median age of 55 (26-85) years. Polyps were detected in 99 of the 138 patients (71.7%) undergoing surgery for GPs; gallbladder stones were detected in the remaining 39. Thus, the false positive rate was 28% in ultrasound evaluation of polyps. Remarkably, the polyps in all of these cases were < 10 mm.

Sixty-six patients (66.6%) did not have any symptoms at the time of presentation; however, 33 patients with polyps were symptomatic. Sixty-two of 66 asymptomatic patients elected to have surgical treatment because of possible future risks. Three of four asymptomatic patients had a cholecystectomy because their polyp increased to > 10 mm in 6 mo; the remaining patient had a cholecystectomy because of their age and sex (65 years old male). On pathological examination, the polyps of these four patients were detected as cholesterol polyps and adenomatous polyps. The 33 symptomatic patients presented with complaints of right upper quadrant pain and dyspepsia, and had surgery upon detection of polyps in USG (Table 1). Gallstones were accompanied with

Table 1 Characteristics of 99 patients diagnosed with gallbladder polyps by histopathological examination

Characteristics		Pseudopolyp	True polyp (n = 22)		P value[1]
Pathology result		(n = 77)	Adenocarcinoma	Adenoma	
Sex	Woman	51	10	1	0.33
	Man	26	11	0	
Age (yr)	< 50	52	1	1	< 0.0001
	⩾ 50	25	20	0	
Symptoms	Yes	24	9	0	0.62
	No	53	12	1	
Number	Multiple	23	0	0	0.01
	Single	54	21	1	
Size (mm)	⩽ 10	73	0	1	< 0.0001
	> 10	4	21	0	

[1]In terms of true polyp incidence between data.

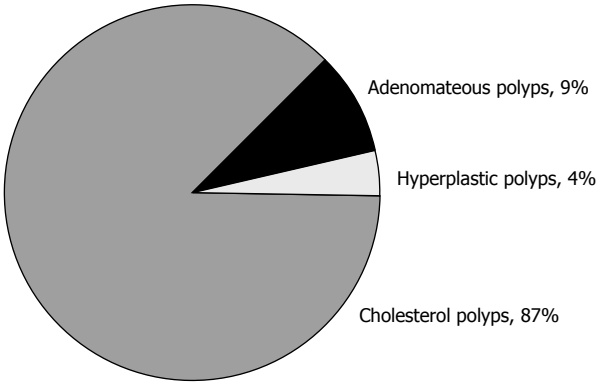

Figure 2 Distribution of pseudopolyp cases.

polyps in 18 (54.5%) of these symptomatic patients. Only in two of the malignant cases was a polypoid structure accompanied by gallstones.

As shown in Table 1, 54.5% of patients were under the age of 50 and 90% of true polyps were seen in patients over 50 years old. In addition, the incidence of polyps was 3.7% under 50 years of age, rising to 44% in patients over 50 years of age ($P < 0.0001$).

Sonograhic characteristics of the patients

While gallstones were detected only in 1 of 26 patients who had lesions of 10 mm diameter in preoperative USG, postoperative diagnoses was true gallbladder polyp in 21 (84.6%) of the remaining 25 patients. The other four patients were reported to have pseudopolyps. Preoperative USG diagnosed 96% of lesions over 10 mm accurately and 84% of them were found to be true polyps (adenoma/adenocarcinoma). Histopathological diagnoses reported polyps only in 74 of 112 patients who had lesions < 10 mm. Thus, the accuracy of USG for polyps < 10 mm decreased to 66% and only one of these 74 cases was a true polyp (adenoma). There was a statistically significant difference in the diagnosis of true polyps between polyps < 10 mm or > 10 mm ($P < 0.0001$).

Histopathological examinations of GP

The mean polyp diameter of the polyps from 99 patients (histopathologically defined as 77 pseudopolyps and 22 true polyps) was 8.8 mm (range 3-19 mm). The most commonly seen GP was a cholesterol polyp (Figure 2). Twenty-one of 22 patients with true polyps were diagnosed with adenocarcinoma, and the other one was adenoma. All the malignant polyps were > 10 mm and solitary. Eleven patients with malignancy were male and the median age was 61 (40-85) years. In our series, the incidence of malignant GP was 21.2% (21 of 99 cases). The incidence dropped to 15.2% when all 138 patients with preoperative diagnoses of polyps were taken into consideration.

Results of malignant patients

Cholecystectomy only was performed in 16 of 21 patients with malignancy, and cholecystectomy with liver S-5 resection and lymph node dissection was performed in the remaining five patients. No additional treatment other than cholecystectomy was performed in 10 patients with T1 tumors limited to the mucosa and submucosa. The other 7 patients did not accept additional treatment. Eight patients received chemotherapy treatment after surgery. Ten of these patients were still alive and 11 of them died. Survival was 14.8 mo (range 4-38 mo).

DISCUSSION

GPs are common gallbladder lesions and should not be ignored because of their association with malignancy. In the literature, the incidence of GP has been reported as between 0.3% and 12.0%[9,10]. In our clinical series of 5832 patients undergoing cholecystectomy, GPs were an indication for cholecystectomy in 2.3% (138 patients) of cases. Thirty-nine of these patients were diagnosed with only cholelithiasis; therefore, the true incidence of GPs was 1.7%.

There are different concepts about the effect of demographic factors such as age and gender on the incidence of GPs. Some studies reported that GPs are more frequently seen in males[2,11-14] or females[9-15], and some studies even suggest that there is no effect of gender on GPs[14-21]. Approximately 2/3 of cases in our study were-women and the true polyp ratio was 29.7% in men and 17.7% in women (Table 1). Ito *et al*[17] reported that the mean age was 59 years in their 417 patients series. Although 53% of patients in our study were under the age of 50, 90% of true polyps were detected in patients over 50 years old. As demonstrated in Table 1, the incidence of true polyps was 3.8% under the age of 50 years and 44% over the age of 50 years ($P < 0.05$).

Ultrasonography is the most frequently used and most valuable diagnostic tool for preoperative evaluation of gallbladder pathologies[8]. One hundred thirty eight patients in our series were diagnosed with GP using USG. Considering that 39% of them were also diagnosed with cholelithiasis, the accurate diagnosis rate of USG was 71.7%. The sensitivity of USG for GPs has beenreported to be between 32% and 90%[5,18]. While USG can

usually detect polyps > 5 mm, it becomes more accurate if the polyp is > 10 mm[19]. Indeed, USG detected almost all polyps > 10 mm accurately (25 of 26 cases) and these polyps were true polyps (adenoma/adenocarcinoma). However, the accuracy of USG diagnosis lesions < 10 mm was 66%. In addition, GPs were detected as < 10 mm in 39 patients who were thought to have GPs preoperatively but in whom no polyps were detected postoperatively. Postoperatively, the pathological diagnoses of these 39 patients were chronic cholecystitis and cholelithiasis. Cholesterolosis occurs as a result of accumulation of esterified cholesterol and triglycerides in macrophages of the lamina propria, and they are often mistaken as small polyps in USG[17]. Gallbladder stones attached to the wall of the gallbladder can easily be interpreted as a polyp in USG[18]. The presence of stones in the gallbladder reduces the success rate of USG in the diagnosis of GPs; USG diagnosis of GPs is to 99% accurate in the absence of any stones. On the other hand, in our patients, GPs did not usually cause any symptoms. Association of stones with GPs may cause symptoms and prompts the patient to consult a doctor, making the diagnosis easier. In our study, gallstones accompanied GPs in only 18.1% (18 patients) of patients. All patients with stones were symptomatic. However, there were no stones in 15 of the 33 symptomatic patients and GPs caused the symptoms in these patients. In our series of patients, being symptomatic did not have any impact on detection of true polyps ($P = 0.71$).

Another important factor associated with malignancy in GPs is the diameter of the polyps[6,20]. Kozuka *et al*[21] reported that the critical limit for differentiation of benign and malignant GPs was 12 mm and suggested cholecystectomy for GPs larger than 12 mm. Kubota *et al*[22] compared postoperative pathological data of 72 patients with GPs and preoperative ultrasound. They reported 22% of neoplastic polyps of the gallbladder as > 10 mm. They also reported that evaluation of the polyp shape may be beneficial, but it is not enough to distinguish cholesterol polyps from adenoma and cancer. Sugiyama *et al*[23] tried to make a distinction between benign and malignant polyps using preoperative USG and endoscopic USG. They detected adenoma or cancer in 14% of polyps with diameters of 6-10 mm in preoperative USG. Zielinski *et al*[2] emphasized that there is a significant increase in the risk of neoplasia in polypoid lesions > 6 mm; they suggest performing cholecystectomy in these patients. In our study, the majority of polyps (73 of 74 cases) < 10 mm were pseudopolyps, and the remaining polyps were adenomas. None of the malignant polypswere < 10 mm. Eight-four percent of polyps > 10 mm were true polyps (adenoma/adenocarcinoma) and all of the these true polyps were found to be adenocarcinoma. This suggests that a limit of 10 mm is very important ($P = 0.0001$). Similarly, no true polyps were detected in the setting of multiple polyps. Remarkably, 28% of single polyps were diagnosed as adenocarcinoma.

The literature suggests that patients over 50 years old, polyps > 10 mm, polyps with a broad base or long pedicle, polyps associated with cholecystitis or cholelithiasis, or irregular thickening of the gallbladder in the setting of biliary colic are indications for cholecystectomy[4,23,24]. In our study, 21 (21.2%) of 99 patients with GPs were diagnosed with malignancy, all of whom were older than 50 years with single polyps > 10 mm. In addition, the success rate of USG for diagnosing GPs > 10 mm was more evident and an important point. Patients had surgery mostly because of their extreme sensitivity and anxiety. We found that surgery was not beneficial in patients with multiple polypoid lesions or polyps < 10 mm. For this reason, the surgical team should reassure and relax the patients and avoid unnecessary cholecystectomies.

In conclusion, being male and over 50 years old with a solitary polyp > 10 mm benefited most from cholecystectomy.

COMMENTS

Background

Gallbladder polyps (GP) are frequently detected incidentally. They are usually misdiagnosed as gallstones in sonographic examinations. There is no consensus for treatment and follow-up of GP because of its particularly rare incidence of malignancy. There are some risk factors associated with high risk of malignancy. Early diagnosis and surgical treatment of GP affects survival of gallbladder carcinomas.

Research frontiers

Many studies have investigated risk factors that increase the incidence of malignancy of GP. Age, gender, polyp size, polyp number, accompanying gallstones and the inflammatory status of the gallbladder are significant risk factors.

Innovations and breakthroughs

In this study, all the malignant polyps were solitary and over 10 mm in size. Malignant polypsweredetermined in 44% of the patients aged over 50. The authors failed to show an association between gender and malignancy for GP. Ultrasonography (US) was more sensitive for polyps over 10 mm. US was more helpful in showing malignancy for cases with polyps under 10 mm.

Applications

This study will facilitate surgeons' decision making for treatment and follow-up of patients with GP.

Terminology

Histopathologically, cholesterol polyps/colestherolosis, hyperplastic and adenomateous polyps are defined as pseudopolyps, while adenomas and adenocarcinomas are defined as true polyps.

Peer review

This manuscript, which was written on a subject of considerable controversy in general surgery, has been generally well designed.

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BRIEF ARTICLE

Newly designed J-shaped tip guidewire: A preliminary feasibility study in wire-guided cannulation

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Abstract

AIM: To perform wire-guided cannulation using a newly designed J-shaped tip guidewire, and to verify feasibility and safety for use.

METHODS: The study was conducted on endoscopic retrograde cholangiopancreatography (ERCP) patients with naïve papilla undergoing diagnosis and treatment of biliary diseases between September 2011 and July 2012. We performed ERCP in a succession of 50 cases with a J-shaped tip guidewire. The first insertion attempt began with a trainee who had 5 min to complete cannulation, followed if necessary by the trainer for another 5 min. We assessed the primary success rate of selective biliary cannulation within 10 min and adverse events such as post-ERCP pancreatitis (PEP), bleeding or perforation.

RESULTS: The primary success rate was 90% (45/50) within 10 min, the initial success rate within 5 min by trainee staff was 76% (38/50). The rate of PEP was 6% (3/50), but all 3 cases were mild pancreatitis. All patients were managed successfully with conservative treatment. There was no bleeding or perforation.

CONCLUSION: A newly designed J-shaped tip guidewire has the possibility to facilitate selective biliary cannulation for ERCP and appears to be safe.

Key words: J-shaped tip guidewire; Wire-guided cannulation; Endoscopic retrograde cholangiopancreatography; Biliary tract; Cannulation technique; Perforation

Core tip: We conducted a feasibility study that performed endoscopic retrograde cholangiopancreatography (ERCP) with a newly designed J-shaped tip guidewire. This new guidewire has a strongly-flexed atraumatic tip with hydrophilic coating; therefore, it may contribute to the improvement of the passage through the intra-duodenal biliary segment and to the decrease of adverse events such as post-ERCP pancreatitis. We assessed the primary success rate of selective biliary cannulation within 10 min and rate of post-ERCP pancreatitis. The primary success rate was 90% (45/50); the rate of post-ERCP pancreatitis was 6% (3/50), but all 3 cases were mild. The J-shaped tip guidewire may facilitate selective biliary cannulation in ERCP.

Omuta S, Maetani I, Shigoka H, Gon K, Saito M, Tokuhisa J, Naruki M. Newly designed J-shaped tip guidewire: A preliminary feasibility study in wire-guided cannulation. *World J Gastroenterol* 2013; 19(28): 4531-4536 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i28/4531.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i28.4531

INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) is used to diagnose and treat biliary disease. Deep cannulation of the common bile duct is required for this procedure, and the current success rate for the relatively difficult conventional contrast-guided cannulation (CGC) technique ranges from 50%-90%[1-5]. Wire-guided cannulation (WGC) is a recently developed alternative to CGC that has been shown to increase primary biliary cannulation rate[6-11], chiefly by reducing incidence of post-ERCP pancreatitis (PEP)[12-20]. However, despite efficiency improvements, the sharp tips of guidewires are sometimes associated with perforation[21-23]. Even without perforation, complications can occur when the guidewire tip hits the fold and flexion of the intra-duodenal biliary segment. While a looped tip guidewire has been developed, its utility in avoiding perforation has not sufficiently been evaluated[24].

Here, we assessed the efficiency of ERCP using a newly designed J-shaped tip guidewire with a strongly flexed atraumatic tip and hydrophilic coating designed to improve passage through intra-duodenal biliary segments and decrease the adverse events, such as PEP, bleeding or perforation.

MATERIALS AND METHODS

Patients

Fifty patients with naïve papilla undergoing diagnosis and treatment for biliary diseases between September 2011 and July 2012 received ERCP using J-shaped tip guidewires. Patients were excluded if only their pancreatic ducts were diagnosed or treated, if they had previously undergone endoscopic sphincteroplasty, or if they had duodenal stenosis or Billroth Ⅱ or Roux-en-Y anastomosis, or refused to provide informed consent.

Patients were sedated *via* intravenous administration of midazolam (5-10 mg) and buprenorphine (0.2 mg). Scopolamine butylbromide (20 mg) or glucagon (1 mg) was injected intravenously to inhibit gastrointestinal peristalsis, and each patient received nafamostat mesilate (20 mg/d) prior to ERCP. Blood samples collected 2 h after ERCP were used to determine complete blood counts and serum amylase levels, and those collected after 18-24 h also measured hepatobiliary enzymes and C-reactive protein. We did not place a pancreatic duct stent for the prevention of pancreatitis in either procedure.

J-shaped tip guidewire

The guidewire (RWHJ-2545A, 0.025-inch; Paiolax Medical Devices, Inc., Kanagawa, Japan) tip was bent to attain a 1-mm radius, and a hydrophilic coating was applied starting 50 mm from the tip. The shaft was covered by a sheath and the jacket coated with water-repellent material (Figure 1).

Endoscopic procedure

Endoscopy was performed with JF-260V (Olympus, Tokyo, Japan) or ED-530XT8 (Fujinon, Tokyo, Japan) endoscopes, after catheters were preloaded with guidewires. In the present study, in general, a regular catheter was chosen except for the case undergoing sphincterotomy. First, catheters (CleverCut3 V, Olympus, Tokyo, Japan; Tamdem XL, Boston Scientific, Natick, MA, United States) were preloaded with guidewires, the guidewire tip was extended 5 mm from the catheter, bent into a "J" shape, and then the guidewire was pulled back into a stand-by position (Figure 2A). Endoscopists controlled the direction parallel to the axis of bile duct of the catheter by inches. Assisting endoscopists participated in the guidewire manipulation in all cases. An assisting endoscopist moved the guidewire back and forth in small motions by using a tactile feedback (in-and-out movement method). No fluoroscope was used during attempts of insertion, but once the guidewire was inserted without resistance then fluoroscopy was used only after insertion to confirm success (Figure 2B). The catheter was then inserted into the biliary system along the guidewire, and contrast medium was injected (Figure 2C). No test injection was performed before successful cannulation.

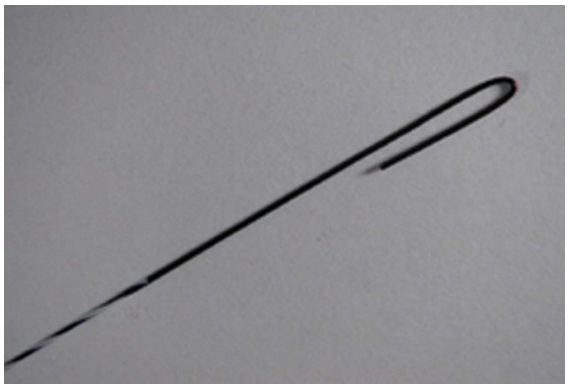

Figure 1 Newly designed J-shaped tip guidewire. The shape of the tip is a radius of 1 mm; 50 mm from the tip is the start of a hydrophilic coating.

The first insertion attempt began with a trainee who had 5 min to complete cannulation, followed if necessary by a trainer with career experience of over 500 ERCPs (Maetani I or Shigoka H or Omuta S) for another 5 min. If both attempts failed, efforts continued with a standard biliary guidewire (Jagwire 0.035 angle type, Boston Scientific) for another 10 min (second attempt) and were repeated as necessary according to the trainers' recommendations (exchange of endoscopist or guidewire, pancreatic duct guidewire placement method, or pre-cutting sphincterotomy).

Definitions

Success was defined as completing cannulation with the J-shaped tip guidewire and obtaining a cholangiogram within 10 min. Cannulation time was defined as from when a tip of the guidewire first touched the orifice of the papilla to the obtainment of cholangiogram. PEP was defined as continued abdominal pain ⩾ 24 h after ERCP, with more than 3 times the normal (upper limit) serum-

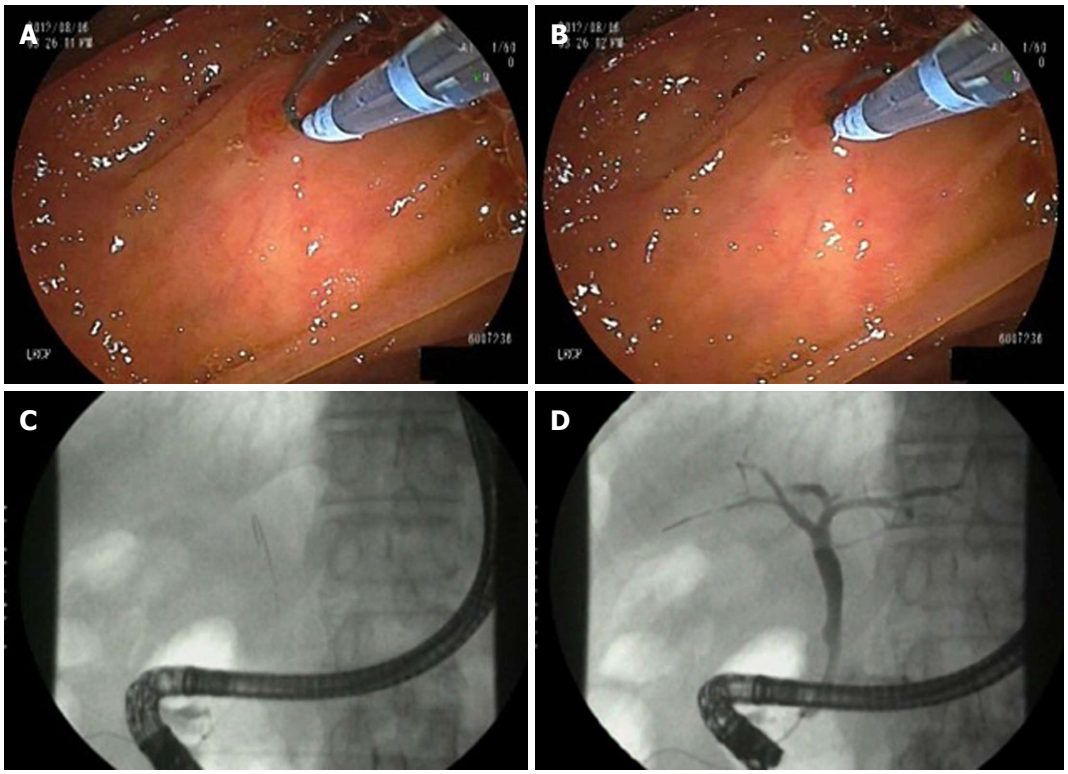

Figure 2 Endoscopic and fluoroscopic images showing the technique with J-shaped tip guidewire. A: Assistant endoscopist extended approximately 5 mm of the guidewire tip and restored it to the original "J" configuration (stand-by position); B: Selective biliary cannulation was attempted under endoscopic control without fluoroscopy; C: The guidewire was moved in an in-and-out motion by an assisting endoscopist. Once the guidewire was advanced without resistance, fluoroscopy was used to confirm successful cannulation; D: Contrast medium was injected after confirmation of successful biliary cannulation.

Table 1 Baseline patient characteristics and indications

Item (n = 50)	Value
Age, yr [median (IQR)]	75.3 (68-83)
Sex (male)	24
Periampullary diverticulum, n (%)	22 (44)
Indications	
Choledocolithiasis (including suspicion)	27
Cholangiocarcinoma	7
Pancreatic cancer	6
Gallbladder cancer	4
Other malignant disease	2
Cholangiocellular carcinoma	1
Suspected biliary SOD	1
Mirrizi syndrome	1
Biliary leak after cholecystectomy	1

IQR: Interquartile rage; SOD: Sphincter of Oddi dysfunction.

amylase level[25]. Pancreatitis severity was classified using the Atlanta International Symposium criteria[26]. Suspected sphincter of Oddi dysfunction was defined according to the revised Milwaukee classification[27]. Sphincter of Oddi manometory was not performed. Hyperamylasemia was defined as 3 times the normal (upper limit) amylase level 18-24 h after ERCP.

Ethics

The protocol adhered to the Helsinki Declaration and was approved in advance by the Institutional Ethical Review Board. The trial was registered with the University hospital Medical Information Network Clinical Trials Registry (UMIN000007526). All participants gave written informed consent beforehand.

Outcome measurement

The primary study endpoint was the success rate of cannulation with the J-shaped tip guidewire performed within 10 min. The secondary endpoints were as follows: (1) the rate of the occurrence of PEP; (2) time to selective biliary cannulation; (3) number of attempts for selective biliary cannulation; and (4) number of accidental pancreatic duct insertions. Data are presented as median and interquartile ranges (IQR).

RESULTS

Baseline characteristics and indications are summarized in Table 1, and details of the endoscopic procedure are given in Table 2.

The overall success rate of endoscopy was 90% (45/50, Table 3), with cannulation achieved within the first 5 min in 38 patients (76%). Cannulation was achieved on the second attempt in 3 patients. The median time to cannulation for these 48 patients was 42.5 s (IQR: 5-262 s). Of the remaining two patients, one required pancreatic duct guidewire placement and the other a pre-cutting sphincterotomy. The median number of attempts was 2.0 (IQR:

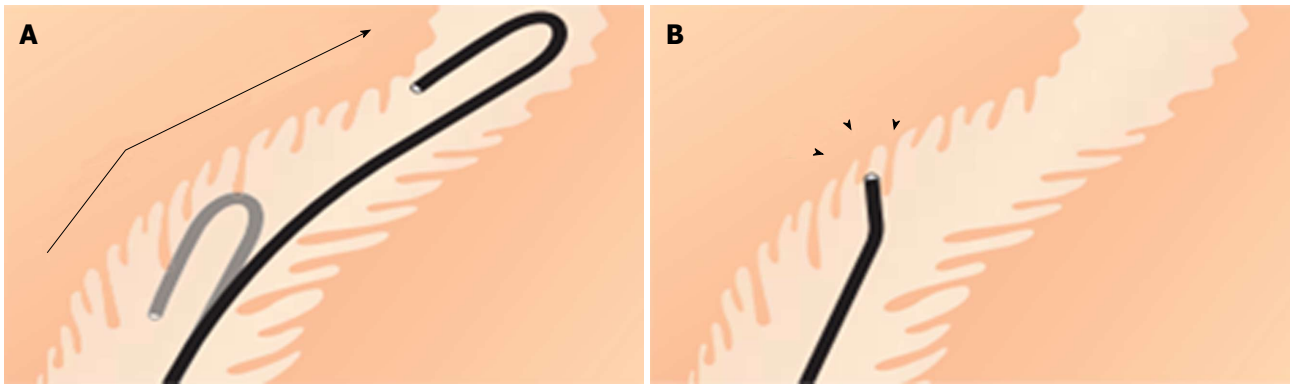

Figure 3 An image *via* intra-duodenal biliary segments of J-shaped tip guidewire (A) and standard guidewire (B). A: Blunted J-shaped tip may facilitate passage through intra-duodenal segment (arrow); B: Normal guidewire tips may be become stuck in epithelial folds or flexion of intra-duodenal biliary segments (arrowheads).

Table 2 Number of patients receiving different procedures

Procedure	*n*
Endoscopic papillary (large) balloon dilation	24
Endoscopic sphincterotomy	19
Endoscopic nasobiliary drainage	20
Endoscopic nasobiliary gallbladder drainage	2
Placement of biliary stent (plastic or metal)	18
Intraductal ultrasonography	7
Aspiration, biopsy	12
Only cholangiogram	0

Table 3 Cannulation outcomes

Item (*n* = 50)	Value
Success, *n* (%)	45 (90)
< 5 min	38 (76)
5-10 min	7 (14)
Time to selective biliary cannulation[1], s	42.5 (5.0-262.0)
No. of attempts[1]	2.0 (1.0-6.0)
No. of accidental pancreatic duct insertion[1]	1.0 (0.0-3.0)
Amylase level[1], IU/L	148 (94-331)
Post-ERCP pancreatitis, *n* (%)	3 (6)
Mild	3
Severe	0
Hyperamylasemia, *n* (%)	4 (8)

[1]Data is shown as median (IQR). IQR: Interquartile range; ERCP: Endoscopic retrograde cholangiopancreatography.

1.0-6.0), and the median number of accidental pancreatic duct insertions was 1.0 (IQR: 0.0-3.0). The median serum-amylase level was 148 IU/L (IQR: 94-331 IU/L), and hyperamylasemia occurred in 4 patients.

Mild PEP occurred in 3 patients (6%); in 2 of these, success was achieved within 5 min after endoscopic papillary large balloon dilation, while the third patient received the pre-cutting sphincterotomy mentioned above. All patients were managed successfully with conservative treatment. There were no other adverse events including bleeding or perforation.

DISCUSSION

The success rate for selective biliary cannulation using a J-shaped tip guidewire was comparable to that found in previous studies[6-16,28,29], and no guidewire-related adverse events such as bleeding or perforation occurred. Although ours was a preliminary study, the atraumatic and blunt tip of the new guidewire may facilitate selective biliary cannulation (Figure 3A) and reduce instances of perforation and bleeding.

Although straight and angled tips are the most common types used in WGC[1-20,28,29], these sharp tips often stick in the intra-duodenal biliary segment (Figure 3B). While the superiority of the J-shaped tip cannot be definitively shown without controls, the success rate, speed of cannulation, and facility of use appear improved compared to other studies. While similar procedures using standard guidewires resulted in a 77.9% overall success rate (trainees and trainer combined)[28], here we achieved a 76% success rate with trainees, and an overall success rate of 90%. Additionally, the 6% PEP rate is similar to that of other studies[14,18,19,20].

WGC was first introduced by Siegel *et al*[30]. Meta-analysis has shown that the reduction of pancreatic duct opacification is another possible advantage over CGC[14,18,19]. Further, WGC has been suggested to decrease the risk of PEP[14,18,19], facilitating its spread across the globe as a potential first-line method.

Usually, when guidewires are extended from the tip of a catheter without enough space for advancement, the wire may act like a needle and pierce the epithelium. The J-shape of the guidewire protrudes from the catheter before approaching the biliary orifice, and reduces this likelihood. We therefore believe our J-shaped design to be the aspect that improved insertion into the biliary system. Limitations to this study include small sample size, no controls, a single institution, and involvement of multiple endoscopists. A randomized comparison is warranted for objective evaluation of its performance. One drawback of the J-shaped tip guidewire is the 1-mm radius, which is wider than a standard guidewire and may hamper selective cannulation through a narrow orifice.

In conclusion, a newly designed guidewire with a

J-shaped tip may facilitate selective biliary cannulation in ERCP. However, a large prospective randomized control trial is necessary to verify the performance of this guidewire in comparison with standard guidewires.

COMMENTS

Background

Selective biliary cannulation is essential for diagnosis and therapeutic endoscopic retrograde retrograde cholangiopancreatography (ERCP) in biliary diseases. Wire-guided cannulation (WGC) increases the primary biliary cannulation rate and decreases the risk of post-ERCP pancreatitis (PEP). Therefore, WGC is now widely performed. However, even experts meet with difficulty and the possible risk of bleeding and perforation due to the guidewire.

Research frontiers

The authors performed ERCP using a newly designed J-shaped tip guidewire. A J-shaped tip guidewire with a strongly flexed atraumatic tip and hydrophilic coating was designed to improve passage through intra-duodenal biliary segments and decrease the adverse events, such as PEP, bleeding and perforation. The authors conducted a feasible study.

Innovations and breakthroughs

This is a single center pilot study. The primary success rate was 90% (45/50) within 10 min. The rate of PEP was 6% (3/50), but all 3 cases were mild pancreatitis. All patients were managed successfully with conservative treatment. There was no bleeding or perforation.

Applications

A newly designed J-shaped tip guidewire may facilitate selective biliary cannulation and the structure of the tip may contribute to decrease PEP and bleeding, or perforation. However, it is necessary to conduct a large prospective randomized control trial to verify the performance.

Peer review

This is a single center pilot study of a newly designed J-shaped tip guidewire for wire-guided cannulation. The authors hypothesized that the J-shaped tip prevented perforation or PEP during cannulation. The limitation of this study is a small sample size without a control group as the authors discussed.

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BRIEF ARTICLE

Survival outcome of patients with spontaneously ruptured hepatocellular carcinoma treated surgically or by transarterial embolization

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Abstract

AIM: To evaluate clinical outcomes of patients that underwent surgery, transarterial embolization (TAE), or supportive care for spontaneously ruptured hepatocellular carcinoma (HCC).

METHODS: A consecutive 54 patients who diagnosed as spontaneously ruptured HCC at our institution between 2003 and 2012 were retrospectively enrolled. HCC was diagnosed based on the diagnostic guidelines issued by the 2005 American Association for the Study of Liver Diseases. HCC rupture was defined as disruption of the peritumoral liver capsule with enhanced fluid collection in the perihepatic area adjacent to the HCC by dynamic liver computed tomography, and when abdominal paracentesis showed an ascitic red blood cell count of > 50000 mm^3/mL in bloody fluid.

RESULTS: Of the 54 patients, 6 (11.1%) underwent surgery, 25 (46.3%) TAE, and 23 (42.6%) supportive care. The 2-, 4- and 6-mo cumulative survival rates at 2, 4 and 6 mo were significantly higher in the surgery (60%, 60% and 60%) or TAE (36%, 20% and 20%) groups than in the supportive care group (8.7%, 0% and 0%), respectively (each, $P < 0.01$), and tended to be higher in the surgical group than in the TAE group. Multivariate analysis showed that serum bilirubin (HR = 1.09, $P < 0.01$), creatinine (HR = 1.46, $P = 0.04$), and vasopressor requirement (HR = 2.37, $P = 0.02$) were significantly associated with post-treatment mortality, whereas surgery (HR = 0.41, $P < 0.01$), and TAE (HR = 0.13, $P = 0.01$) were inversely associated with post-treatment mortality.

CONCLUSION: Post-treatment survival after surgery or TAE was found to be better than after supportive care, and surgery tended to provide better survival benefit than TAE.

Key words: Ruptured hepatocellular carcinoma; Surgery; Transarterial embolization

Core tip: We have shown here that overall survival rates of patients with ruptured hepatocellular carcinoma (HCC) is significantly higher in patients with surgery or transarterial embolization (TAE) than in those with supportive care, and tended to be higher in patients with surgery than in those with TAE. To date, there has been a dearth of reliable clinical evidence on the merits of surgical treatment versus those of TAE, in the context of survival benefit in patients with a spontaneous HCC rupture. Therefore, the present study may provide useful information for clinicians to determine the most appropriate treatment option for spontaneously ruptured HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the worldwide health problem and the third leading cause of cancer-related death globally[1-3]. Despite recent considerable advances in the understanding of tumor biology and the continued progression and development of diagnostic and therapeutic tools[4-7], the overall prognosis of HCC remains disappointing. In particular, due to its hypervascularity, HCC can exhibit rapid progression with direct invasion of surrounding tissues or it can invoke spontaneous tumor rupture[8]. HCC rupture is one of the life-threatening complications of HCC, and therefore, the most efficient treatment modality should be selected and rapidly applied to patients with ruptured HCC.

The incidence of spontaneous HCC rupture has decreased due to the earlier detection of HCC. Nevertheless, its incidence has been reported to be high as 3%-15% and its in-hospital mortality rate to range from 25% to 75% in the acute phase[9-12]. Open surgery was the main method used to treat HCC rupture from the 1960s to the 1980s[13-15]. Recently, survival benefit by transarterial embolization (TAE) has been reported[16-18]. However, to the best of our knowledge, no definite recommendation has been issued regarding optimal treatment of HCC rupture, and the comparative survival benefits of surgery and TAE remain unclear.

Therefore, in this retrospective study, we undertook to evaluate survival outcomes according to treatment modalities, that is, surgery, TAE, or supportive care, in patients with a spontaneously ruptured HCC, and sought to identify the factors that predispose post-treatment mortality in these patients.

MATERIALS AND METHODS

Study subjects

Between August 2003 and February 2012, 1765 consecutive patients were initially diagnosed as having HCC at Inha University Hospital. Of these 1765 patients, 61 (3.5%) patients were clinically diagnosed as having spontaneously ruptured HCC. No patient had recent history of HCC treatment such as surgery or transarterial chemoembolization (TACE) within one month prior to the diagnosis of HCC rupture. HCC was diagnosed according to the diagnostic guidelines issued by the American Association for the Study of Liver Diseases[19]. HCC rupture was defined as disruption of the peritumoral liver capsule with enhanced fluid collection in the perihepatic area adjacent to the HCC by dynamic liver computed tomography (CT)[20], and when abdominal paracentesis showed an ascitic red blood cell count of > 50000 mm^3/mL in bloody fluid[21,22].

Of the 61 patients, 4 patients were excluded because they underwent two-staged surgical treatment after TAE for ruptured HCC (n = 3) and they had concurrent malignancy (gastric cancer, n = 1). Three patients were also excluded because they did not meet the diagnostic criteria of a ruptured HCC although HCC rupture was clinically suspected based on right upper quadrant abdominal pain and a reduced serum hemoglobin level. Therefore, 54 patients finally constituted the study cohort and their retrospective database was analyzed.

Evaluation of patients with ruptured HCC

Database information at time of diagnosis of ruptured HCC was reviewed: age, gender; vital signs; medical history; white blood cell count, hemoglobin, and platelet count; international normalized ratio (INR); serum alanine aminotransferase, bilirubin, albumin, and creatinine; viral hepatitis findings including hepatitis B surface antigen, and anti-hepatitis C virus antibody findings; serologic tests for human immunodeficiency virus; alpha-fetoprotein; and vasopressor requirement. Furthermore, we evaluated HCC tumor statuses namely tumor number, size, presence of portal vein tumor thrombosis, and presence of extra-hepatic metastasis. Intrahepatic HCC lesion size was recorded as the longest diameter of the largest lesion in at least one dimension. Liver cirrhosis was diagnosed based on clinical evidence of portal hypertension (encephalopathy, esophageal varices, ascites, splenomegaly, or platelet count < 100000/mm^3)[23] or by previously performed ultrasonography[24]. Child-Turcotte-Pugh (CTP) and Model for End-stage Liver Disease (MELD) scores were assessed, and HCC staging was performed using the Barcelona Clinic Liver Cancer (BCLC) staging system[25].

Treatment of ruptured HCCs

Immediately following a diagnosis of HCC rupture, patients were transferred to an intensive care unit. At time of HCC rupture, liver functions can be much more ag-

gravated by hemorrhage or shock than after the condition was relatively well controlled. Therefore, frequent assessments of liver function were required concurrently with volume replacement and coagulopathy correction, and liver function was closely monitored before definite treatment decision making. Norepinephrine was infused intravenously as the primary vasopressor if shock event developed, and all 54 patients were administered 3rd generation cephalosporin antimicrobial therapy.

The need for emergency hemostasis, such as, open surgery or TAE, was explained to all patients in the absence of a contraindication and their family members. They were informed of the risks and benefits of emergency surgery or TAE in detail. To avoid any coercion, written informed consent was obtained from all patients and a family member before intervention of hemostasis. The follow were viewed as surgical contraindication: the presence of poorly controlled chronic ascites; the presence of poorly controlled chronic hepatic encephalopathy; the presence of a poor liver function; or a poor performance status. Of the 54 patients enrolled, 6 (11.1%) underwent surgery and 25 (46.3%) TAE, and the remaining 23 (42.6%) patients received supportive care without hemostatic intervention (Figure 1). Successful control of hemorrhage was defined as hemodynamic stabilization, a normal hemoglobin level, and no requirement for further transfusion. During follow-up period after treatment, dynamic liver CT images and serum alpha-fetoprotein levels were obtained every 1-3 mo.

TAE group: In hemodynamically unstable patients with an obvious continuous hemorrhage, TAE was considered if reserved liver function was relatively good regardless of the correction of coagulopathy. Briefly, the tumor location, the active bleeding site, and portal vein patency were determined angiographically. Thereafter, embolization of the feeding artery was performed with gelfoam, which is the small cube of approximately 1 mm^3 sized absorbable gelatin sponge particles. Two patients who underwent TACE were included in the TAE group. However, TACE/TAE was not performed if the main portal vein was completely occluded by tumor thrombus.

Surgical group: After stabilizing hemodynamic status by volume replacement and transfusion, patients underwent a full clinical assessment to evaluate the possibility of surgical treatment. Segmentectomy with perihepatic packing (n = 3, 50%), lobectomy (n = 2, 33.3%), or liver wedge resection with feeding artery ligation (n = 1, 16.7%) were performed depending on circumstances (Figure 1).

Supportive care group: Patients contraindicated for surgery or TACE/TAE received only vigorous and careful conservative treatments with replacement of blood or albumin, correction of coagulopathy, antimicrobial therapy, and analgesics, diuretics, *etc*.

The study protocol was approved by the Institutional Review Board at Inha University Hospital, Incheon 400-711, South Korea.

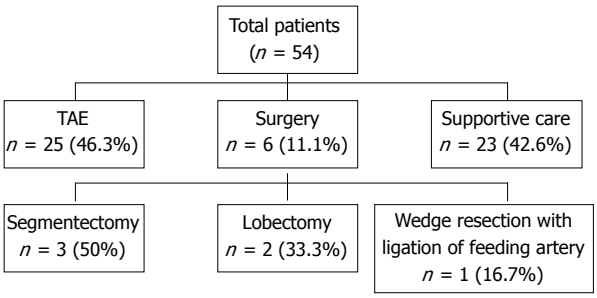

Figure 1 Flow diagram showing the treatment of 54 patients. Of the 54 patients enrolled, 6 (11.1%) underwent surgery and 25 (46.3%) transarterial embolization (TAE), and the remaining 23 (42.6%) patients received supportive care without hemostatic intervention.

Statistical analysis

The baseline characteristics of patients are expressed as medians (ranges) and frequencies. Differences between categorical or continuous variables were analyzed using the χ^2 test, Fisher's exact test, or the Student's t test. Post-treatment cumulative mortality rates were analyzed using Kaplan-Meier survival curves, and group differences were compared using the log-rank test. In patients that received supportive care, survival was defined from diagnosis of HCC rupture to patients' death. Multivariate analysis was performed using a Cox regression hazard model to identify predictors of post-treatment mortality in patients with spontaneously ruptured HCC. Two-tailed P values of less than 0.05 were considered statistically significant in all analyses. Statistical analysis was performed using SPSSv18.0 (SPSS Inc, Chicago, IL, United States).

RESULTS

Baseline characteristics of patients

The baseline characteristics of the 54 patients are summarized in Table 1. Median age was 54 years (range, 30-87 years) and 47 (87.0%) were male. The most common etiology of HCC was hepatitis B virus infection, which was observed in 36 (66.7%) patients. Of the 54 patients, 6 (11.1%) were of CTP class A, 23 (42.6%) were of CTP class B, and 5 (46.3%) were of CTP class C. Eleven (20.4%) of the 54 patients had a single HCC and 43 (79.6%) patients had multiple HCC. Median tumor size was 8.5 cm (range, 2.9-25.5 cm), and 4 (7.4%) patient had HCCs within Milan criteria. Forty-seven (87.7%) patients had nodular type HCC. Before treatment, 0 (0%), 5 (9.3%), 9 (16.7%), 15 (27.8%), and 25 (46.3%) patients were found to have BCLC 0, A, B, C, or D stage HCC, respectively. Median alpha-fetoprotein concentration at diagnosis of HCC rupture was 1158 ng/mL (range, 3.0×10^4-6.1×10^4 ng/mL). Thirteen (24.1%) patients required a vasopressor due to shock at presentation. Ruptured HCC was located on the surface of liver in all the patients.

Comparison of clinical parameters by treatment modality

Clinical variables in the three treatment groups are summarized in Figures 2 and 3, and Table 2. Mean tumor

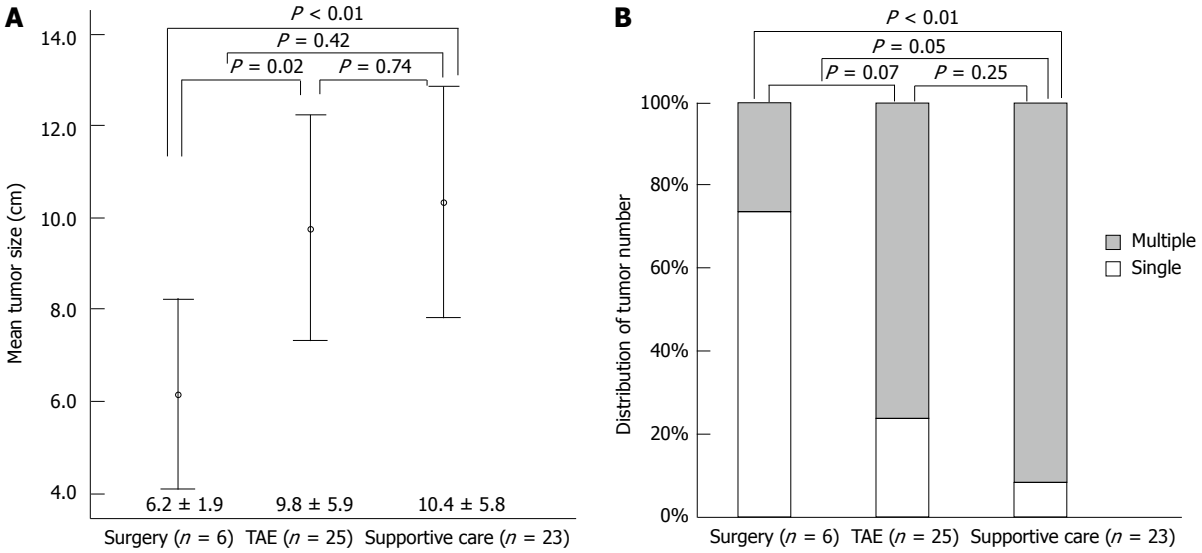

Figure 2 Comparison of clinical parameters in the three treatment groups. A: Mean tumor size was significantly smaller in the surgical group than in the transarterial embolization (TAE) (P = 0.02) or supportive care (P < 0.01) groups; B: The single tumor rate was significantly higher in the surgical group than in the supportive care group (P < 0.01).

Table 1 Baseline clinical characteristics of the 54 patients with ruptured hepatocellular carcinoma *n* (%)

Variable	Total (n = 54)
Age[1], yr	54 (30-87)
Gender (male)	47 (87.0)
Etiology	
HBV/HCV/alcohol/others	36 (66.7)/6 (11.1)/7 (13.0)/5 (9.3)
CTP classification	
A/B/C	6 (11.1)/23 (42.6)/25 (46.3)
Tumor size[1], cm	8.5 (2.9-25.5)
Tumor number	
Single/multiple	11 (20.4)/43 (79.6)
Tumor type	
Nodular/infiltrative	47 (87.0)/7 (13.0)
Within Milan criteria	4 (7.4)
BCLC stage	
0/A/B/C/D	0 (0.0)/3 (5.6)/8 (14.8)/15 (27.8)/28 (51.9)
Vasopressor requirement	13 (24.0)
Alpha-fetoprotein[1], ng/mL	1158 (3.0×10^4-6.1×10^4)

[1]Median (range). HBV: Hepatitis B virus; HCV: Hepatitis C virus; CTP: Child-Turcotte-Pugh classification; BCLC: Barcelona Clinical Liver Cancer.

size was significantly smaller in the surgical group than in the TAE (P = 0.02) or supportive care (P < 0.01) groups (Figure 2A). The single tumor rate was significantly higher in the surgical group than in the supportive care group (P < 0.01) (Figure 2B). Furthermore, the surgical group had better reserve hepatic function (CTP class) than the other two groups (both P < 0.01) (Figure 3A). Mean MELD score was higher in the supportive care group than in the other two groups (both P = 0.01), but was not different in the surgical and TAE groups (P = 0.24) (Figure 3B).

Serum platelet count (P = 0.03), total bilirubin (P < 0.01), and creatinine levels (P < 0.01) were significantly lower in surgical group than supportive care group (Table 2). The other clinical parameters including age, gender, and tumor type showed no difference among three treatment groups. Incomplete hemostasis occurred in 1 (16.7%) patient in the surgical group and in 5 (20%) patients in the TAE group (P = 1.00), and post-treatment liver failure occurred in 0 (0%) patient in the surgical group and in 1 (5%) patient in the TAE group (P = 0.31). Rebleeding after complete hemostasis was observed in 0 (0%) patients in the surgical group and in 1 (5%) patient in the TAE group (P = 1.00), and they received supportive care (Table 2).

Cumulative overall survival of ruptured HCC patients according to treatment types

One-month overall cumulative mortality for the 54 study subjects was 63.8%. Cumulative survival rates at 2-, 4- and 6-mo were 60.0%, 60.0% and 60.0%, respectively, in the surgical group and 36.0%, 20.0% and 20.0%, respectively in the TAE group, and 8.7%, 0% and 0%, respectively in the supportive care group (each, P < 0.01). Cumulative survival rates at 2-, 4- and 6-mo were tended to be higher in the surgical group than in the TAE group despite the statistical insignificance (P = 0.14) (Figure 4A). Cumulative survival rates at 2-, 4- and 6-mo were 50.2%, 40.1% and 33.1%, respectively in the intervention group such as surgery or TAE, and 8.7%, 0% and 0%, respectively, in the supportive care group (P < 0.01) (Figure 4B).

Multivariate analysis for predictors of post-treatment mortality

Multivariate analysis showed that surgery (HR = 0.41, P < 0.01), and TAE (HR = 0.13, P = 0.01) were inversely associated with post-treatment mortality in ruptured HCC patients. Serum bilirubin (HR = 1.09, P < 0.01), creatinine (HR = 1.46, P = 0.04), and vasopressor use (HR = 2.37, P = 0.02) were positively associated with post-treatment mortality. Age, gender, INR, albumin, tumor

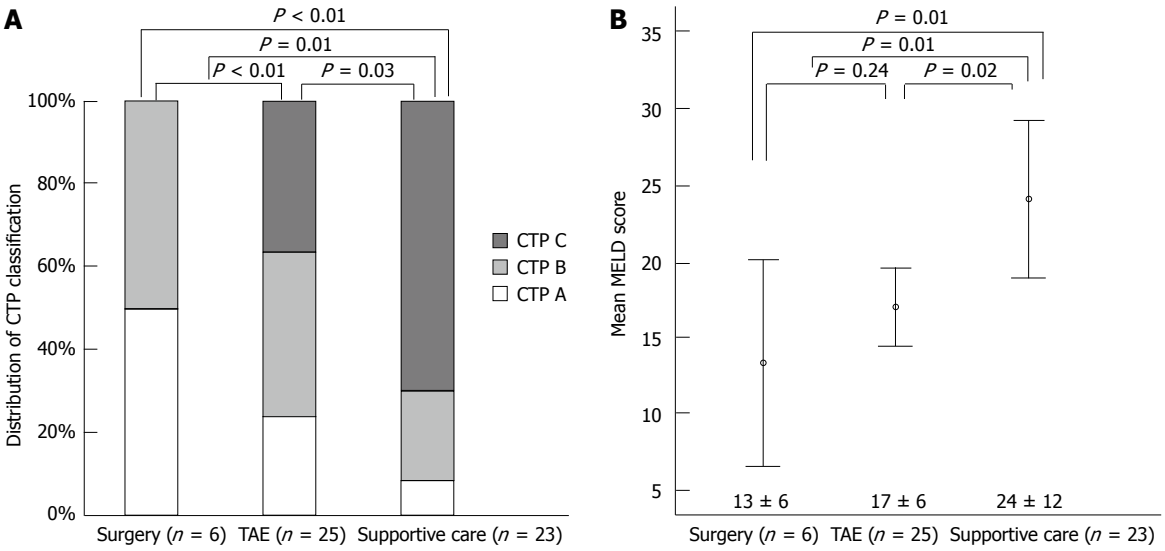

Figure 3 Comparison of Child-Turcotte-Pugh and Model for End-stage Liver Disease scores in the three treatment groups. A: The surgical group had better reserve hepatic function [Child-Turcotte-Pugh (CTP) class] than the other two groups (both $P < 0.01$); B: Mean Model for End-stage Liver Disease (MELD) score was higher in the supportive care group than in the other two groups (both $P = 0.01$), but was not different in the surgical and transarterial embolization (TAE) groups ($P = 0.24$).

Table 2 Clinical parameters of patients according to treatment modality *n* (%)

Variables	Resection	TAE	Supportive	*P* value
	6 (11.1)	25 (46.3)	23 (42.6)	
Age, yr	59 (42-79)	54 (30-83)	53 (36-87)	NS
Gender (male)	5 (83.3)	22 (88.0)	20 (86.9)	0.95
Hb[1], g/dL	8.2 (5.1-12.1)	7.6 (4.5-11.2)	8.1 (2.9-13.6)	NS
Platelet[1], × 10^3/mm^3	127.5 (80-236)	139 (11-534)	191 (86-606)	0.03[4], NS
Prothrombin time[1], INR	1.4 (0.7-3.0)	1.3 (1.0-2.3)	2.2 (1.0-6.6)	NS
Albumin[1], mg/dL	2.6 (0.4-3.5)	2.9 (1.8-3.7)	2.6 (1.2-4.3)	NS
Total bilirubin, mg/dL	2.3 (0.5-6.2)	1.2 (0.4-5.4)	6.7 (0.6-23.0)	< 0.01[4], NS
Creatinine[1], mg/dL	1.2 (0.8-2.0)	1.1 (0.6-1.2)	1.9 (0.9-4.9)	< 0.01[4], 0.01[5]
Tumor type				
Nodular/infiltrative	5/1 (83.3/16.7)	21/4 (84/16)	21/2 (91.3/8.7)	0.72
BCLC stage A/B/C/D	3/2/1[2]/0 (50.0/33.3/16.7/0)	0/4/9/12 (0/16/36/48)	0/2/5/16 (0/8.7/21.7/69.6)	< 0.01
Alpha-fetoprotein[1], ng/mL	33.9 (3-3.6 × 10^4)	1345 (3-6.1 × 10^4)	1389 (19-6.1 × 10^4)	NS
Vasopressor requirement	1 (16.7)	7 (28.0)	5 (21.7)	0.79
Incomplete hemostasis	1 (16.7)	5 (20)	NA	1.00[3]
Post-treatment liver failure	0 (0)	6 (24)	NA	0.31[3]
Rebleeding	0/5 (0)	1/20 (5.0)	NA	1.00[3]

[1]Median (range); [2]1 patient received palliative resection; [3]*P* value between resection and TAE group; [4]*P* value between resection and supportive group; [5]*P* value between TAE and supportive group. TAE: Transarterial embolization; BCLC: Barcelona Clinical Liver Cancer; NA: Not available; NS: Not significant.

size, and tumor number were not found to be associated with post-treatment mortality (Table 3).

DISCUSSION

We have shown here that overall survival rates of patients with ruptured HCC is significantly higher in patients with surgery or TAE than in those with supportive care, and tended to be higher in patients with surgery than in those with TAE. Furthermore, high serum bilirubin and creatinine levels, and vasopressor requirement were found to be significantly associated with post-treatment mortality. To our knowledge, this is the first study to investigate the survival benefit of surgery *vs* TAE. Although several previous studies have evaluated the therapeutic efficacy of surgery or TAE, in patients with spontaneous HCC rupture, direct comparative information is little available regarding survival outcomes after surgery and TAE in such patients.

Spontaneous HCC rupture is likely to occur in patients with advanced staged HCC with reported incidences of 10.0% in Japan[15], 12.4% in Thailand[10], and about 3.0% in the United Kingdom[26]. In the present study, the estimated incidence of spontaneous HCC rupture was 3.5%, and the overall 1-mo mortality was a high as 64% in our cohort, which are the similar to the outcomes of previous studies[10,11,27]. However, despite its high mortality, survival benefits for surgical treatment[13-15] and for TAE[16-18] have been reported in patients with a spontaneous HCC rupture. Likewise, in the present study, the

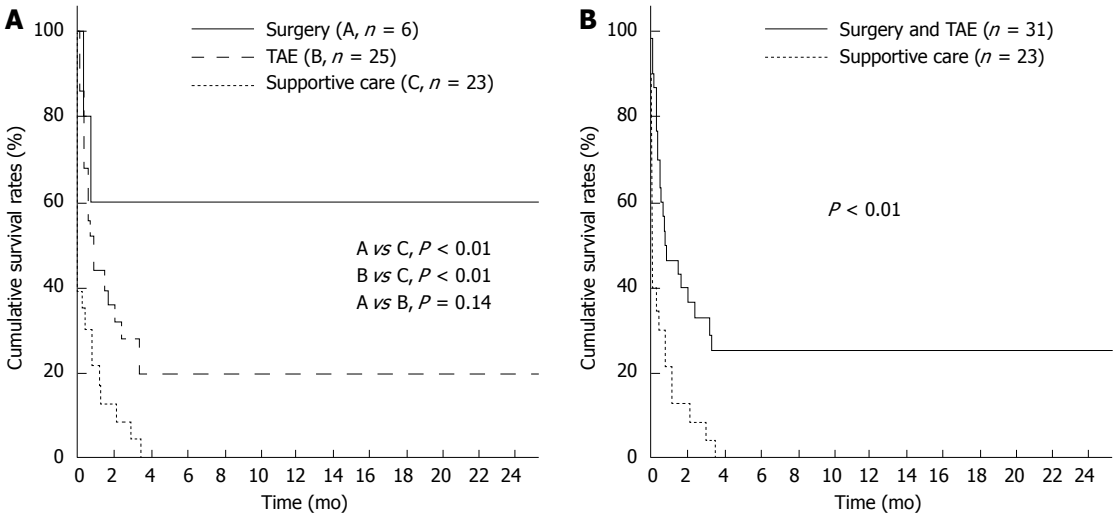

Figure 4 Cumulative overall survival according to treatment types. A: Cumulative survival rates at 2-, 4- and 6-mo in the surgical group or in the transarterial embolization (TAE) group were significantly higher in the supportive care group (each, $P < 0.01$); B: Cumulative survival rates at 2-, 4- and 6-mo were significantly higher in the intervention group such as surgery and TAE than in the supportive care group ($P < 0.01$).

Table 3 Significant predictive factors of post-treatment mortality in spontaneously ruptured hepatocellular carcinoma patients

Variables	Univariate analysis			Multivariate analysis[1]		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age, yr	0.99	0.96-1.01	0.18	0.98	0.95-1.01	0.14
Gender (male)	0.88	0.37-2.09	0.77	-	-	-
INR	1.77	1.30-2.41	< 0.01	0.92	0.49-1.70	0.79
Total bilirubin, mg/dL	1.13	1.07-1.19	< 0.01	1.09	1.13-1.15	< 0.01
Albumin, mg/dL	0.73	0.42-1.03	0.07	0.81	0.49-1.32	0.39
Creatinine, mg/dL	1.86	1.33-2.61	< 0.01	1.46	1.01-2.13	0.04
AFP, ng/mL	1.00	1.00-1.01	0.21	-	-	-
Tumor number multiple *vs* single	2.45	1.09-5.54	0.03	1.14	0.46-2.83	0.78
Tumor size, cm	1.01	0.96-1.05	0.88	-	-	-
Vasopressor requirement	1.96	1.87-3.29	0.01	2.37	1.13-4.96	0.02
Treatment type						
Supportive care (control)	-	-	-	-	-	-
TACE/TAE	0.44	0.03-0.64	0.01	0.13	0.03-0.66	0.01
Surgery	0.15	0.24-0.80	< 0.01	0.41	0.21-0.79	< 0.01

Subjects, $n = 54$; event, patient death after treatment ($n = 47$). [1]Cox proportional hazards model. CTP: Child-Turcotte-Pugh classification; AFP: Alpha-fetoprotein; TACE: Transarterial chemoembolization; TAE: Transarterial embolization; INR: International normalized ratio.

overall survivals of patients with surgery or TAE were significantly higher than that in those with supportive care, especially in those in a hemodynamically stable state with a low serum bilirubin level, and good renal function. However, to date, there has been a dearth of reliable clinical evidence on the merits of surgical treatment versus those of TAE, in the context of survival benefit in patients with a spontaneous HCC rupture. Therefore, the present study may provide useful information for clinicians to determine the most appropriate treatment option for spontaneously ruptured HCC.

Ruptured HCC is a catastrophic disorder characterized by fatal complications, such as, coagulopathy, hemodynamic instability, or liver insufficiency. Thus, treatment should be considered carefully based on adequate information. As has been found in previous studies[13-15], surgical treatment was found to provide significant survival benefit as compared with supportive care in the current study. Furthermore, surgical group had relatively better hepatic function reserve, smaller tumors, and smaller numbers of tumors than the supportive care groups, although bias might have been introduced by selection for surgery. Nonetheless, the cumulative overall survival rate was higher in surgical group than in the supportive care group, and surgical treatment was found to be independent predictor of post-treatment survival by multivariate analysis. Although not all patients could have undergone surgery due to a poor hepatic function or an unstable vital status, surgical hemostasis can be considered if hepatic dysfunction or hemodynamic instability can be maximally corrected immediately after initial rupture of a hepatoma. Furthermore, post-surgical complications need to be considered before treatment decision-making despite the absence of an immediate severe complication after surgical intervention in the present study.

It has been reported that TAE is effective in achiev-

ing immediate hemostasis for ruptured HCC. In the present study, the overall survival rate was better in the TAE group than in the supportive care group. Advanced angiographic techniques enable the tumor location, active bleeding focus, and portal vein patency to be assessed, but life-threatening complications, such as, liver failure can developed after TAE, at rates ranging from 12% to 34%[12,17]. In the present study, post-TAE liver failure and technical failure for immediate hemostasis was observed in 6 (24%) and 5 (20%) patients of the TAE group, respectively, which suggests that TAE should be selectively administered in patients with good reserved hepatic function, tolerable coagulopathy, and a patent main portal vein.

In terms of comparison of survival benefits between the surgical and TAE groups, the current study failed to show a significant difference, although the cumulative overall survival rate tended to be higher in the surgical treatment group. Although TAE is less invasive than open surgical hemostasis, we suppose that open surgical treatment offer a higher chance of successfully achieving hemostasis by removing the bleeding focus or by allowing complete ligation of feeding artery. In previous studies, the 30-d mortality rate after TAE group has been reported to be lower than that after open surgical group[12,17,28]. However, in the present study, cumulative overall 1-mo survival rates were statistically similar between two groups. Moreover, at 1 mo after operative treatment, cumulative survival was clinically higher in the surgical group than in the TAE group despite the statistical insignificance (Figure 4). However, it should be borne in mind that this lack of significance may have been due to small patient numbers. Therefore, large number of patients who underwent surgical treatment need to be evaluated in the comparative study in the future.

Patients with a poor liver function reserve cannot tolerate surgical resection or aggressive angiographic intervention. Therefore, CTP class or MELD score, which reflect reserved hepatic function, could be important pretreatment factors. However, in the present study, of variables comprising the CTP class, only serum bilirubin was found to be independently associated with post-treatment survival. Likewise, of the variables comprising the MELD scores, only serum bilirubin and creatinine were found to be independent factors. Therefore, we estimated individual variables of CTP class or MELD score to avoid overestimation of the other factors in the multivariate analysis of post-treatment survival in patients with spontaneous HCC rupture.

Elevated serum creatinine and vasopressor requirements are likely to reflect multiorgan failure[29], and decreased effective circulating volume or the presence of superimposed infection may induce alterations in organ perfusion and in hemodynamic stability. Moreover, inflammatory reactions triggered by hepatocellular necrosis after HCC rupture may contribute to liver insufficiency, and subsequent multiorgan failure. Although decreased serum albumin level and a prolonged prothrombin time suggest reduced liver synthetic function, they can be corrected by albumin and coagulation factor replacement. On the other hand, serum bilirubin level cannot be rapidly and artificially corrected immediately after parenchymal liver damage. Therefore, serum bilirubin may be an independent factor of patient survival, unlike serum albumin or INR. Furthermore, the importance of serum bilirubin level in patients with HCC rupture has been previously reported[17,30]. However, during treatment decision-making, age, INR, albumin, and tumor size and number may also be clinically important variables despite their lack of statistical significance in the present study.

Our study has several limitations. First, the study is inherently limited by its retrospective study design. However, we enrolled all eligible patients. Second, the varied clinical and tumor statuses of patients and the critical disease status prevented randomization, and probably introduced bias. Furthermore, it takes long time to collect the prospective database of patients due to low incidence of the disease. Third, the absolute number of patients who underwent surgery was small, and therefore, there might be no significant difference in cumulative survival rate between surgery and TAE groups. Accordingly, we suggest a large-scale study be conducted to confirm our study.

In conclusions, the present study suggests that the post-treatment outcomes of surgery or TAE are better than that of supportive care in patients with spontaneous HCC rupture, and that surgical hemostasis might provide better survival benefit than TAE. However, we advise that serum bilirubin, creatinine, and hemodynamic status should be considered during treatment decision making. Regardless of its shortcomings, we believe that the present study would provide important information that aids decision making in patients with spontaneous HCC rupture.

COMMENTS

Background

Hepatocellular carcinoma (HCC) rupture is one of the life-threatening complications of HCC, and therefore, the most efficient treatment modality should be selected and rapidly applied to patients with ruptured HCC.

Research frontiers

Recently, survival benefit by transarterial embolization (TAE) has been reported. However, no definite recommendation has been issued regarding optimal treatment of HCC rupture, and the comparative survival benefits of surgery and TAE remain unclear.

Innovations and breakthroughs

The present study suggests that the post-treatment outcomes of surgery or TAE are better than that of supportive care in patients with spontaneous HCC rupture, and that surgical hemostasis might provide better survival benefit than TAE.

Applications

This study may provide useful information for clinicians to determine the most appropriate treatment option for spontaneously ruptured HCC.

Peer review

It is a rare situation specially for Western Europe, so it is good to know the experience of the group.

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BRIEF ARTICLE

Different regional distribution of *SLC25A13* mutations in Chinese patients with neonatal intrahepatic cholestasis

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Abstract

AIM: To investigate the differences in the mutation spectra of the *SLC25A13* gene mutations from specific regions of China.

METHODS: Genetic analyses of *SLC25A13* mutations were performed in 535 patients with neonatal intrahepatic cholestasis from our center over eight years. Unrelated infants with at least one mutant allele were enrolled to calculate the proportion of *SLC25A13* mutations in different regions of China. The boundary between northern and southern China was drawn at the historical border of the Yangtze River.

RESULTS: A total of 63 unrelated patients (about 11% of cases with intrahepatic cholestasis) from 16 provinces or municipalities in China had mutations in the *SLC25A13* gene, of these 16 (25%) were homozygotes, 28 (44%) were compound heterozygotes and 19 (30%) were heterozygotes. In addition to four well described common mutations (c.851_854del, c.1638_1660dup23, c.615+5G>A and c.1750+72_1751-4dup17insNM_138459.3:2667 also known as IVS16ins3kb), 13 other mutation types were identified, including three novel mutations: c.985_986insT, c.287T>C and c.1349A>G. According to the geographical division criteria, 60 mutant alleles were identified in patients from the southern areas of China, 43 alleles were identified in patients from the border, and 4 alleles were identified in patients from the northern areas of China. The proportion of four common mutations was higher in south region (56/60, 93%) than that in the border region (34/43, 79%, χ^2 = 4.621, P = 0.032) and the northern region (2/4, 50%, χ^2 = 8.288, P = 0.041).

CONCLUSION: The *SLC25A13* mutation spectra among the three regions of China were different, providing a basis for the improvement of diagnostic strategies and interpretation of genetic diagnosis.

Key words: Citrin deficiency; Mutation spectrum; Intrahepatic cholestasis; *SLC25A13*

Core tip: Genetic testing of *SLC25A13* gene was performed in individuals from southern, border and northern regions of China. The proportion of four common mutations was significant higher in southern region than in the border region and the northern region, so mutation screening for the common 4 mutations an appropriate test in the southern region. In the border and northern region, DNA sequencing is probably more practical.

Chen R, Wang XH, Fu HY, Zhang SR, Abudouxikuer K, Saheki T, Wang JS. Different regional distribution of *SLC25A13* mutations in Chinese patients with neonatal intrahepatic cholestasis. *World J Gastroenterol* 2013; 19(28): 4545-4551 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i28/4545.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i28.4545

INTRODUCTION

Citrin deficiency is estimated to be the most common urea cycle disorder in the world. It is an autosomal recessive disorder which includes adult-onset type Ⅱ citrullinemia (CTLN2; OMIM #60347)[1,2] and neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD; OMIM #605814)[3-5]. Most NICCD patients show symptoms which ameliorate by 1 year of age[6], but some patients may progress to liver failure and even require liver transplantation during infancy[7-10]. Others may develop CTLN2 more than a decade later[11,12]. Dietary treatment has shown to ameliorate symptoms and may prevent the need for transplant[13,14]. Therefore, prompt diagnosis and appropriate management are important for achieving a favorable long term prognosis for this disease.

Citrin deficiency is caused by mutations in the *SLC25A13* gene[1,15]. The protein product of the *SLC25A13* gene is citrin, a polypeptide of 675 amino acid residues with a molecular weight of 74 kDa. Citrin contains four EF-hand domains and six mitochondrial transmermbrane (TM)-spanning domains, and resides in the mitochondrial inner membrane[1]. Citrin is expressed in the liver and functions as calcium (Ca^{2+})-stimulated aspartate-glutamate carrier (AGC) for cytosolic glutamate and protons[16]. Over 60 different functional proved mutations in the human *SLC25A13* gene have been identified. These show significant differences in their racial distribution[13,17-20]. In China, the carrier frequency of 4 most common known mutations shows significant regional difference[19,20]. The estimated carries in population are 1/48 in south of the Yangtze River and 1/940 are carries of the river in the North[19].

Currently common mutation screening is used for rapid molecular diagnosis[21]. However, the appropriateness of use in specific populations needs to be established in that population. Therefore, we undertook the present study to investigate the regional distribution of *SLC25A13* mutations spectrum in Chinese patients with neonatal intrahepatic cholestasis. Our results will facilitate the design of appropriate screening strategies for this disorder in different regions.

MATERIALS AND METHODS

Subjects

Between June 2003 and December 2011, patients with cholestasis who were referred to the pediatric liver center of Children's Hospital of Fudan University for conjugated hyperbilirubinemia were enrolled. The inclusion criteria included the onset of conjugated jaundice before 6 mo of age; serum total bilirubin < 5 mg/dL and conjugated bilirubin > 1 mg/dL, or total bilirubin > 5 mg/dL and conjugated bilirubin > 20%[22]. We excluded other diseases that may affect the extrahepatic biliary system, such as biliary atresia, choledochal cyst, tumor, inspissated bile, or hemangioma, by imaging the hepatobiliary system. The imaging procedures included ultrasound scanning and hepatobiliary iminodiacetic acid (HIDA) scintigraphy in each case and laparotomic cholangiography in selected cases. Cases (n = 535) met the inclusion criteria and written informed consent was obtained from their parents.

The study protocol conforms to the ethical guidelines of the Declaration of the Helsinki of 1975 and was approved by the Ethics Committee on human research of the Children's Hospital of Fudan University.

Mutation identification

DNA was extracted from peripheral blood samples, which were obtained from each participant and his or her parents using the Tiangen Blood Genomic DNA Isolation Kit according to the manufacturer's instructions (Tiangen Biotech, Shanghai, China). Four common mutations (c.851_854del, c.1638_1660dup23, c.615+5G>A and c.1750+72_1751-4dup17insNM_138459.3:2667 also known as IVS16ins3kb)[1,20,23] were screened in all subjects. In patients for whom only one mutation was identified by the above screening or who had hyperaminoacidemia were subject to DNA sequencing as described previously[24]. Selection process of patients with mutant allele for analysis is given in Figure 1. The mutation alleles were verified in their parents by the target sequencing to establish segregation. Genomic sequences were obtained at the National Center for Biotechnology Information with RefSeq NM_014251.2 as *SLC25A13* reference. Nomenclature of *SLC25A13* variants was assigned following the guidelines of Human Genome Variation Society (http://www.hgvs.org/mutnomen)[25].

Geographical division

The population boundary between northern and southern China is drawn at the historical border of the Yangtze River during early Neolithic times (3000-7000 years ago)[26]. According to this criteria, Zhejiang, Jiangxi, Fujian, Guangdong, Hunan, Guizhou, Taiwan are classified as southern areas as they are south of Yangtze River; the provinces of Jiangsu, Shanghai, Anhui, Hubei, Sichuan and Chongqing are classified as border areas as they are in the basin of the Yangtze River; and the provinces of Henan, Liaoning, Shanxi, Jilin, Shandong, Hebei and Ningxia are classified as northern areas as they are in north of Yangtze River (Figure 2).

Patients with at least one mutated *SLC25A13* allele were selected. To calculate the mutation spectra, the mutations observed in the related family members were counted only once. If the patient was a heterozygote or a compound heterozygote and the parents were from

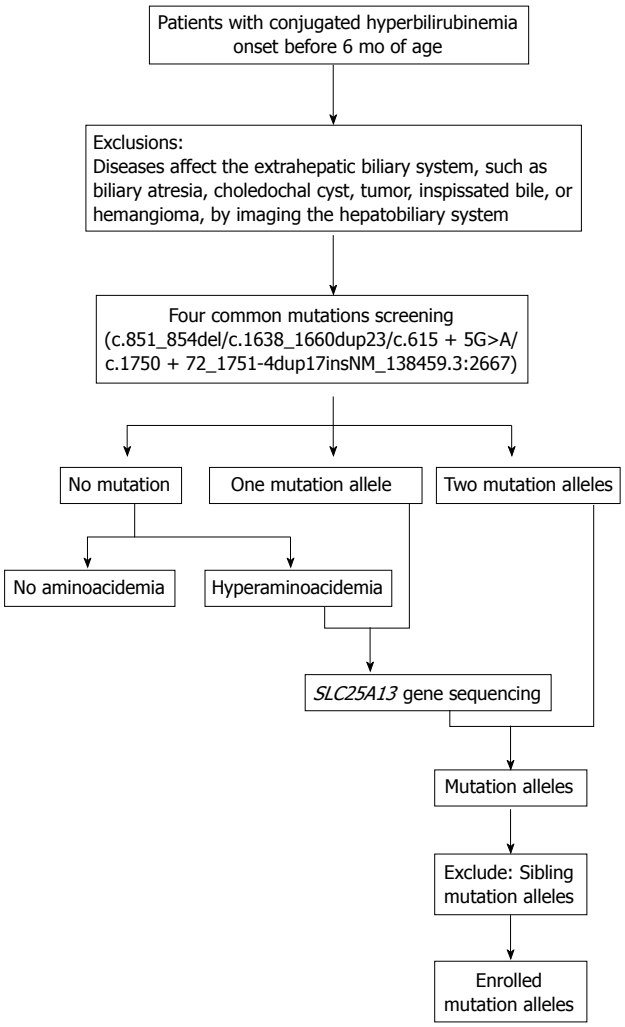

Figure 1 Selection process of patients with mutant allele for analysis.

different regions, the parent's sample was tested to determine the origin of the allele(s). Except for two patients who were born from consanguineous parents, all other infants were to our knowledge unrelated.

Homology and structural predictions

The homology between the mutated Citrin protein and the human reference, as well as Citrin from other species, were surveyed using Clustal X software (European Bioinformatics Institute, Hinxton, Saffron Walde, United kingdom). PolyPhen-2 (Polymorphism Phenotyping version2.2.2), which is available at http://genetics.bwh.harvard.edu/pph2/, was used to predict the possible impact of an amino acid substitution on the structure and function of the Citrin protein. MutationTaster was used to evaluate the disease-causing potential of sequence alterations, at http://mutationtaster.org/MutationTaster/index.html. A *P* value close to 1 indicates a high 'security' of the prediction. MutationTaster employs a Bayes classifier to eventually predict the potential of an alteration causing disease. The Bayes classifier is fed with the outcome of all tests and the features of the alterations and calculates probabilities for the alteration to be either disease causing or not.

Statistical analysis

Statistical tests on the distribution of mutant genotypes in the three areas of China were assessed by performing a $2 \times 2\ \chi^2$ test with the SPSS version 17.0 software (University of Chicago, Chicago, IL, United States) package. A P value < 0.05 was considered to be statistically significant. When there are small expected values in the 2×2 table, the result of fisher's exact test was used.

RESULTS

General information

Among the 535 patients, 183 originated from the southern area, 291 were from the border area and 61 were from the northern area. Sixty-nine patients with at least one *SLC25A13* gene mutation were found, including 6 sibling pairs. These sixty-three unrelated patients, including 25 females and 38 males, were further analyzed. Sixteen (25%) were found to be homozygotes for one mutation, 28 (44%) were compound heterozygotes and 19 (30%) heterozygotes for only one mutation. The distribution of carriers according to the state of origin is depicted in Figure 2.

Mutation types

A total of 17 mutations, including 14 mutations that had been previously reported by us and others (c.851_854del, c.1638_1660dup23, c.615+5G>A, c.1750+72_1751-4dup17insNM138459.3:2667, c.1019_1177del, c.1801G>A, c.550C>T, c.1078C>T, c.955C>T, c.1754G>A, c.775C>T, c.1092_5delT, c.615+1G>A, c.254T>C)[17,20,24,27-30] and 3 novel mutations (c.985_986insT, c.287T>C, c.1349A>G),were observed in the present investigation (Table 1).

Analysis of 3 previously unreported variants

The c.287T>C mutation in exon 4 is predicted to result in the substitute of phenylalanine to serine at position 96 (p.F96S). This mutation was found in a compound heterozygote state with the mutations c.851_854del. p.F96S is located between the second and third EF-hand domain, which is highly conserved in different species (Table 2). The Polymorphism Phenotyping for the variant amino acid p.F96S from Polyphen 2 is 1.000, indicating that the missense mutation has a high chance of affecting protein function. The *P* value from MutationTaster is 0.997, suggesting that is most likely a disease-causing mutation.

Mutations c.985_986insT and c.1349A>G were found in compound heterozygote state in a patient. The mutation c.985_986insT was found to be derived from this patient's paternal allele and predicted to result in a frame shift and the introduction of a premature stop codon at position 372. Mutation c.1349A>G (p.E450G) was derived from the patient's maternal allele, which is located in the loop between the TM3 and TM4 spanning regions. Conservation analysis in different species indicated that the amino acid in this position is highly conserved (Table 2). The Polymorphism Phenotyping for the variant amino

Figure 2 Distribution of mutant alleles enrolled in this study. As shown in the map, the provinces were separated into three parts by the Yangtze River. The numbers in parentheses are the number of mutation c.851_854del/c.1638_1660dup23/c.615+5G>A/c. 1750+72_1751-4dup17ins NM_138459.3:2667/other alleles.

Table 1 Regional distribution of mutant *SLC25A13* alleles and frequencies in China *n* (%)

Mutation	South	Border	North	Nucleotide change	Protein change	Ref.
Common	56 (93)	34 (79)[1]	2 (50)[2]			
851del4	41 (68)	22 (51)	2 (50)	c.851_854del	p.M285fsX286	Kobayashi *et al*[1]
1638ins23	9 (15)	4 (9)		c.1638_1660dup23	p.A554fsX570	Kobayashi *et al*[1]
IVS6+5G>A	2 (3)	4 (9)		c.615 + 5G>A	-	Saheki *et al*[23]
IVS16ins3kb	4 (7)	4 (9)		c. 1750+72_1751-4dup17ins NM_138459.3:2667	p.A584fsX585	Tabata *et al*[20]
Other	4 (7)	9 (21)	2 (50)			
IVS11+1G>A			1 (25)	c.1019_1177del	-	Kobayashi *et al*[1]
E601K	1 (2)	1 (2)		c.1801G>A	p.E601K	Yamaguchi *et al*[17]
R184X		1 (2)		c.550C>T	p.R184X	Lu *et al*[19]
R360X			1 (25)	c.1078C>T	p.R360X	Tabata *et al*[20]
R319X		1 (2)		c.955C>T	p.R319X	Song *et al*[29]
IVS6+1G>A		2 (5)		c.615+1G>A	-	Fu *et al*[24]
L85P		1 (2)		c.254T>C	p.L85P	Fu *et al*[24]
R585H		1 (2)		c.1754G>A	p.R585H	Song *et al*[28]
1092_5delT	1 (2)			c.1092_5delT	p.R319X	Fu *et al*[24]
Q259X		1 (2)		c.775C>T	p.Q259X	Wen *et al*[30]
985insT	1 (2)			c.985_986insT	p.A329fsX372	Present study
F96S		1 (2)		c.287T>C	p.F96S	Present study
E450G	1 (2)			c.1349A>G	p.E450G	Present study

Allele counts for the mutations are given together with their relative frequencies expressed as percentage value (in brackets). Novel mutations found in this study are indicated by bold letters. Variation in allele proportion (counts) between the three regions: $^1\chi^2 = 4.621$, $P = 0.032$, common mutation in south *vs* in border; $^2\chi^2 = 8.288$, $P = 0.041$, common mutation in south *vs* in north. GenBank reference sequences were NT_079595 and NM_014251.2.

acid p.E450G was 1.000, indicating a high chance of affecting protein function. The *P* value from MutationTaster is more than 0.999, suggesting that the mutation might affect the protein's features.

Distribution of SLC25A13 gene mutations

The distribution of *SLC25A13* mutations in carriers originating from different regions of China is given in Figure 1. Sixty (56%) mutant alleles originated from the southern region with Zhejiang, Jiangxi, Fujian, Guangdong, Hunan, Guizhou, and Taiwan accounting for 27, 15, 9, 1, 4, 3 and 1 mutant allele, respectively. Forty-three mutant alleles (40%) originated from the border region, with Jiangsu, Shanghai, Anhui, Hubei, Sichuan, and

Table 2 Conservation analysis of *SLC25A13* gene mutations F96S and E450G among different species

F96S																						
Human	L	C	A	P	D	A	L	F	M	V	A	F	Q	L	F	D	K	A	G	K	G	E	V
Canis	L	C	A	P	D	A	L	F	M	V	A	F	Q	L	F	D	K	A	G	K	G	E	V
Bos	L	C	A	P	D	A	L	F	M	V	A	F	Q	L	F	D	K	A	G	K	G	E	V
Equus	L	C	A	P	D	A	L	F	M	V	A	F	Q	L	F	D	K	A	G	K	G	E	V
Pan	L	C	A	P	D	A	L	F	M	V	A	F	Q	L	F	D	K	A	G	K	G	E	V
Mus	L	C	A	P	D	A	L	F	M	V	A	F	Q	L	F	D	K	A	G	K	G	E	V
Gallus	L	C	A	P	D	A	L	F	M	V	A	F	Q	L	F	D	K	A	G	K	G	E	V
Xenopus	L	C	A	P	D	A	L	F	M	V	A	F	Q	L	F	D	K	A	G	K	G	E	V
E450G																							
Human	G	G	S	Q	V	I	F	T	N	P	L	E	I	V	K	I	R	L	Q	V	A	G	E
Canis	G	G	S	Q	V	I	F	T	N	P	L	E	I	V	K	I	R	L	Q	V	A	G	E
Bos	G	G	S	Q	V	I	F	T	N	P	L	E	I	V	K	I	R	L	Q	V	A	G	E
Equus	G	G	S	Q	V	I	F	T	N	P	L	E	I	V	K	I	R	L	Q	V	A	G	E
Pan	G	G	S	Q	V	I	F	T	N	P	L	E	I	V	K	I	R	L	Q	V	A	G	E
Mus	G	G	S	Q	V	I	F	T	N	P	L	E	I	V	K	I	R	L	Q	V	A	G	E
Gallus	G	M	C	Q	V	V	F	T	N	P	L	E	I	V	K	I	R	L	Q	T	A	G	E

Chongqing accounting for 14, 6, 12, 4, 5 and 2, respectively. In the northern region, only four mutant alleles (4%) were found, including 1 from Henan, 1 from Liaoning and 2 from Shanxi provinces.

The *SLC25A13* mutation spectra among the three regions of China presented significant differences. The four common mutations exhibited maximal relative frequencies in the southern China (56/60, 93%), and four other mutations were detected, including two novel mutations. On the contrary, in the area bordering the Yangtze River, there was a wide range of mutation types. The four common mutations accounted for 79% (34/43) of the mutant alleles, and eight other mutation types were found, including one novel mutation. In northern China the mutation c.851_854del accounted for 50% (2/4) of the mutant alleles. The other two mutations were c.1019_1177del and c.1078C>T. The proportion of the four common mutations was higher in south region (93%) than that in the border region (79%, χ^2 = 4.621, P = 0.032) and that in the north (50%, χ^2 = 8.288, P = 0.041) (Table 1).

Among the four common mutations, c.851_854del was the most. Proportion accounts for 68% (41/60) in southern region, 51% (22/43) in the border region and 50% (2/4) in the northern region. The difference of the proportion between the southern and border part of China was marginally significant (χ^2 = 3.109, P = 0.078).

DISCUSSION

The mutation spectra for the *SLC25A13* gene differ within the Asian population[18-20]. A different carrier rate for the common mutations between different parts of China has been reported[19,30]. Here we demonstrate that the mutation spectrum of *SLC25A13* gene varies considerably among specific regions of China with common mutations having a higher proportion in the southern region than in the border and northern regions.

In the southern region of China, four common mutations accounted for 93% and c.851_854del is the predominant mutation accounting for 68%. This is consistent with the published data[19,30]. The c.851_854del mutation is a common ancestral mutation, and the frequency difference between various regions of China may be associated with ancient migration.

The mutations found in patients from the border region exhibited significant variety. The total mutant allele number in patients from the border was less than that in patients from the southern region (43 *vs* 60), but the mutation types were much greater than that in the southern area (12 *vs* 8). In total, seven private mutations were found in patients from the border, compared with three private mutations found in patients from the southern region. This divergence may reflect the ethnic diversity of this area. Previously, the data on *SLC25A13* mutations in this region were very limited. This study is the first paper providing an estimate for the border region and significantly increases the data on southern and northern China. Considering the high proportion of uncommon mutations, the previously reported *SLC25A13* mutation carrier rate in this region may be underestimated and sequencing may be a prefect method for testing.

The c.851_854del mutation was the only one of the four common mutations detected in the northern region. The explanation for this may be that other common mutations are rare in that part of China. Two mutant c.851_854del alleles were found among the 4 known mutant alleles, suggesting mutation 851del4 is the frequent mutation in this region.

Since variants c.287T>C and c.1349A>G have not undergone functional testing, so we analyzed the data without these and statistical significance was reached even when those two variants were removed from the analysis. The proportion of the four common mutations was also higher in southern region (95%) than the border (81%, χ^2 = 4.929, P = 0.048) and the northern region (50%, χ^2 = 10.343, P = 0.029). Thus the primary conclusion remains valid.

This paper is the first study conducted the *SLC25A13* mutation spectrum in neonatal intrahepatic cholestasis

from different parts of China. The previous study evaluated the population frequency for the common mutations and conducted that the carrier frequency in China is 1/79-1/65[17,19]. Conversely only 94% (59/63) of cases with suspected citrin deficiency in our study had the common mutations. This suggests that point mutation testing alone is not sufficient to exclude citrin deficiency even in cases from the southern region but may be a cost effective way of confirming the diagnosis as the first step.

There were limitations of this study. Firstly, only a small number of patients came from the north of the Yangtze River, and only limited cases were reported from that area, so the sampling bias needs to be considered. The current literature has not shown a significant difference between *SLC25A13* mutation types and the phenotype observed, so the smaller sample size is not likely to lead to referral bias in favor of null or missense mutations in this study. Secondly, for 19 of the 126 alleles, we could not find any mutations. One possible explanation could be that the patients with one detected mutant allele are carriers and this may be a risk factor for cholestasis or they may have an alternate cause for cholestasis. Alternatively, as previously described[31,32] they may have a second mutation not detected by Sanger sequencing or the targeted test for the IVS16ins3kb rearrangement such as intronic mutations or large rearrangements.

In conclusion, the mutation spectra of the *SLC25A13* gene are significantly different among patients with neonatal intrahepatic cholestasis from different parts of China. These differences should be considered when establishing a molecular diagnostic strategy or interpreting their results.

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COMMENTS

Background

SLC25A13 gene mutations lead to Citrin deficiency, which includes adult-onset type Ⅱ citrullinemia and neonatal intrahepatic cholestasis caused by citrin deficiency. The carrier frequency is high in Asian populations, and the mutation spectrum of *SLC25A13* gene in Chinese population (most came from southern China) was found to be different from that of other population groups in East Asia.

Research frontiers

The mutant alleles of *SLC25A13* gene was conducted in southern, border and northern regions of China, providing a basis for the improvement of diagnostic strategies and interpretation of genetic diagnosis.

Innovations and breakthroughs

The proportion of four common mutations was higher in the southern region (56/60, 93%) than that in the border region (34/43, 79%, χ^2 = 4.621, P = 0.032) and the northern region (2/4, 50%, χ^2 = 8.288, P = 0.041). Three novel mutations were found, which has expanded the *SLC25A13* mutation spectrum.

Applications

The mutation spectra of the *SLC25A13* gene are significantly different among patients with neonatal intrahepatic cholestasis from different parts of China. These differences should be considered when establishing a molecular diagnostic strategy or interpreting their results.

Terminology

An allele is a single copy of a gene. For autosomal genes, an individual inherits two alleles at each locus, with one from each parent. Genotypes are described as homozygous if the two alleles are the same and as heterozygous if the alleles are different. The mutant allele is the mutated form of a gene.

Peer review

This is a retrospective study aimed at investigating the regional distribution of *SLC25A13* mutations in Chinese patients with neonatal intrahepatic cholestasis. The topic is relevant, since biochemical diagnosis of citrin deficiency is not widely available and mutation analysis of the *SLC25A13* gene is crucial to diagnosis. The study was well-conducted and the manuscript is reasonably well written with good scientific value.

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BRIEF ARTICLE

Efficacy of capecitabine and oxaliplatin regimen for extrahepatic metastasis of hepatocellular carcinoma following local treatments

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Abstract

AIM: To investigate the efficacy and safety of capecitabine and oxaliplatin (CapeOx) for extrahepatic metastasis after local treatment of hepatocellular carcinoma (HCC).

METHODS: Thirty-two patients with extrahepatic metastasis of HCC after local treatment were prospectively enrolled. The CapeOx regimen consisted of capecitabine 1000 mg/m^2 taken orally twice daily on days 1-14, and oxaliplatin was administered at a total dose of 100 mg/m^2 on day 1. The treatment was repeated every 3 wk until disease progression or unacceptablle toxicity. Efficacy and safety were assessable for all enrolled patients. The primary objective of this study was to assess the overall response rate. The secondary objectives were to evaluate the overall survival (OS), the time to tumor progression (TTP) and the toxicity profile of the combined strategy. TTP and OS were assessed by the Kaplan-Meier method and differences between the curves were analyzed using the log-rank test. The statistical software SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, United States) was used for statistical analysis. All *P* values were 2-tailed, with statistical significance defined by $P \leqslant 0.05$.

RESULTS: Thirty-two patients were assessable for efficacy and toxicity. The median follow-up duration was 15 mo (range, 12-20 mo). At the cut-off date of March 31, 2012, 27 patients died due to tumor progression and one patient died of myocardial infarction. Four patients were still alive (three patients with disease progression). OR was 21.9% (n = 7), the stabilization rate was 40.6% (n = 13), and the disease control rate was 62.5%. The responses lasted from 4 to 19 mo (median, 6 mo). Median TTP was 4.2 mo (95%CI: 2.5-7.4), and the median OS time was 9.2 mo (95%CI: 6.5-17.8). The 1-year survival rate was 43.6% (95%CI: 29.0-66.0). In a multivariate analysis, OS was significantly longer in patients with a Child-Pugh class A compared with class B patients (P = 0.014), with a median OS of 10.1 mo *vs* 5.4 mo, and there were trends towards longer OS (P = 0.065) in patients without portal vein tumor thrombosis. There were no significant effects of age, gender, performance status, cirrhosis, metastatic sites, and level of alpha fetoprotein (AFP) or hepatitis B virus-DNA on OS. Among the 22 patients with elevated AFP levels at baseline ($\geqslant$ 400 ng/mL), the level fell by more than 50% during treatment in 6 patients (27.3%). The most frequent treatment-related grade 3 to 4 toxicities included leucopenia/neutropenia, transient elevation of aminotransferases, hand-foot syndrome and fatigue.

CONCLUSION: CapeOx showed modest anti-tumor activity in metastatic HCC. However, the manageable

toxicity profile and the encouraging disease control rate deserve further study for these patients.

Key words: Hepatocellular carcinoma; Extrahepatic metastasis; Capecitabine; Oxaliplatin; Local treatments

Core tip: Distant metastases are still obstacles in improvement of outcome in hepatocellular carcinoma (HCC) patients after local treatment. Although, sorafenib is used as a standard systemic treatment for those patients, it is not suitable for patients with intermediate HCC who were not eligible to or failed in the locoregional therapy. This study reports the capecitabine and oxaliplatin regimen for extrahepatic metastasis after local treatment of HCC. The objective response rate was 21.9%, and 40.6% of patients had stable disease, and the median overall survival and the time to tumor progression were 4.2 and 9.2 mo, respectively. Furthermore, the result of this study showed that toxicity profile was tolerated well.

He SL, Shen J, Sun XJ, Zhu XJ, Liu LM, Dong JC. Efficacy of capecitabine and oxaliplatin regimen for extrahepatic metastasis of hepatocellular carcinoma following local treatments. *World J Gastroenterol* 2013; 19(28): 4552-4558 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i28/4552.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i28.4552

INTRODUCTION

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths worldwide, with the incidence on the rise[1]. The overall 5-year survival rate for all HCC patients has remained no more than 5%[2]. Surgical resection, local ablation, transarterial chemoembolization (TACE) and liver transplantation are the mainstay of treatment of localized HCC, but local recurrence and distant metastasis are still obstacles in the further improvement of outcome in HCC patients after local treatments. Sorafenib, a small molecule multikinase inhibitor, was the first systemic agent used to prolong survival of patients with advanced HCC, as demonstrated in two phase Ⅲ trials and it is now the reference standard for systemic treatment of these patients[3,4]. However, its efficacy and safety have not been demonstrated in patients with poor liver function (Child-Pugh class B)[5]. Moreover, patients with extrahepatic metastasis had a greater risk of death than those with intrahepatic disease treated by sorafenib[6]. Systemic treatment with oral targeted therapy may be lifelong and expensive. In addition, sorafenib is not covered in the scope of health insurance for advanced HCC in China. Therefore, systemic treatment options remain to be defined in patients with extrahepatic metastasis of HCC after local treatments.

Capecitabine is a rationally designed, orally administered, tumor-selective fluoropyrimidine that mimics continuous infusion of 5-fluorouracil (5-FU). Capecitabine was found to be safe in patients with cirrhosis and provided an 11% response rate (RR) including radiologically confirmed complete response (CR) in one patient[7]. Oxaliplatin has consistently shown preclinical and clinical anti-tumor activity against gastrointestinal cancers. In metastatic colorectal cancer, oxaliplatin in combination with 5-FU resulted in response rates of 20%-50% and median progression-free survival (PFS) of approximately 7.5-9.0 mo in randomized trials[8]. A phase Ⅲ study of 5-FU/oxaliplatin conducted in Asian patients suffering from inoperable or metastatic HCC showed the feasibility and demonstrated its superior efficacy compared with doxorubicin[9].

Response evaluation for intrahepatic lesions in patients with advanced HCC is difficult because of variability of both tumor growth pattern and results of previous local treatments including TACE, ablation or radiation therapy[10]. Therefore, this study selected advanced HCC patients with at least one measurable extrahepatic metastatic lesion. The regimen of capecitabine and oxaliplatin (CapeOx) for patients with extrahepatic metastatic HCC was based on (1) the synergy of these two drugs in patients with advanced or metastatic solid tumors[11]; (2) the regimen of oxaliplatin and 5-FU with a manageable toxicity profile in cirrhotic Child-Pugh class A-B or liver transplanted patients[12]; (3) the clinical activity and favorable toxicity profile of capecitabine alone and in combination with oxaliplatin in advanced or metastatic colorectal cancer[13,14]; (4) no dose adjustment required for capecitabine and oxaliplatin due to hepatic dysfunction[15]; and (5) the feasibility and efficacy of CapeOx alone or in combination with antiretroviral therapy in patients with human immunodeficiency virus- (and hepatitis C virus-co-) infection and HCC[16]. This study aims to evaluate the efficacy and safety of CapeOx regimen in patients with extrahepatic metastasis following local treatment.

MATERIALS AND METHODS

Patients

From March 2009 to March 2012, we enrolled 32 patients with extrahepatic metastasis. Eligibility criteria included the following: (1) initially received surgery, thermal ablation, TACE or TACE combined with radiotherapy; (2) at least one measurable extrahepatic lesion; (3) no previous systemic treatment; (4) World Health Organization (WHO) performance status (PS) 0-2; (5) Child-Pugh class of A or B; and (6) age between 18-70 years and adequate bone marrow, renal and hepatic function (absolute neutrophil count $\geqslant 1.5 \times 10^9$/L and platelet count $\geqslant 80 \times 10^9$/L; serum creatinine $\leqslant$ 1.5 mg/dL; aspartate aminotransferase and alanine aminotransferase $\leqslant 2.5 \times$ upper limits of normal; total bilirubin $\leqslant 1.5 \times$ upper limits of normal). Study entry required a complete medical history, physical examination, complete blood cell with a differential count, biochemistry panel, and a coagulation panel

and serum alpha-fetoprotein (AFP), chest or abdominal computed tomography (CT) scan or magnetic resonance imaging (MRI). Main exclusion criteria were Child-Pugh class C, previous systemic treatment, central nervous system metastases, severe cardiac and/or respiratory failure, concurrent malignancy, and baseline sensitive peripheral neuropathy; pregnant or lactating females. This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. The study was approved by the local ethics committee. Informed consent was obtained from all participants.

Before registration, complete blood cell and platelet counts were examined weekly, and physical examination, biology [serum alpha-fetoprotein (AFP), transaminases, alkaline phosphatases, bilirubin, lactate dehydrogenase, γ-glutamyltransferase, albumin, prothrombin time, and creatinine], and safety assessments were performed before each cycle of chemotherapy. Analysis of AFP level and tumor assessment by CT scan or MRI were undertaken every two cycles. Objective response (OR) was confirmed by a second evaluation 4 wk later. Objective and discordant responses were reviewed by an independent radiologist. Treatment was discontinued because of either disease progression and unacceptable toxicity, or patient's refusal. Other treatments were proposed in the event of disease progression.

Treatment protocol

CapeOx regimen was administered in a 3-wk cycle. In each cycle, oxaliplatin (ELOXATIN®, Sanofi-Aventis, Hangzhou, China) was administered at a total dose of 100 mg/m^2 as a 2-h *iv* infusion on day 1, and capecitabine (XELODA®, Shanghai Roche Shanghai, China) 1000 mg/m^2 was taken orally twice daily (total daily dose 2000 mg/m^2) on days 1-14. Hepatitis B surface antigen positive patients were treated with lamivudine (HEPTODIN®, GlaxoSmithKline, Suzhou, China) 100 mg/d before the first CapeOx cycle to prevent severe hepatitis during treatment. All patients with bone metastases received bisphosphonates treatment once a month. Depending on the severity of side effects, chemotherapy was paused or the dose was reduced. A 20% dose reduction was required based on predefined criteria. Briefly, capecitabine dose was reduced by 20% due to recurrence of grade 3 or 4 diarrhea or hand/foot syndrome. Oxaliplatin dose was reduced by 20% in case of grade 1 or 2 peripheral neuropathy, whereas in case of grade 3 or 4 neuropathy (defined as permanent functioning discomfort), oxaliplatin was discontinued and capecitabine was administered alone as initially scheduled. Patients were considered assessable for toxicity if they had received a minimum of one cycle of treatment.

Assessment of responses

Baseline evaluation included physical examination, assessment of medical history, evaluation of performance status, and blood counts. During treatment, patients were evaluated before each cycle of therapy with the above parameters. Response was assessed after every two cycles of chemotherapy by CT scan or MRI using the Response Evaluation Criteria in Solid Tumors 1.1 (RECIST 1.1) criteria[17]. CR was defined as the disappearance of all target and non-target lesions compared to baseline. Partial response (PR) was defined as at least a 30% decrease in the longest diameters of all target lesions, taking as a reference the baseline sum of the diameters with no new lesions appearing. Patients were considered to have progressive disease (PD) if any new lesions appeared, if the tumor size increased by at least 20% in the diameters of the target lesions, taking as reference the smallest sum on study, or if there was unequivocal progression of existing non-target lesions. A patient who did not meet the definition of CR, PR or PD was classified as having stable disease. The percentage of patients who had the best responses (other than PD) according to the RECIST 1.1 criteria, and had those responses maintained for at least 28 d after the first radiologic evaluation, was defined as the disease control rate (DCR). AFP and hepatitis B virus (HBV)-DNA levels were determined every 2 mo. Body weight, PS, and symptoms were recorded before each cycle. Toxic effects of chemotherapy were evaluated according to the National Cancer Institute-Common Terminology Criteria for Adverse Events version 3.0. This specific scale was used to assess oxaliplatin neurotoxicity[18].

Statistical analysis

The primary objective of this study was to assess the overall response rate. The secondary objectives were to evaluate the overall survival (OS), the time to tumor progression (TTP) and the toxicity profile of the combined strategy. TTP was the interval from the starting date of therapy to the date of progression; OS was defined as the time interval between the first cycle of chemotherapy and death due to any cause or the last clinical follow-up. TTP and OS were assessed by the Kaplan-Meier method and differences between the curves were analyzed using the log-rank test. For the statistical analysis, the statistical software SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, United States) was used. All P values were 2-tailed, with statistical significance defined by $P \leqslant 0.05$.

RESULTS

Thirty-two patients (21 men and 11 women) were enrolled between March 2009 and March 2012. Median age of the patients was 59 years (range 19-70 years). Chronic HBV infection was the most common etiology of underlying liver disease (23 patients, 71.9%). Two patients (6.3%) had a history of alcohol abuse. Twenty-two (68.8%) patients belonged to Child-Pugh class A and 10 (31.2%) to Child-Pugh class B. Cirrhosis was present in 12 patients (37.5%), and 22 patients (68.8%) had serum AFP (≥ 400 ng/mL). Four patients received curative HCC resection and 19 patients were treated with TACE, 4 patients were treated by TACE combined with radiotherapy after diagnosis. Five patients underwent ablation of HCC. Patients' other baseline characteristics are summarized in Table 1.

Table 1 Patient and tumor characteristics at baseline (n = 32)

Characteristics	Patients, n (%)
Age (yr), median (range)	56 (19-70)
Gender	
Male	21 (65.6)
Female	11 (34.4)
ECOG performance status	
0	16 (50)
1	11 (34.4)
2	5 (15.6)
Underlying liver disease	
HBV	23 (71.9)
Alcohol	2 (6.3)
Other	7 (21.8)
Prior therapy	
Surgery	4 (12.5)
Ablation	5 (15.6)
TACE	19 (59.4)
TACE + radiotherapy	4 (12.5)
Child Pugh score	
A	22 (68.8)
B	10 (31.2)
Cirrhosis	
No	20 (62.5)
Yes	12 (37.5)
HBV-DNA	
< 1.0e3 cps/mL	16 (69.6)
⩾ 1.0e3 cps/mL	7 (30.4)
Portal vein thrombosis	
No	25 (78.1)
Yes	7 (21.9)
Median AFP (ng/mL)	
⩾ 400	22 (68.8)
< 400	10 (31.2)
Metastasis	
Lung	9 (28.1)
Bone	6 (18.8)
Adrenal gland	9 (28.1)
Lymph node	3 (9.4)
Peritoneum	3 (9.4)
Other	2 (6.2)

ECOG: Eastern Cooperation Oncology Group; HBV: Hepatitis B virus; TACE: Transarterial chemoembolization; AFP: Alpha fetoprotein.

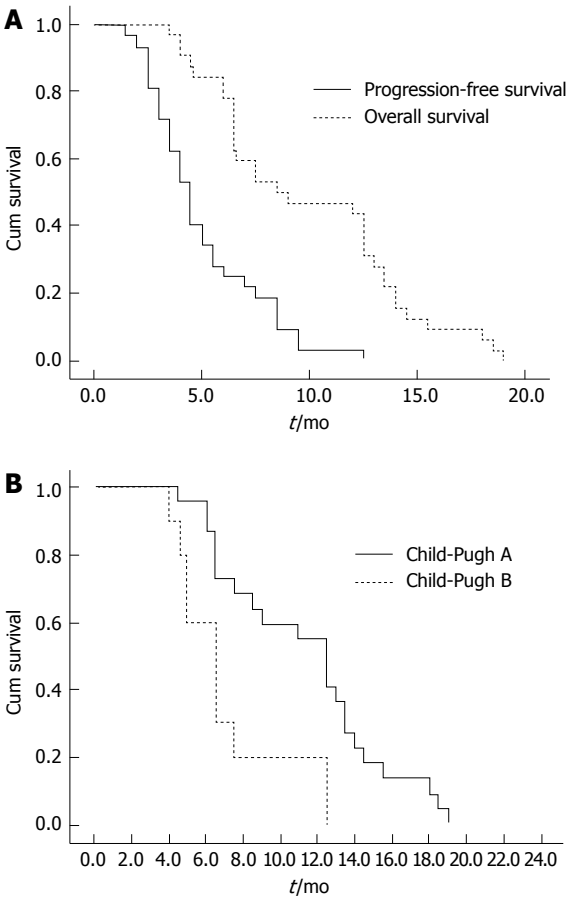

Figure 1 Kaplan-Meier estimation. A: Progression-free survival and overall survival (n = 32); B: Overall survival by Child-Pugh class group (n = 32).

In total, 142 cycles of CapeOx were administered, with a median of four cycles (range 1-9 cycles) per patient. Dose reductions including oxaliplatin in 9/32 patients (28.1%) were due to grade 1/2 toxicities, and capecitabine in 9/32 patients (28.1%) because of grade 3/4 toxicities. Oxaliplatin was discontinued in 2 patients with grade 3 neurotoxicity. Thirty-two patients were assessable for efficacy and toxicity. The median follow-up duration was 15 mo (range 12-20 mo). At the cut-off date of March 31, 2012, 27 patients died due to tumor progression and one patient died of myocardial infarction. Four patients were still alive (3 patients with disease progression). OR was 21.9% (n = 7), the stabilization rate was 40.6% (n = 13), and the DCR was 62.5%. The responses lasted 4-19 mo (median, 6 mo). Median TTP was 4.2 mo (95%CI: 2.5-7.4), and the median OS time was 9.2 mo (95%CI: 6.5-17.8; Figure 1A). The 1-year survival rate was 43.6% (95%CI: 29-66). In a multivariate analysis, OS was significantly longer in patients with a Child-Pugh class A compared with class B patients (P = 0.014), with a median OS of 10.1 mo *vs* 5.4 mo (Figure 1B), and there were trends towards longer OS (P = 0.065) in patients without portal vein tumor thrombosis. There were no significant effects of age, gender, PS, cirrhosis, metastatic sites, and level of AFP or HBV-DNA on OS (data not shown). Among the 22 patients with elevated AFP levels at baseline (⩾ 400 ng/mL), the level fell by more than 50% during therapy in 6 patients (27.3%). Moreover, 2 of the 5 patients whose initial PS was equal to 2, improved to 1 after two cycles of treatment. Three of 23 patients treated with lamivudine therapy switched to entecavir therapy because the level of HBV-DVA had exceeded the baseline level (⩾ 1.0e3 cps/mL) during treatment.

Safety

Toxicities are summarized in Table 2. Treatments were generally well tolerated in the majority of patients, and there were no treatment-related deaths. Thirty-two patients were assessable in toxicity. Grade 3-4 toxicity occurred in 11 patients (34.4%). Hematologic toxicity was the most common severe toxicity, including thrombocytopenia (6.3%; no bleeding events) and neutropenia (6.3%; fever in only one case). Grade 3 neurotoxicity was the most common severe non-hematologic toxicity, affecting 2 patients (6.39%), whereas grades 1 and 2 neurotoxicity

Table 2 Treatment-related toxicities in 32 patients

Adverse event	Grade 1	Grade 2	Grade 3	Grade 4
Neutropenia	2	2	1	1
Thrombocytopenia	4	3	1	1
Anemia	5	1	0	0
Nausea/vomiting	6	3	1	0
Mucositis	4	2	0	0
Stomatitis	3	2	1	0
Diarrhoea	0	1	1	0
Transaminases	7	3	1	0
Hyperbilirubinemia	2	0	0	0
Neurotoxicity	5	8	2	0
Hand-foot syndrome	4	2	1	0

Hepatitis B virus DNA level (real-time polymerase chain reaction, Abbott, Wiesbaden, Germany).

occurred in 6 (18.8%) and 3 (9.4%) patients, respectively.

Additional treatments

Nine patients had received additional treatments due to tumor progression. Six patients with bone metastasis received local palliative radiotherapy, and three patients received sorafenib therapy.

DISCUSSION

Sorafenib is currently considered standard of care systemic therapy for patients with advanced HCC. The use of sorafenib is based on phase Ⅱ and phase Ⅲ data in patients with metastatic HCC, with the treatment group showing close to a 3-mo survival advantage over the non-treated group in Child-Pugh class A[3,19]. In contrast, Child-Pugh class B patients did not seem to derive any benefit from sorafenib in phase Ⅲ trials[5,20]. Similarly, in a series of Asian patients, only patients with a score of B7 seemed to benefit from sorafenib, at the cost of higher rates of bleeding events[21]. National Institute for Health and Clinical Excellence does not recommend sorafenib for patients with advanced hepatocellular carcinoma, because it does not provide enough benefit to patients to justify its high cost[22]. In addition, the results of SOFIA study showed that only dose-adjusted, but not full-dose sorafenib was a cost-effective treatment compared to best supportive care in intermediate and advanced HCC. There was no cost-effective treatment for patients with intermediate HCC who were not eligible to or failed locoregional therapy even if they were treated with dose-adjusted sorafenib[23].

The survival rates of HCC patients have risen greatly concomitant with the progress in diagnostic and treatment methods. However, the survival prognosis for treatment-resistant progressive liver cancers is extremely poor[24]. Although surgical resection was used as treatment for pulmonary metastasis from HCC, the treatment might be only beneficial for patients with few than three lung lesions[25]. Chemotherapy used in combination with interferon is considered to be effective but lacks adequate scientific evidence[26].

From general point of view and in line with previous reports[12,27,28], CapeOx seems feasible and suitable for palliative care in patients with advanced HCC. With lack of renal toxicity of oxaliplatin[29], the low incidence of myelosuppression observed with capecitabine[30], the synergistic anti-tumor activity and safety of capecitabine and oxaliplatin combination in advanced HCC[31], and the absence of dose adjustment required for both agents in case of hepatic dysfunction, make the CapeOx regimen attractive in advanced HCC patients with cirrhosis or chronic HBV infection[15,32]. A multicenter, open-label, phase Ⅱ study of CapeOx reported a response rate of 6% and a disease control rate of 72%[33], however, patients who had not undertaken local therapies were eligible for this study. For patients with extrahepatic metastasis from HCC, systemic chemotherapy of carboplatin and 5-FU had demonstrated a statistically significant improvement in OS (10.7 mo *vs* 5.1 mo) in comparable patients with non-chemotherapy[34].

For these patients who had extrahepatic metastasis after local treatments and who had no significant alteration of their liver function, palliative chemotherapy can be delivered with tolerable toxicity[35]. Recently, research combining the use of CapeOx and cetuximab for advanced HCC reported an RR of 12.5%, TTP of 3.3 mo and overall survival of 4.4 mo[36]. Another phase Ⅱ trial of CapeOx with bevacizumab for advanced HCC in 2011 showed tumor response and disease control rates of 20% and 77.5%, respectively[31]. The median OS and PFS were 9.8 and 6.8 mo, respectively. In our study, although only the cytotoxic chemotherapy drugs oxaliplatin and capecitabine were used for patients with extrahepatic metastasis, the result was encouraging for both efficacy and toxicity. Partial response was seen in 21.9% patients, and 62.5% had their disease controlled. The study also showed a median TTP of 4.2 mo and a median OS of 9.2 mo in a patient population of 50% with Eastern Cooperation Oncology Group PS 1-2, and more than 31% of the patients with Child- Pugh class B disease status.

Obviously the underlying liver cirrhosis increases the risk of severe adverse events as many chemotherapeutic drugs are metabolized or eliminated *via* the liver. Moreover, severe complications might occur if a cytotoxicity-related side effect appears on a cirrhotic liver. Certain causes of the underlying cirrhosis, such as hepatitis B virus infection, may be reactivated after chemotherapy induced immunosuppression, producing an additive toxic effect[37].

In conclusion, palliative chemotherapy can be delivered to patients with extrahepatic metastasis from HCC following local treatments with tolerable toxicity. However, the efficacy was not satisfactory. More effective systemic chemotherapy regimens are needed for this subgroup of patients.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths worldwide. The overall 5-year survival rate for all HCC patients has remained no more than 5%. Sorafenib is the first agent to demonstrate a survival advantage over supportive care in HCC. Nevertheless, in a relatively fit group of sorafenib-treated patients (95% Childs-Pugh A), median survival was only 10.7 mo. The purpose of this study was to evaluate the safety and efficacy of the capecitabine-oxaliplatine combination (CapeOx) in patients with extrahepatic metastatic HCC following local treatments.

Research frontiers

Local recurrence and distant metastasis are still obstacles in further improvement of outcome in HCC patients after local treatments. Doxorubicin, until recently considered the standard chemotherapeutic for HCC, is associated with an objective response rate of approximately 10%. In this study, the authors aimed to valuate the efficacy and safety of CapeOx regimen in patients with extrahepatic metastasis from HCC following local treatments.

Innovations and breakthroughs

Sorafenib is currently considered standard of care systemic therapy for patients with advanced HCC, but results from recent several studies showed it not suitable in some patients with advanced HCC. In this study, CapeOx regimen showed modest anti-tumor activity in metastatic HCC and tolerated toxicities.

Applications

This study may represent a future strategy for therapeutic intervention in the treatment of patients with extrahepatic metastasis from HCC following local treatments even if with liver cirrhosis.

Terminology

Capecitabine is an orally administered, tumor-selective fluoropyrimidine that mimics continuous infusion of 5-fluorouracil. Oxaliplatin s a new platinum complex, diaminocyclohexane compound, which is thought to result from inhibition of DNA synthesis in cancer cells. The combination of oxaliplatin and capecitabine has also shown some promise in HCC because both drugs are tolerated in the setting of hepatic dysfunction.

Peer review

This study is an uncontrolled phase 2 evaluation of capecitabine and oxaliplatin for locally controlled HCC with extrahepatic metastases involving 32 patients, the majority of whom were hepatitis B virus infected and non-cirrhotic. The majority of patients' metastases were pulmonary or intra-abdominal with 6/32 being confined to bone. Twenty-eight percent of patients required dose reduction of capecitabine due to grade 3/4 toxicity but only 2 grade 3 oxaliplatin toxicities occurred. Ninety-seven percent of patients died or manifested tumor progression. Median time to tumor progression was 4.2 mo and median overall survival was 9.2 mo. This is an interesting prospective study on efficacy of CapeOx combination regimen for extrahepatic metastasis of HCC following local treatments, and gives a practical point of view in management of these patients.

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BRIEF ARTICLE

Surgical management of patients with bowel obstructions secondary to gastric cancer

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Abstract

AIM: To assess whole-body fluorodeoxyglucose (FDG) positron emission tomography/computed tomography (PET/CT) in the management of small bowel obstructions (SBOs) secondary to gastric cancer and its role in treatment strategies.

METHODS: The medical records of all of the patients who were admitted for an intestinal obstruction after curative resection for gastric cancer were retrospectively reviewed. PET/CT was performed before a clinical treatment strategy was established for each patient. The patients were divided into 2 groups: patients with no evidence of a tumor recurrence and patients with evidence of a tumor recurrence. Tumor recurrences included a local recurrence, peritoneal carcinomatosis or distant metastases. The primary endpoint was the 1-year survival rate, and other variables included patient demographics, the length of hospital stay, complications, and mortality.

RESULTS: The median time between a diagnosis of gastric cancer and the detection of a SBO was 1.4 years. Overall, 31 of 65 patients (47.7%) had evidence of a tumor recurrence on the PET/CT scan, which was the only factor that was associated with poor survival. Open and close surgery was the main type of surgical procedure reported for the patients with tumor recurrences. R0 resections were performed in 2 patients, including 1 who underwent combined adjacent organ resection. In the group with no evidence of a tumor recurrence on PET/CT, bowel resections were performed in 7 patients, adhesiolysis was performed in 7 patients, and a bypass was performed in 1 patient. The 1-year survival curves according to PET/CT evidence of a tumor recurrence *vs* no PET/CT evidence of a tumor recurrence were significantly different, and the 1-year survival rates were 8.8% *vs* 93.5%, respectively. There were no significant differences (P = 0.71) in the 1-year survival rates based on surgical *vs* nonsurgical management (0% with nonoperative treatment *vs* 20% after exploratory laparotomy).

CONCLUSION: ^{18}F-FDG PET/CT can be used to identify the causes of bowel obstructions in patients with a history of gastric cancer, and this method is useful for planning the surgical management of these patients.

Key words: Positron emission tomography/computed tomography; Small bowel obstructions; Gastric cancer; Clinical treatment strategy

Core tip: The management of patients who present with a small bowel obstruction (SBO) after treatment of primary carcinoma challenges the clinical judgement of

even the most experienced surgeons when the feared cause is metastatic disease. It is difficult to predict whether the quality and/or the quantity of life in this group of patients will be improved by surgery. This study evaluated the clinical role of ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography/computed tomography (PET/CT) in identifying SBOs and its role in subsequent clinical treatment strategies. We found that ^{18}F-FDG PET/CT is an appropriate method to identity the causes of bowel obstructions secondary to gastric cancer, and this method is useful for the surgical management of these patients.

Wu WG, Dong P, Wu XS, Li ML, Ding QC, Zhang L, Yang JH, Weng H, Ding Q, Tan ZJ, Lu JH, Gu J, Liu YB. Surgical management of patients with bowel obstructions secondary to gastric cancer. *World J Gastroenterol* 2013; 19(28): 4559-4567 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i28/4559.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i28.4559

INTRODUCTION

An intestinal obstruction is a common problem in patients with an advanced malignancy. Approximately 3%-15% of all terminal cancer patients will develop an intestinal obstruction[1,2]. In advanced abdominal and pelvic malignancies, 5%-51% of patients with ovarian malignancies and 10%-28% of patients with gastrointestinal cancers will develop an intestinal obstruction[3-8]. An intestinal obstruction may be due to intra-abdominal adhesions, intra-abdominal hernias, local cancer recurrences, peritoneal carcinomatosis, or distant metastases from other tumors. Small bowel obstructions (SBOs) secondary to malignant disease are often a sign of end-stage disease and are associated with poor survival. The treatment of such patients presents a dilemma for the surgeon. Inappropriate surgery will not significantly improve morbidity and mortality outcomes and often has limited success in relieving symptoms. Nonoperative treatment is often ineffective at restoring bowel function, and when relief is obtained, early reobstruction frequently occurs[9,10]. The management of patients who present with a bowel obstruction after treatment of primary carcinoma challenges the clinical judgment of even the most experienced surgeons when the feared cause is metastatic or recurrent disease[11]. The management of these patients is difficult, and it is unclear which patients will benefit from surgery and which patients will have similar outcomes from medical management because many patients may have diffuse peritoneal metastatic disease and/or adhesions from previous surgery. It is difficult to predict whether the quality and/or the quantity of life in this group of patients will be improved by surgery because these patients have a poor prognosis at the time of presentation[12]. In addition, the management of these patients presents an additional difficulty because the intestinal obstruction may be due to more than one physiopathological process, such as an intraluminal obstruction from polypoid lesions that occlude the bowel lumen, an intramural obstruction from the infiltration of a tumor within the muscular coat of the bowel wall, and an extramural obstruction from mesenteric and omental masses and extrinsic compression from malignant adhesions.

Positron emission tomography (PET) with ^{18}F-fluorodeoxyglucose (FDG) detects the increased utilization of glucose by malignant cells to provide diagnostic information and is more accurate than conventional diagnostic methods in cases of primary and recurrent gastrointestinal tumors[12-15]. To date, the usefulness of integrated FDG PET/computed tomography (CT) in the treatment decisions for patients with bowel obstructions secondary to malignant disease has not been investigated. This study evaluated the clinical role of whole-body FDG PET/CT in the management of SBOs secondary to gastric cancer and its role in the formulation of subsequent clinical treatment strategies.

MATERIALS AND METHODS

Patients

This retrospective chart review was approved by the institutional review board and was performed at the Department of General Surgery, Xinhua Hospital, School of Medicine, Shanghai Jiaotong University. A retrospective review of our electronic database was conducted to find all patients with a history of curative resection for gastric cancer who were admitted for an intestinal obstruction from August 1, 2008 to January 1, 2010. Adult patients with discharge diagnoses of bowel obstructions and gastric cancer were enrolled. Patients whose cancer was first diagnosed with the bowel obstruction and patients without radiographic confirmation of an obstruction were excluded. Patients with an early postoperative bowel obstruction, which is generally defined as a mechanical obstruction that occurs within 1 mo of abdominal surgery, were excluded. No patient with a bowel obstruction before a cancer diagnosis was included in this study. The diagnosis of a bowel obstruction was based on a combination of clinical signs and symptoms and radiologic findings. The enrolled patients had at least one of the following symptoms along with radiographic confirmation of an obstruction: nausea and vomiting, colicky pain, abdominal bloating, obstipation, or an inability to tolerate PO intake. Radiographic confirmation of the obstruction was usually by either plain abdominal films or a CT scan. In addition, at least one of the following findings was required: dilated loops of bowel with a paucity of air in the colon, air/fluid levels, or a transition from a dilated bowel to a decompressed bowel[16]. PET/CT was performed for each patient before the clinical treatment strategy was established. Based on the PET/CT results, the patients were divided into 2 groups: patients with no evidence of a tumor recurrence and patients with evidence of a tumor recurrence, which included a local recurrence, peritoneal carcinomatosis or distant metastases.

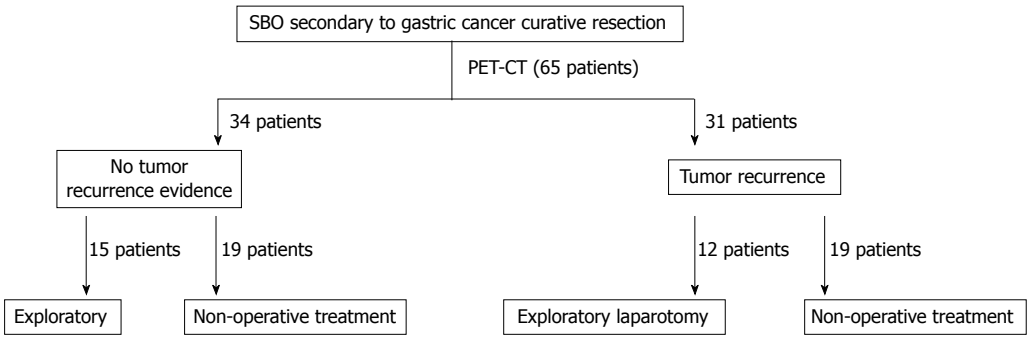

Figure 1 The treatment decision-making process. PET/CT: Positron emission tomography/computed tomography; SBO: Small-bowel obstruction.

PET/CT

PET/CT imaging was performed using a GE Discovery ST 8-slice scanner. The patients were scanned after 6 h of fasting. Blood glucose levels were checked immediately before the scan. An average of 296-370 MBq (*i.e.*, 8-10 mCi) FDG was injected intravenously, and whole-body images were obtained 1 h later. Low-dose CT images were used for attenuation correction. An oral contrast agent was administered to all of the patients for PET/CT imaging. A semiquantitative and visual analysis was made. The images were evaluated by 2 nuclear medicine specialists, and a consensus was required to prevent interobserver variability. The FDG uptake was defined as qualitatively positive when the focal FDG uptake was higher than the normal biodistribution of background FDG activity. In addition, to exclude the physiologic uptake, the FDG uptake in the bowel was considered positive only when wall thickening of the same bowel was simultaneously detected by CT. The PET/CT images were analyzed for the number and the sites with positive FDG uptake, and the standardized uptake value value of all of the positive FDG uptake values was measured.

Operative and non-operative management

The patients with an obstruction were divided into 2 treatment groups: patients who received conservative treatment and patients who underwent surgical management. The standard nonoperative management of small bowel obstructions consisted of fluid and electrolyte replacement, bowel rest, and tube decompression. A nonoperative course may be followed for 24-48 h. If the obstruction has not resolved within that time period, it is unlikely that the obstruction will ever resolve and laparotomy is usually advised. In the patients who underwent surgery for a bowel obstruction after curative resection of gastric cancer, the type of operation was determined by 3 expert gastrointestinal surgeons depending on the overall medical status of the patient, the wishes of the patient, and the abdominal examination. The primary endpoint of the analysis of surgical *vs* non-surgical treatment for the bowel obstructions in this study was the 1-year survival rate, and other recorded variables included patient demographics, the length of hospital stay, complications, and mortality. The modified Clavien system was used to grade any postoperative complications. In-hospital mortality was defined as the percentage of patients who died before hospital discharge. The length of hospital stay was defined as the number of days from the index procedure to hospital discharge. This study was approved by the Human Research Review Committee of our hospital.

Statistical analysis

Statistical analyses and graphics were generated using the SPSS 13.0 statistical package for Windows (SPSS, Inc., Chicago, IL, United States). If the P value was < 0.05, the results were considered statistically significant. Patency after palliation and the overall survival were estimated using the Kaplan-Meier actuarial method, and the curves were compared using the log-rank test. To identify the independent factors that influenced clinical success and the risk factors that were associated with the 1-year overall survival, univariate and multivariate analyses were performed. The results were expressed as the mean ± SD or as the percentages.

RESULTS

There were 72 cases of bowel obstructions in patients with a history of curative surgery for gastric cancer at our institution during the study period. Seven patients declined PET/CT imaging and were excluded from the analysis. The remaining 65 patients were all admitted for a SBO and were included in the analysis. The surgical decision-making process is shown in Figure 1. The average age at the time of the primary gastric cancer diagnosis was 62.5 ± 17.1 years. The mean age at admission for a SBO was 63.9 ± 15.6 years.

The median time between curative resection of gastric cancer and the detection of a SBO was 1.4 years. The clinicopathological data of the patients are listed in Table 1. Each patient underwent PET/CT before the final clinical treatment strategy was determined. PET/CT indicated that 31 patients (47.7%) had evidence of a tumor recurrence, including a local recurrence, peritoneal carcinomatosis, and distant metastases (Table 2; Figure 2A). The remaining 34 (52.3%) patients had no evidence of a tumor recurrence (Figure 2B). Both the univariate

Table 1 Factors on presentation associated with a malignant etiology of the small-bowel obstruction

Factors	Value
All	65 (100.0)
Sex	
Female	11 (16.39)
Male	54 (83.1)
Age (yr)	
< 70	45 (69.2)
⩾ 70	20 (30.8)
Site	
Lower	41 (63.1)
Middle	8 (12.3)
Upper	16 (24.6)
Diffuse	0 (0.0)
Comorbidities	
Yes	42 (64.6)
No	23 (35.4)
Surgery	
Subtotal gastrectomy	33 (50.8)
Total gastrectomy	19 (29.2)
Extended total gastrectomy	13 (20.0)
Types of digestive reconstruction	
Billroth Ⅰ	20 (30.8)
Billroth Ⅱ	15 (23.1)
Roux-en-Y	30 (46.1)
Grading	
Well differentiated	22 (33.8)
Moderately differentiated	22 (33.8)
Poorly differentiated	21 (32.4)
Undifferentiated	0 (0.0)
T stage	
T1	0 (0.0)
T2	21 (32.3)
T3	38 (58.5)
T4	6 (9.2)
No. metastatic nodes	
N0	8 (12.3)
N1	22 (33.8)
N2	23 (35.4)
N3	12 (18.5)
M stage	
M0	65 (100.0)
M1	0 (0.0)
Intra-abdominal chemotherapy	
Yes	35 (53.8)
No	30 (46.2)
Postchemotherapy	
Yes	55 (84.6)
No	10 (15.4)
Recurrence in PET/CT	
Yes	31 (47.7)
No	34 (52.3)
Re-surgery	
Yes	27 (41.5)
No	38 (58.5)

PET/CT: Positron emission tomography/computed tomography.

and multivariate analyses for factors that may have correlated with survival revealed that the only factor that was associated with poor survival was PET/CT evidence of a tumor recurrence (Table 3).

In patients who received surgical treatment, the type of operation was determined by 3 expert gastrointestinal surgeons based on abdominal examinations and the general condition of the patient. A total of 27 patients (12 in the group with PET/CT evidence of a tumor recurrence and 15 with no evidence of a tumor recurrence) underwent laparotomy. The types of surgical procedures that were performed are summarized in Table 4. Open and close surgery was the main type of surgical procedure reported for the patients with tumor recurrences. R0 resections were performed in 2 patients, including 1 who underwent combined adjacent organ resection. In the group with no evidence of tumor recurrences on PET/CT, bowel resections were performed in 7 patients, adhesiolysis was performed in 7 patients, and a bypass was performed in 1 patient. The overall incidence of postoperative complications was 44.4% (12 of 27 patients). There were 7 patients with Clavien grade I complications, including 3 with wound infections and 4 with pleural effusions. Another 3 patients were classified as having grade Ⅱ complications, including 1 with an anastomotic leakage and 2 with pneumonia. One patient had the grade Ⅲb complication of an abdominal abscess, and 1 patient had the grade V complication of multiple organ failure and died in the hospital 1 wk after surgery.

Table 2 Recurrence site, number and standardized uptake value of recurrence in positron emission tomography/computed tomography

Variables		*n*	SUV mean (range)
Recurrence	Yes	31	7.3 (2.6-28.3)
	No	34	/
Recurrence site	Locoregional recurrence (Remnant stomach or anastomosis site)	2	6.8 (4.1-16.2)
	Distant metastasis	29	7.9 (2.6-28.3)
	Lymph-node	16	7.5 (2.6-28.3)
	Liver	8	8.0 (2.8-15.5)
	lung	6	7.1 (2.6-14.2)
	Other site (bone, skin, *etc.*)	10	6.2 (3.2-12.5)
	Peritoneum	12	5.4 (2.7-11.2)

SUV: Standardized uptake value.

The 1-year survival curves according to the PET/CT findings are shown in Figure 3A. There was a significant difference in the survival between patients with and without evidence of recurrences on PET/CT, and the 1-year survival rates were 8.8%, and 93.5%, respectively (P = 0.00). The 1-year survival curves according to exploratory laparotomy and nonoperative treatment are shown in Figure 3B. There were no significant differences (P = 0.71) in the 1-year survival based on surgical *vs* nonsurgical management (0% with nonoperative treatment *vs* 20% after exploratory laparotomy). The 1-year survival curves according to evidence of a tumor recurrence on PET/CT are shown in Figure 3C.

Other variables in the analysis included 30-d readmission, the length of hospital stay, complications, and the mortality rates. These variables are listed in Table 5. In both the PET/CT-positive and -negative groups, exploratory laparotomy resulted in a shorter mean length of hospital stay than nonsurgical management (P < 0.05).

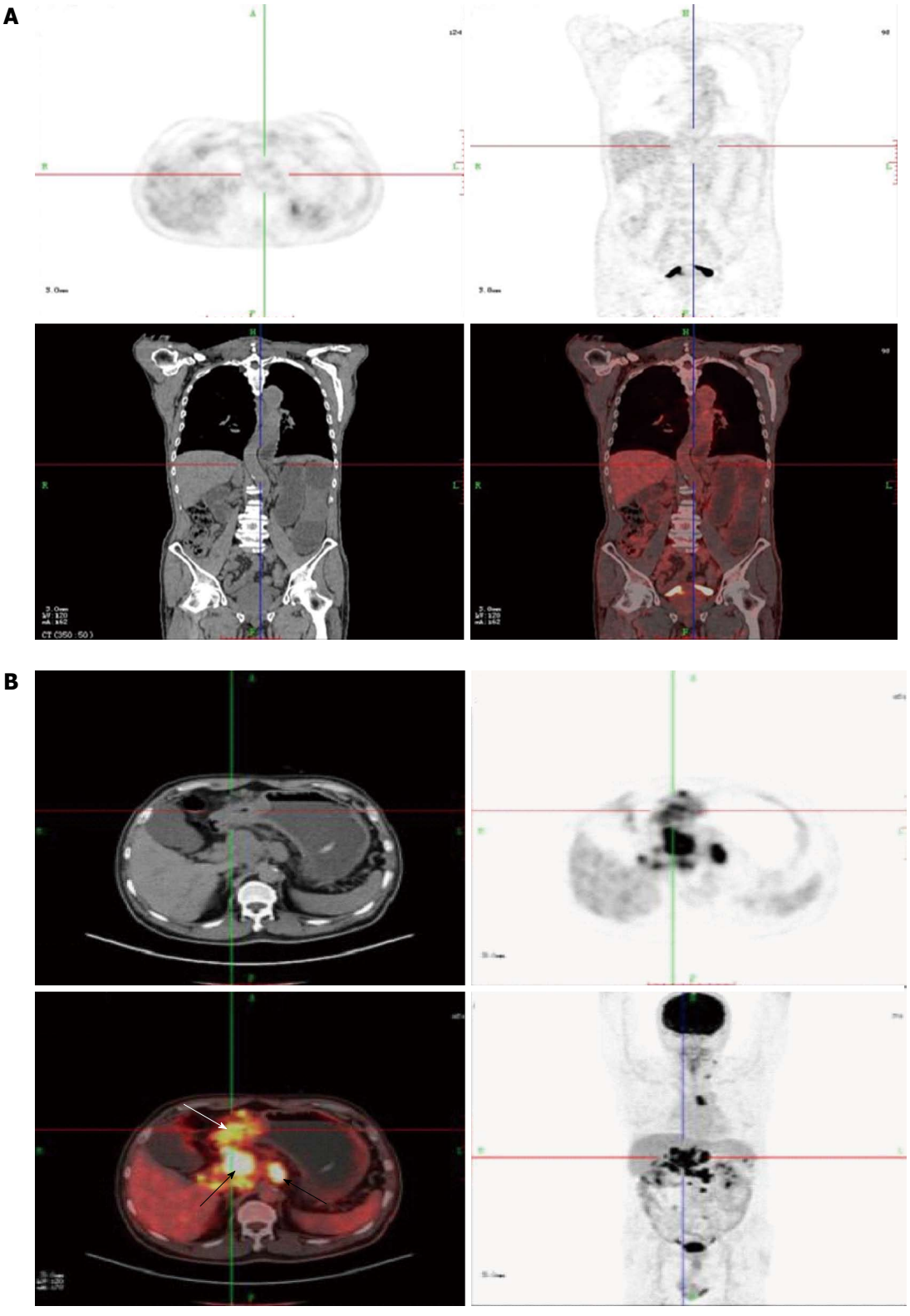

Figure 2 Patients who had had gastric cancer resection underwent positron emission tomography/computed tomography because of small-bowel obstruction. A: A 68-year-men who had had gastric cancer resection 2 years previously underwent positron emission tomography (PET)/computed tomography because of small-bowel obstruction. Whole body PET projection image and axial PET image showed no focal hypermetabolic activity; B: A 38-year-female who had had gastric cancer resection 1 years previously underwent positron emission tomography/computed tomography because of small-bowel obstruction. Whole body PET projection image and axial PET image showed the remnant stomach (white arrow) and lymph-node (black arrow) focal hypermetabolic activity.

Table 3 The clinicopathologic factors of the 65 patients

Factors	Survive (*n*)	Death (*n*)	Univariate analysis *P* value	Multivariate analysis *P* value
Sex			0.751	
Female	6	5		
Male	26	28		
Age (yr)			0.180	
< 70	25	20		
⩾ 70	7	13		
Site			0.336	
Lower	20	21		
Middle	1	7		
Upper	11	5		
Diffuse	0	0		
Comorbidities			0.798	
Yes	20	22		
No	12	11		
Surgery			0.683	
Subtotal gastrectomy	17	16		
Total gastrectomy	10	9		
Extended total gastrectomy	5	8		
Types of digestive reconstruction			0.446	
Billroth Ⅰ	12	8		
Billroth Ⅱ	6	9		
Roux-en-Y	14	16		
Grading			0.241	
Well differentiated	11	11		
Moderately differentiated	8	14		
Poorly differentiated	13	8		
Undifferentiated	0	0		
T stage			0.447	
T1	0	0		
T2	8	13		
T3	21	17		
T4	3	3		
No. metastatic nodes			0.105	
N0	1	7		
N1	11	11		
N2	14	19		
N3	6	6		
Intra-abdominal chemotherapy			0.459	
Yes	19	16		
No	13	17		
Postchemotherapy			0.511	
Yes	26	29		
No	6	4		
Recurrence in PET/CT			0.000	0.000
Yes	2	29		
No	31	3		
Re-surgery			0.804	
Yes	14	13		
No	18	20		

PET/CT: Positron emission tomography/computed tomography.

DISCUSSION

An intestinal obstruction is a common problem in patients with advanced cancers. Approximately 3%-15% of all terminally ill cancer patients will suffer from an intestinal obstruction and 10%-28% of patients with gastrointestinal cancers will develop an intestinal obstruction. Obstructions in patients with a history of gastric cancer may be secondary to a malignant process, either extrinsic or intrinsic to the bowel, or an underlying benign etiology, such as an intra-abdominal hernia or intraperitoneal adhesions. The current treatment options for patients with a bowel obstruction secondary to malignant disease include surgery to bypass/remove the obstruction, gastrointestinal decompression *via* a nasogastric tube, and medications (*e.g.*, octreotide)[17]. In inoperable cases, decompression *via* a nasogastric tube may be the only treatment available. Nasogastric tube decompression can provide symptomatic relief but may cause mucosal erosion, esophagitis, or aspiration pneumonia, which further diminish quality of life. However, surgical treatment is often contraindicated because of the poor physical status of the patient, and many patients with gynecological malignancies, especially ovarian cancer, are not candidates for surgery because of the presence of diffuse intraperitoneal carcinomatosis, multiple partial obstruction points, ascites, and/or a history of previous radiotherapy. A critical step in the management of patients with a bowel obstruction and a history of curative resection of gastric cancer is to determine whether a malignant process is present. Identifying the underlying etiology of the bowel obstruction, malignant or benign, will significantly impact management decisions. Additionally, distinguishing between a malignant obstruction and a benign obstruction is a key measure in deciding which patients should undergo early operation.

Table 4 Types of surgery performed for small-bowel obstruction

Procedure	*n*
Open and close	10
R0 resection	2
Bypass	1
Bowel resection	7
Adhesiolysis	7

It is well documented that the complete surgical removal of gastric tumors with lymph node dissection is the only curative treatment that is currently available; however, disease recurrence after radical surgery still occurs in approximately 22%-48% of patients, and its prognosis is poor[18-21]. Tumor marker evaluation, endoscopy, and imaging studies have previously been used to monitor patients for gastric cancer recurrences; however, there are several limitations to tumor markers and endoscopy. Tumor markers cannot be used to determine the site of recurrence, and endoscopy cannot detect extraluminal recurrences[22]. The most important limitation of CT in the detection of locally recurrent gastric cancer is the lack of specificity because the diagnostic ability of CT depends on the morphological changes of the involved organs and distorted anatomical features. In addition, CT uses size criteria. These factors result in difficulties in image interpretation, and CT cannot precisely identify the presence and the quality of tumors.

Whole-body ^{18}F-FDG PET detects increased glucose metabolism in malignant cells to produce diagnostic evidence and can be widely applied for staging, re-

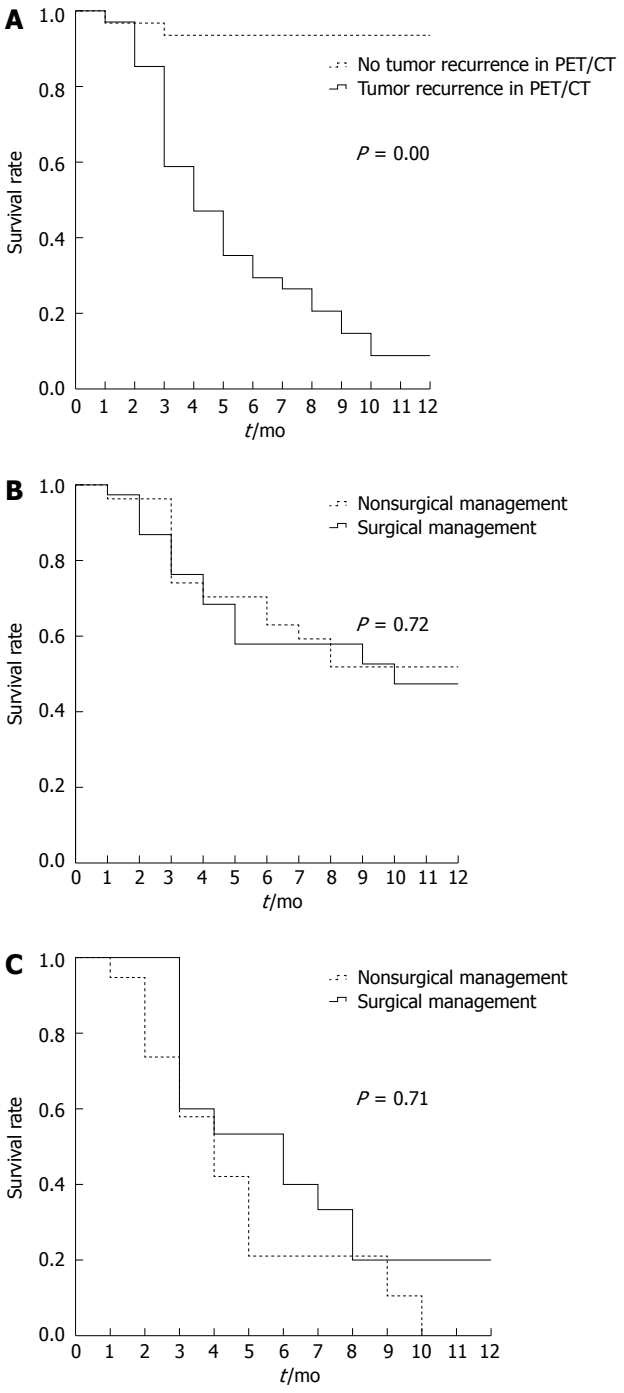

Figure 3 1-year survival curves. A: With respect to no tumor recurrence and tumor recurrence group in positron emission tomography/computed tomography. There was significant difference between two subgroups (P = 0.00). The 1-year survival rate in positron emission tomography/computed tomography (PET/CT) tumor recurrence group is 8.8%, while 93.5% in no tumor recurrence group; B: With respect to exploratory laparotomy and nonoperative treatment group. There was no difference in 1-year survival based on type of surgical *vs* nonsurgical management (P = 0.72); C: With respect to tumor recurrence group in positron emission tomography/computed tomography. The 1-year survival rates for patients in each subgroup were, respectively, 0.0% for tumor nonoperative treatment, and 20% for exploratory laparotomy group. There is also no significant difference between two subgroups (P = 0.71).

staging, and monitoring therapy-induced tumor changes and response to therapy in patients with various cancers. The usefulness of integrated ^{18}F-FDG PET/CT for the diagnosis of recurrences in patients with gastric cancer has been investigated in previous studies, which have indicated that ^{18}F-FDG PET/CT is an effective and helpful diagnostic method in the evaluation of recurrences. Other trials have studied the impact of ^{18}F-FDG PET/CT on the clinical decision-making process[23-25], FDG-PET results led to a radical change in the clinical management of 20% of the patients who were analyzed for resection of colorectal liver metastases. FDG-PET was considered a decisive technique for determining whether to perform surgery, and management was changed in 29% of the patients. This study has confirmed the critical role of whole-body ^{18}F-FDG PET/CT during the clinical course of patients with a bowel obstruction and a history of gastric cancer.

Table 5 Outcomes variables of the 65 patients

Variables	No recurrence in PET/CT		Recurrence in PET/CT	
	Surgical management	Non-surgical management	Surgical management	Non-surgical management
Mean length of stay (d)	10.5 ± 2.3	18.2 ± 8.7	8.2 ± 3.1	19.1 ± 9.6
30-d re-admission	0.00%	21.10%	25.00%	26.10%
In-hospital mortality	0.00%	0.00%	0.10%	0.00%
Overall complications	53.30%	0.00%	33.30%	0.00%

PET/CT: Positron emission tomography/computed tomography.

Several patients in our study with a SBO and a history of gastric cancer did not have end-stage disease that was associated with poor survival. Distinguishing between a malignant obstruction and a benign obstruction is a key measure in deciding which patients should undergo early operation. Patients with metastatic cancer who develop a bowel obstruction have a short median survival time (approximately 3 mo)[16], and decisions regarding the treatment of bowel obstructions must be carefully weighed in these patients. Surgery can offer good palliative benefits for these patients; however, surgery may result in complications that reduce quality of life and cause patients to spend an excessive amount of time in the hospital, which could have been avoided. Therefore, optimal palliation may result from the nonoperative and medical management of symptoms and lead to a potential decrease in the length of hospital stay. Attempting surgery in these patients may not be the best decision, and the finding in our study that there were no differences in the survival of patients with recurrent disease based on the type of management shifts the focus of care for these patients from a selection process to surgical *vs* nonsurgical management.

Multiple specialists are usually involved in the treatment of these patients, including gastroenterologists, interventional and diagnostic radiologists, radiation oncologists, and medical and surgical oncologists. These specialists may have divergent opinions regarding definitive individualized treatment. Our results suggest that patients who had evidence of a tumor recurrence on a

PET/CT scan face an end-of-life scenario and optimal symptom control is the goal for these patients. In addition, FDG PET/CT is a superior post-therapy surveillance modality for the diagnosis of recurrent gastric cancer compared with other imaging methods after initial surgery. In addition, FDG PET/CT has been specifically helpful in optimizing treatment plans and may play an important role in treatment stratification in the future[26]. Miller *et al*[11] compared operative therapy with nonoperative therapy in patients with small bowel obstructions secondary to malignant disease and found a rate of reobstruction that was 15% higher in the nonoperative group. Additionally, they reported shorter times to a reobstruction in patients who had received conservative nonoperative therapy, and they observed that a palpable abdominal mass was an important predictor of poor outcomes in their series. In this study, we concluded that patients with a history of gastric cancer who present with a SBO and who have no evidence of a tumor recurrence on PET/CT will receive benefits in both survival and quality of life after surgery to relieve the obstruction.

In conclusion, ^{18}F-FDG PET/CT can be used to identify the causes of bowel obstructions in patients with a history of curative resection of gastric cancer, and this method is useful for planning the surgical management of these patients. Surgical intervention in a patient who has an obstruction after curative resection of gastric cancer and who has no evidence of a tumor recurrence on a PET/CT examination benefits the quality of life of the patient. Patients with poor survival, including patients with PET/CT evidence of a local recurrence, peritoneal carcinomatosis or distant metastases, would not benefit from surgery.

COMMENTS

Background

The management of patients who present with a small bowel obstruction (SBO) after treatment of primary carcinoma challenges the clinical judgement of even the most experienced surgeons when the feared cause is metastatic disease. It is difficult to predict whether the quality and/or the quantity of life in this group of patients will be improved by surgery because these patients have a poor prognosis at the time of presentation. Positron emission tomography (PET) with ^{18}F-fluorodeoxyglucose (FDG) detects the increased utilization of glucose by malignant cells and is more accurate than conventional diagnostic methods for the diagnosis of primary and recurrent gastrointestinal tumors.

Research frontiers

The management of SBOs after treatment of primary carcinoma is difficult, and it is unclear which patients will benefit from surgery and which patients will have similar outcomes from medical management because many patients may have diffuse peritoneal metastatic disease and/or adhesions from previous surgery. It is difficult to predict whether the quality and/or the quantity of life in this group of patients will be improved by surgery because these patients have a poor prognosis at the time of presentation. In addition, the management of these patients presents an additional difficulty because the intestinal obstruction may be due to more than one physiopathological process, such as an intraluminal obstruction from polypoid lesions that occlude the bowel lumen, an intramural obstruction from the infiltration of a tumor within the muscular coat of the bowel wall, and an extramural obstruction from mesenteric and omental masses and extrinsic compression from malignant adhesions.

Innovations and breakthroughs

^{18}F-FDG PET/computed tomography (CT) can be used to identify the causes of bowel obstructions in patients with a history of curative resection of gastric cancer, and this method is useful for planning the surgical management of these patients. Surgical intervention in a patient who has an obstruction after curative resection of gastric cancer and who has no evidence of a tumor recurrence on a PET/CT examination benefits the quality of life of the patient. Patients with poor survival, including patients with PET/CT evidence of a local recurrence, peritoneal carcinomatosis or distant metastases, may not benefit from surgery.

Applications

^{18}F-FDG PET/CT can be used to identify the causes of bowel obstructions in patients with a history of gastric cancer, and this method is useful for planning the surgical management of these patients.

Terminology

PET with ^{18}F-FDG detects the increased utilization of glucose by malignant cells to provide diagnostic information and is more accurate than conventional diagnostic methods in cases of primary and recurrent gastrointestinal tumors.

Peer review

This article is interesting. The authors present their experience of using whole-body PET/CT in the surgical management of patients with bowel obstructions secondary to gastric cancer. PET/CT significantly improves survival because of its ability to identify the causes of bowel obstructions. Overall, the paper is well written and acceptable for publication in its current form.

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BRIEF ARTICLE

Fibroblast growth factor receptor 4 Gly388Arg polymorphism in Chinese gastric cancer patients

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Abstract

AIM: To investigate the contribution of the fibroblast growth factor receptor 4 (FGFR4) Gly388Arg polymorphism as a genetic risk factor for gastric cancer (GC) and to investigate any associations between this polymorphism and clinicopathological parameters and survival.

METHODS: Tumors and matched adjacent non-cancer tissues were collected from 304 GC patients, and 5 mL of venous blood was collected from 62 GC patients and 392 age- and sex-matched healthy controls without cancer history from the same ethnic population. DNA was extracted, and direct sequencing analyses were performed to genotype the *FGFR4* Gly388Arg polymorphism in all the samples. Differences in the genotype frequencies of the *FGFR4* Gly388Arg polymorphism between GC patients and healthy controls were estimated using the χ^2 test. Binary logistic regression was used for all analysis variables to estimate risk as the ORs with 95%CIs. The relationships between the *FGFR4* genotype and clinicopathological parameters were tested with the χ^2 test. The Kaplan-Meier product-limit method, the log-rank test, and the Cox regression model were applied to evaluate the effect of the *FGFR4* genotype on the overall survival of patients with GC.

RESULTS: In the present GC cohort, 118 patients (38.8%) were homozygous for the Gly388 allele, 124 patients (40.8%) were heterozygous, and 62 patients (20.4%) were homozygous for the Arg388 allele. The frequencies of the Gly/Gly, Gly/Arg, and Arg/Arg genotypes in the healthy controls were 33.6%, 48.0%, and 18.4%, respectively. The distributions of genotypes (χ^2 = 3.589, P = 0.166) and alleles (χ^2 = 0.342, P = 0.559) of the FGFR4 Gly388Arg polymorphism were not different between the GC patients and the healthy controls. Although we observed no correlation between the *FGFR4* Gly388Arg polymorphism and clinicopathological parameters or survival in the total cohort of GC patients, the presence of the Arg388 allele was associated with shorter survival time in patients with GC if the tumor was small (log rank χ^2 = 5.449, P = 0.020), well differentiated (log rank χ^2 = 12.798, P = 0.000), T1 or T2 stage (log rank χ^2 = 4.745, P = 0.029), without lymph node involvement (log rank χ^2 = 6.647, P= 0.010), and at an early clinical stage (log rank χ^2 = 4.615, P = 0.032).

CONCLUSION: Our results suggest that the *FGFR4* Gly388Arg polymorphism is not a risk factor for GC cancer initiation but that it is a useful prognostic marker for GC patients when the tumor is relatively small, well differentiated, or at an early clinical stage.

Key words: Fibroblast growth factor receptor 4; Gly388Arg; Genetic susceptibility; Single nucleotide polymorphism; Gastric cancer

Core tip: This study investigated the contribution of the fibroblast growth factor receptor 4 (FGFR4) Gly388Arg polymorphism as a genetic risk factor for gastric cancer (GC) and any associations between this polymorphism and clinicopathological parameters such as age, gender, clinical stage, tumor grade, human epidermal growth factor receptor 2 status and survival. The results suggested that the FGFR4 Gly388Arg polymorphism was not a risk factor for GC cancer initiation but that it was a useful prognostic marker for GC patients when the tumor was relatively small, well differentiated, or at an early clinical stage.

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INTRODUCTION

Gastric cancer (GC) is one of the most common cancers in the world, and it has a complex etiology. Disease development is related to environmental factors, such as diet and *Helicobacter pylori* (*H. pylori*) infection, as well as genetic predisposition. In the last decade, GC incidence rates have steadily declined owing to changing diets and the application of antibiotics to treat *H. pylori* infection[1]. However, the treatment of patients with advanced GC remains a significant challenge. Surgical resection and chemotherapy are only effective for a fraction of patients, and their prognoses remain very poor. Recently, a number of molecularly targeted agents modulating different signal transduction pathways have been researched in clinical trials for many cancer types[2]. Trastuzumab, a monoclonal antibody against human epidermal growth factor receptor 2 (HER2; also known as ERBB2) was demonstrated to be effective in advanced HER2-positive GC. The addition of trastuzumab to chemotherapy improved survival in patients with advanced GC or gastroesophageal junction cancer compared with chemotherapy alone in a Trastuzumab for Gastric Cancer trial[3]. However, only 7% to 34% of GC cases are HER2 positive[4-6], meaning that only a fraction of GC patients can benefit from trastuzumab. Accordingly, there is an urgent need to better understand the genesis of GC to establish a sound basis for future treatments.

Fibroblast growth factor receptor 4 (FGFR4) is a member of the receptor tyrosine kinase family. These receptors have highly conserved structures containing an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain. The FGFR4 protein interacts with specific growth factors, especially acidic fibroblast growth factor, and is critically involved in cell growth, differentiation, migration, angiogenesis, and tumorigenesis. Several prior studies have investigated the role of the FGFR4 signaling pathway in GC. Notably, a soluble variant of FGFR4 was first detected in human gastrointestinal epithelial cells and cancer cells in a study by Takaishi *et al*[7]. Shin *et al*[8] and Ye *et al*[9]. also demonstrated the upregulation of FGFR4 mRNA and protein in GC, suggesting the possibility that FGFR4 signaling could play a role in gastric carcinogenesis.

Recently, a single nucleotide polymorphism (SNP) at codon 388 (cDNA 1162) from G to A, which results in a change of amino acid from glycine to arginine, was identified in the transmembrane domain of the *FGFR4* gene[10]. Significant scientific effort has been put into the investigation of this *FGFR4* Gly388Arg polymorphism in cancer progression, and the results showed that patients with the Arg/Arg or Gly/Arg genotype (compared to those with a Gly/Gly genotype) had a shorter survival time or a higher proportion of nodal involvement in many types of cancer, including breast, lung, colon, prostate, soft tissue sarcoma, melanoma, and head and neck squamous cell carcinoma[10-19]. However, several researchers have opposed the association between this polymorphism and poor outcomes or lymph node involvement[20-23]. Furthermore, it has been reported that the *FGFR4* Arg388 polymorphism may not be involved in tumor initiation in several different tumor types with the exception of prostate cancer[13,14,18,24]. Xu *et al*[25] conducted a meta-analysis of 2618 patients and 2305 controls and demonstrated that the *FGFR4* Gly388Arg polymorphism was associated with both progression and risk in prostate cancer .

Recently, Ye *et al*[26] reported that the FGFR4 Arg allele was an independent prognostic factor in Chinese patients with GC. To our knowledge, this is the only report on the association between the *FGFR4* Gly388Arg polymorphism and the progression of GC in Chinese patients, and it therefore needs further confirmation. Moreover, no study has been conducted on the correlation between this polymorphism and the risk of GC. In the present study, we expanded the sample sizes by enrolling 304 patients with GC and 392 healthy controls and genotyped every sample for the FGFR4 Gly388Arg polymorphism to confirm the findings of Ye *et al*[26] and investigate the association between the FGFR4 Gly388Arg polymorphism and the risk of GC.

MATERIALS AND METHODS

Patients and healthy controls

A total of 304 Chinese patients diagnosed with GC were recruited and underwent surgery between 2007 and 2009 at Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University. All GC cases were pathologically confirmed, and patient records were used to obtain clinical data. The study included a total of 223 males and 81

Table 1 General characteristics of gastric cancer patients and healthy controls *n* (%)

Characteristics	GC *n* = 304	Healthy controls *n* = 392	Pearson's χ^2 value	*P* value
Age (yr)			2.548	0.110
< 60	125 (41.12)	138 (35.20)		
⩾ 60	179 (58.88)	254 (64.80)		
Sex			0.115	0.735
Male	223 (73.36)	292 (74.49)		
Female	81 (26.64)	100 (25.51)		
Smoker			0.314	0.575
Yes	92 (30.26)	111 (28.32)		
No	212 (69.74)	281 (71.68)		
Hypertension			0.012	0.931
Yes	78 (25.66)	102 (26.02)		
No	226 (74.34)	290 (73.98)		
Diabetes			2.591	0.108
Yes	52 (17.11)	50 (12.76)		
No	252 (82.89)	342 (87.24)		

GC: Gastric cancer.

females. The age of the patients at the time of surgery ranged from 22 to 87 years, with a median age of 63.5 years. The clinical stage was determined according to the Union for International Cancer Control TNM staging system, and the tumor grade was based on the World Health Organization classification. The expression or amplification of HER2 in GC was detected using standard methods, including immunohistochemistry and fluorescence in situ hybridization. The HER2 status was determined according to the Hofmann HER2 scoring system[6]. Follow-up was performed regularly. The median follow-up time for patients still alive at analysis was 49 mo (range, 20-61 mo).

Tumors and matched adjacent non-cancer tissues were snap frozen in liquid nitrogen immediately after surgical removal and stored at -80 ℃. Each tumor sample was evaluated microscopically, and macro-dissection was performed to ensure that more than 70% of the sample was tumor tissue before DNA extraction. Five milliliters of venous blood was collected from 62 GC patients and 392 age- and sex-matched healthy controls without cancer history from the same ethnic population. Informed consent was obtained from all the patients and controls for the use of their specimens and clinicopathologic data for this study. The study was approved by the Institutional Human Ethics Committee.

Genotyping of the FGFR4 Gly388Arg polymorphism

DNA samples from tumor and normal adjacent tissue were prepared with the Puregene DNA extraction kit (Qiagen, Valencia, CA, United States). Genomic DNA was extracted and purified from the blood of GC patients and the healthy control group using the QIAamp DNA Blood Midi kit (Qiagen, Germany). Sanger sequencing was applied to genotype the *FGFR4* Gly388Arg polymorphism with the following PCR primers: 5'-GCGGCCAGTCTCACCACTGAC-3' and 5'-TGGAGTCAGGCTCTTCCGGCA-3'. Each primer was tagged with the M13 primer as a uniform sequencing primer for individual PCR products to facilitate the sequencing process. All PCR assays were carried out in a 25 μL volume containing 10 ng genomic DNA, 0.1 μmol/L of each primer, and 1 × AmpliTaq Gold Pre Mix (AB). The PCR conditions were as follows: 95 ℃ for 10 min; 30 cycles of 95 ℃ for 30 s, 58 ℃ for 30 s, and 72 ℃ for 30 s; and 5 min at 72℃. The PCR product was 355 bp and was sequenced in both directions using the BigDye Terminator kit 3.1 (Applied Biosystems; Foster City, CA, United States) and ABI Genetic Analyzer3730 × l (Applied Biosystems) according to the manufacturer's instructions. Sequence traces were analyzed for the polymorphism after assembly and quality calling with SeqScape2.5 sequence analysis software (Applied Biosystems).

Statistical analysis

Differences in the general characteristics and genotype frequencies of the *FGFR4* Gly388Arg polymorphism between GC patients and healthy controls were estimated using the χ^2 test. Hardy-Weinberg equilibrium analyses were performed to compare observed and expected genotype frequencies using the χ^2 test. Binary logistic regression was used for all analysis variables to estimate risk as the ORs with 95%CIs. The relationships between the *FGFR4* genotype and clinicopathological parameters were tested with the χ^2 test. The Kaplan-Meier product-limit method, the log-rank test, and the Cox regression model were applied to evaluate the effect of the *FGFR4* genotype on the overall survival of patients with GC. The covariates included in the models were age, gender, clinical stage, tumor grade, HER2 status and *FGFR4* genotype. All analyses were carried out with SPSS for Windows software (SPSS, Chicago, IL, version 16.0). A P value < 0.05 was considered to be statistically significant.

RESULTS

FGFR4 genotype in healthy controls and GC patients

Data from the 62 patients who provided both tumor tissue and blood samples showed that the Gly388Arg genotypes were identical between tumor tissue and blood from the same individual. This result confirms that there were no somatic mutations at this locus in any of these patients.

The clinical characteristics of all the subjects are shown in Table 1. There were no significant differences in age, gender, smoking, hypertension, or diabetes status between the GC patients and healthy controls. The frequencies of the *FGFR4* Gly388Arg polymorphism genotypes are summarized in Table 2. Among the 304 GC patients, 118 patients (38.8%) were homozygous for the Gly388 allele, 124 were heterozygous (40.8%), and 62 were homozygous (20.4%) for the Arg388 allele. In the healthy controls, the frequencies of the Gly/Gly, Gly/Arg, and Arg/Arg genotypes and the Arg allele were 33.6%, 48.0%, 18.4% and 42.3%, respectively. The fre-

Table 2 Distribution and regression analysis of the fibroblast growth factor receptor 4 Gly388Arg genotype in gastric cancer patients and healthy controls *n* (%)

Gly388Arg	GC *n* = 304	Healthy controls *n* = 392	OR (95%CI)	*P* value
Genotype				
GG	118 (38.8)	132 (33.6)	1	
AG	124 (40.8)	188 (48.0)	0.738 (0.527-1.033)	0.076
AA	62 (20.4)	72 (18.4)	0.963 (0.633-1.466)	0.862
AA + AG	186 (61.2)	260 (66.3)	0.800 (0.586-1.093)	0.161
Allele				
G	360 (59.2)	452 (57.7)	1	
A	248 (40.8)	332 (42.3)	0.938 (0.756-1.163)	0.559

FGFR4: Fibroblast growth factor receptor 4; GC: Gastric cancer.

Table 3 Association analysis of the fibroblast growth factor receptor 4 Gly388Arg polymorphism and clinicopathological parameters in gastric cancer patients

Variables	Total *n* = 304	Gly/Gly *n* = 118	Gly/Arg + Arg/Arg *n* = 186	Pearson's χ^2 value	*P* value
Age (yr)				0.013	0.909
< 60	125	49	76		
≤ 60	179	69	110		
Sex				1.398	0.237
Male	223	91	132		
Female	81	27	54		
Tumor size				0.198	0.656
≤ 3 cm	58	24	34		
> 3 cm	246	94	152		
Differentiation				2.122	0.145
G1 + G2	100	33	67		
G3 + G4	204	85	119		
Invasion depth				0.000	0.995
T1 + T2	49	19	30		
T3 + T4	255	99	156		
N stage				0.640	0.200
N0	82	27	55		
N1 + N2 + N3	222	91	131		
M stage				1.089	0.297
M0	274	109	165		
M1	30	9	21		
Clinical stage				0.980	0.322
Ⅰ + Ⅱ	103	36	67		
Ⅲ + Ⅳ	201	82	119		
HER2 status				0.391	0.532
Negative	180	63	117		
Positive	45	18	27		

FGFR4: Fibroblast growth factor receptor 4; GC: Gastric cancer; HER2: Human epidermal growth factor receptor 2.

quencies were consistent with the Hardy-Weinberg equilibrium in the healthy controls. The genotype (χ^2= 3.589, P = 0.166) and allele frequencies (χ^2 = 0.342, P = 0.559) of the *FGFR4* Gly388Arg polymorphism were not different between the GC patients and the healthy controls. Binary logistic regression analysis indicated that the ORs for the carriers of Gly/Arg, carriers of Arg/Arg, and carriers of the Gly/Arg or Arg/Arg genotypes were 0.738 (95%CI: 0.527-1.033), 0.963 (95%CI: 0.633-1.466) and 0.800 (95%CI: 0.586-1.093), respectively. No differences were observed between patients and healthy controls. The distribution of the Gly388 and Arg388 alleles was not different between patients and healthy controls (OR = 0.938, 95%CI: 0.756-1.163). These results suggest that the *FGFR4* Gly388Arg polymorphism is not an independent risk factor for GC in Chinese patients.

FGFR4 Gly388Arg polymorphism is not associated with any clinicopathological parameters in GC patients

Based on previous reports and due to the requirements for accurate statistical analysis, the 304 GC patients were divided into two groups: patients with the Gly/Gly genotype and patients with the Arg/Arg or Arg/Gly genotypes. As shown in Table 3, no correlation was observed between the *FGFR4* Gly388Arg polymorphism and any of the following clinicopathological parameters: age at diagnosis, gender, tumor size, clinical stage, differentiation and HER2 status.

Impact of the *FGFR4* Gly388Arg polymorphism on GC survival

When analyzing the entire patient cohort using Kaplan-Meier survival analysis, no difference was observed in survival between patients with the Gly/Gly genotype and patients with the Gly/Arg or Arg/Arg genotypes (log rank χ^2 = 0.047, P = 0.829). When the patient population was stratified by clinicopathological parameters, such as age at diagnosis, gender, tumor size, differentiation, clinical stage, HER2 status and chemotherapy history (5-fluorouracil and cisplatinum), we found that the presence of the Arg388 allele was associated with a shorter survival time in GC patients if the tumor was small (less than or equal to 3 cm in size) (log rank χ^2 = 5.449, P = 0.020, Figure 1A), well differentiated (log rank χ^2 = 12.798, P = 0.000, Figure 1B), of T1 or T2 stage invasion depth (log rank χ^2 = 4.745, P = 0.029, Figure 1C), without lymph node involvement (log rank χ^2 = 6.647, P = 0.010, Figure 1D), and at an early clinical stage (log rank χ^2 = 4.615, P = 0.032, Figure 1E). No survival differences were observed in any of the other subgroups (Table 4). In addition, the Cox proportional hazard analysis of survival demonstrated that the *FGFR4* Gly388Arg polymorphism was not an independent prognostic factor for GC patients (data not shown).

DISCUSSION

In the present study, the overall frequencies of the Gly/Gly, Gly/Arg, Arg/Arg genotypes and Arg388 allele in healthy controls were 33.6%, 48.0%, 18.4% and 42.3%, respectively. As shown in Table 5, the frequencies of the Arg allele or Arg/Arg genotypes in our study were similar to those of the Chinese patient populations reported by Chen *et al*[27], Zhu *et al*[28], Ma *et al*[29]and Yang *et al*[30]. Interestingly, the Arg388 allele frequencies in the Chinese population are much higher than those in Caucasian cohorts. In a meta-analysis by Xu *et al*[31], the Arg

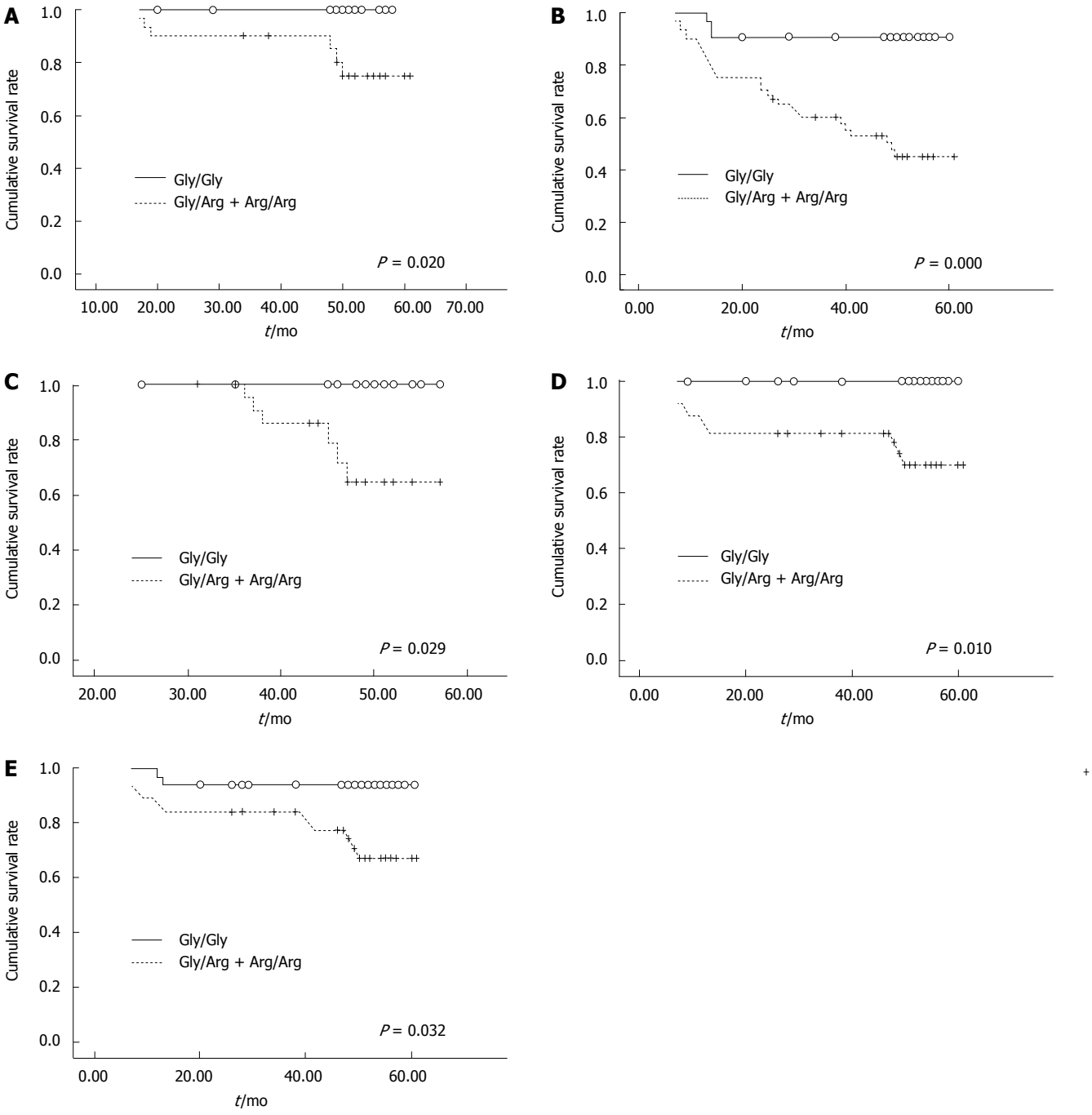

Figure 1 A considerable difference was found between patients with the Gly/Gly genotype and patients with Gly/Arg or Arg/Arg genotypes after stratified Kaplan-Meier survival analysis. A: Patients with tumor size ≤ 3 cm; B: Patients with well-differentiated gastric cancer (grades Ⅰ and Ⅱ); C: Patients classified as stage T1 or T2; D: Patients with no lymph node involvement; E: Patients at an early clinical stage (Ⅰ/Ⅱ).

allele was more highly represented among controls of Asian descent than controls of European and African-American descent. Our findings also support this result, which is contrary to a previous study that reported approximately 50% homo- or hetero-zygous carriers of the Arg allele in healthy controls independent of ethnic background[26].

For the first time, we report the distribution of the *FGFR4* Gly388Arg genotypes and alleles in both GC patients and matched healthy controls. No differences were found between GC patients and healthy controls. Our findings suggest that this polymorphism is not a risk factor for GC initiation in the Chinese population. This result is consistent with previous reports on several cancer types, including breast cancer and lung cancer, in different races[10,18,24]. However, it has been reported that the Arg388 allele is associated with an increased risk of prostate cancer[13,14]. Moreover, this polymorphism also plays a role in some types of non-cancer disease initiation. Zhu *et al*[28] and Ma *et al*[29] found that the *FGFR4* Gly388Arg polymorphism can act as a protective factor against coronary artery disease in the Chinese popula-

Table 4 Influence of fibroblast growth factor receptor 4 Gly388Arg polymorphism on gastric cancer survival

	Total *n* = 257	Gly/Gly *n* = 100	Gly/Arg + Arg/Arg *n* = 157	Log rank χ^2 value	*P* value
Age (yr)					
< 60	98	37	61	1.459	0.227
⩾ 60	159	63	96	1.734	0.188
Sex					
Male	188	79	109	0.041	0.839
Female	69	21	48	0.018	0.894
Tumor size					
⩽ 3 cm	55	24	31	5.449	0.020
> 3 cm	202	76	126	0.308	0.579
Differentiation					
G1 + G2	94	33	61	12.798	0.000
G3 + G4	163	67	96	2.637	0.104
Invasion depth					
T1 + T2	46	19	27	4.745	0.029
T3 + T4	211	81	130	0.037	0.848
N stage					
N0	73	24	49	6.647	0.010
N1 + N2 + N3	184	76	108	0.024	0.876
M stage					
M0	235	94	141	0.027	0.869
M1	22	6	16	0.139	0.710
Clinical stage					
Ⅰ + Ⅱ	91	33	58	4.615	0.032
Ⅲ + Ⅳ	166	67	99	0.048	0.827
Chemotherapy (5-fluorouracil and cisplatinum)					
Yes	156	55	101	0.019	0.891
No	51	21	30	0.442	0.506
HER2 status					
Negative	157	54	103	0.458	0.499
Positive	36	15	21	1.014	0.314

FGFR4: Fibroblast growth factor receptor 4; GC: Gastric cancer; HER2: Human epidermal growth factor receptor 2.

Table 5 Frequency of the codon 72 genotype

	Gly/Gly	Gly/Arg	Arg/Arg
This study (China)	132 (33.6)	188 (48.0)	72 (18.4)
Chen *et al*[27] (China)	133 (29.1)	229 (50.1)	95 (20.8)
Zhu *et al*[28] (China)	231 (33.4)	346 (50.0)	115 (16.6)
Ma *et al*[29] (China)	243 (33.2)	368 (50.3)	121 (16.5)
Yang *et al*[30] (China)	123 (32.0)	195 (50.6)	67 (17.4)
Ma *et al*[14] (Japan)	67 (37.4)	87 (48.6)	25 (14.0)
Morimoto *et al*[15] (Japan)	39 (38.2)	50 (49.0)	13 (12.7)
Ho *et al*[34] (Singapore)	30 (34.1)	38 (43.2)	20 (22.7)
Bange *et al*[10] (Italy)	55 (44.7)	60 (48.9)	8 (6.5)
Spinola et al[11] (Italy)	112 (50.9)	83 (37.7)	25 (11.4)
Ho *et al*[35] (United Kingdom)	150 (51.5)	117 (40.2)	24 (8.2)
Wang *et al*[36] (United States-European)	53 (54.6)	40 (41.2)	4 (4.1)
Wang *et al*[36] (United States-African)	76 (80.9)	18 (19.1)	0 (0.0)
FitzGerald *et al*[13] (United States-European)	631 (50.4)	496 (39.6)	124 (9.9)
FitzGerald *et al*[13] (United States-African)	60 (75.0)	18 (22.5)	2 (2.5)

tion. Based on a recent case-control study, the *FGFR4* Gly388Arg polymorphism is also considered to be a genetic risk factor that contributes to the aggravation of gallstone disease[27]. Therefore, these findings may reflect a tissue-specific effect of this polymorphism.

In our study, we found a significant difference following stratification by tumor size, differentiation, invasion depth, lymph node involvement or clinical stage ($P < 0.05$). Clinical stage depends on invasion depth and lymph node involvement. Therefore, our results demonstrate that the *FGFR4* Gly388Arg polymorphism is a prognostic factor in relatively small (less than 3 cm), well-differentiated (grades Ⅰ and Ⅱ) and early-stage GC (stages Ⅰ and Ⅱ) tumors but not in the total cohort of GC patients, which is in contrast to previous reports by Ye *et al*[26]. Because this study (and that of Ye *et al*[26]) focused on patients of Chinese origin, we suggest two possibilities to explain the conflicting results. The first possibility is the sample size. One major strength of our study is its large size. Our study included a higher proportion of patients with large tumors and late-stage disease than the study by Ye *et al*[26]. Thus, our results are more reliable given the isolation of the target patients from a pool of varied cases. Second, another possible explanation for the discord is that different DNA analysis methods were used in these two studies. Our study used a direct sequencing approach for genotype analysis, which was a more reliable method than the PCR-RFLP approach used by Ye *et al*[26]. Moreover, we detected the association between HER2 status and the FGFR4 Gly388Arg polymorphism in GC for the first time. No correlation was observed between HER2 status and the FGFR4 genotype ($P = 0.532$), which was consistent with previous reports in breast cancer[32].

The biochemical function of the *FGFR4* Gly388Arg polymorphism in GC is still unclear. No correlation was found between the *FGFR4* genotype and mRNA expression by Ye *et al*[26], and therefore, we do not attribute the polymorphism's effect to FGFR4 up-regulation. The FGFR4 Arg388 allele may be in linkage disequilibrium with other genetic changes that contribute to poor prognosis in GC. A previous study indicated that 39 head and neck cancer cell lines harboring the FGFR4 Arg388 allele exhibited an increased sensitivity to cisplatinum[33]. In breast cancer, no significant survival differences between *FGFR4* genotypes were found in patients without adjuvant systemic therapy. However, with adjuvant systemic therapy, breast cancer patients with the Gly/Gly genotype exhibited better disease-free survival and overall survival duration. Notably, this association seemed to be attributable to relatively poor therapy response in Arg388 carriers[32]. In our study, 156 patients underwent chemotherapy (5-fluorouracil and cisplatinum) following surgery. However, the median survival time of patients with the Gly/Arg and Arg/Arg genotypes did not differ from that of patients with the Gly/Gly genotype who underwent chemotherapy. Therefore, our data do not provide any evidence to support the theory that the FGFR4 Gly388Arg polymorphism is associated with sensitivity to chemotherapy.

In conclusion, our results demonstrate that the *FGFR4* Gly388Arg polymorphism plays no role in the initiation

of GC in Chinese patients, but it may be a factor in the survival of patients harboring relatively small, well-differentiated tumors in early GC stages.

COMMENTS

Background

Gastric cancer (GC) is one of the most common cancers in the world. Surgical resection and chemotherapy are only effective for a limited number of patients, and their prognoses remain very poor. Accordingly, there is an urgent need for a better understanding of the genesis of GC to establish a sound basis for future treatments.

Research frontiers

A germline polymorphism in the fibroblast growth factor receptor 4 (FGFR4) gene resulting in an amino acid change from glycine to arginine was identified several years ago. The presence of the FGFR4 Arg388 allele is associated with decreased disease-free survival in many cancers, including breast, lung, colon, prostate, soft tissue sarcoma, melanoma, and head and neck squamous cell carcinoma. Recently, Ye *et al* reported that the FGFR4 Arg388 allele was an independent prognostic factor in Chinese patients with GC. To our knowledge, this is the only report on the association between the FGFR4 Gly388Arg polymorphism and the progression of GC in Chinese patients, and this association needs further confirmation. Moreover, no study has been conducted on the correlation between this polymorphism and the risk of GC.

Innovations and breakthroughs

The correlation between the *FGFR4* Gly388Arg polymorphism and the risk of GC was investigated for the first time, and the authors suggested that this polymorphism was not a risk factor for GC cancer initiation. Another interesting finding was that the Arg388 allele frequency was much higher in this Chinese population than in Caucasian cohorts. The authors also investigated the associations between the *FGFR4* Gly388Arg polymorphism and clinicopathological parameters and survival in a larger sample series using more reliable methods. This result demonstrates that this polymorphism may contribute to the survival of patients with relatively small, well-differentiated tumors in the early stages of GC.

Applications

The FGFR4 Gly388Arg polymorphism is helpful for predicting the prognosis of early-stage GC patients with relatively small, well-differentiated tumors.

Terminology

Single nucleotide polymorphism (SNP): Genetic polymorphisms are natural variants in the genomic DNA sequence that are present in more than 1% of the population. One SNP represents the DNA variations in a single nucleotide. SNPs are widely used to better understand disease processes, thereby paving the way for genetic-based diagnostics and therapeutics.

Peer review

The results are interesting and may convey a useful prognostic marker for some GC patients.

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BRIEF ARTICLE

Helicobacter pylori and Crohn's disease: A retrospective single-center study from China

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Abstract

AIM: To investigate the association between *Helicobacter pylori* (*H. pylori*) infection and the prevalence of Crohn's disease (CD).

METHODS: Subjects were selected from patients admitted the gastrointestinal (GI) department at The First Affiliated Hospital School of Medicine (Zhejiang University) for abdominal pain, hematochezia, diarrhea and other GI symptoms between January 2008 and September 2012. CD was diagnosed by endoscopy and biopsy. *H. pylori* infection was detected by a ^{14}C-urea breath test and culturing of the biopsy sample. Demographic, anthropometric and serologic data were collected for each patient. *H. pylori* infection rate was compared between CD and control groups, followed by a subgroup analysis based on extent and severity of CD. Student's *t*, Mann-Whiney *U*, and χ^2 tests were used to analyze the data.

RESULTS: A total of 447 patients were analyzed, including 229 in the CD group and 248 in the control group. There were no significant differences in age, sex, and rates of hypertension or diabetes. However, the CD group showed significantly higher rates of smoking history (34.9% *vs* 18.1%), alcohol intake (17.4% *vs* 8.1%), white blood cell count (9.7 ± 2.9 × 10^9/L *vs* 4.3 ± 0.9 × 10^9/L), and C-reactive protein (36.3 ± 20.8 mg/L *vs* 5.5 ± 2.3 mg/L) but lower body mass index (24.5 ± 2.0 kg/m^2 *vs* 26.0 ± 2.2 kg/m^2) than the control group. The *H. pylori* infection rate in the CD group was 27.1%, significantly lower than that of 47.9% in the control group. Furthermore, the *H. pylori* infection rates in patients with colonic, small intestine, ileocolonic and extensive CD were 31.1%, 28.9%, 26.8% and 25.9% respectively, all of which were significantly lower than in the control group. Finally, the *H. pylori* infection rates in patients with remission, moderate and severe CD were 34.3%, 30.7% and 22.0% respectively, which were also significantly lower than in the control group.

CONCLUSION: Lower *H. pylori* infection in CD patients suggests a correlation between bacterial infection and CD, suggesting caution when considering *H. pylori* eradication in CD patients.

Key words: Crohn's disease; *Helicobacter pylori*; Urea breath test; Biopsy; Pathogenesis; Inflammatory bowel disease; Bacteria

Core tip: The association between *Helicobacter pylori* (*H. pylori*) infection and Crohn's disease (CD) prevalence is still unclear. In this retrospective study, we collected 229 CD patients and 248 control subjects to investigate the risk factors for prevalence, extent and severity of CD. Through extensive analysis, we found significantly lower *H. pylori* infection rates in CD patients having different disease extent and severity, providing evidence for bacteria involvement in CD pathogenesis and serving as a reminder for clinicians to remain cautious when considering *H. pylori* eradication in CD patients.

Xiang Z, Chen YP, Ye YF, Ma KF, Chen SH, Zheng L, Yang YD, Jin X. *Helicobacter pylori* and Crohn's disease: A retrospective single-center study from China. *World J Gastroenterol* 2013; 19(28): 4576-4581 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i28/4576.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i28.4576

INTRODUCTION

Crohn's disease (CD) is a chronic and relapsing inflammatory disease affecting any part of the intestine, most commonly involving the distal ileal, ileocecal and colonic sections[1]. CD is more common in northern Europe and America than in southern Europe and developing countries, and its prevalence has increased since the mid-1970s[2,3]. The pathogenesis of CD is unclear and increasing evidence supports the hypothesis of combinational involvement of genetic predisposition, immune response, and the environment, especially the gut bacteria and antigens[4]. Though several pathogens have been considered as infectious agents in CD, conclusive data is still lacking[5]. Therefore, identifying the correlation between a potential bacteria and CD pathogenesis is of clinical importance.

Helicobacter pylori (*H. pylori*) belongs to the family of curved or spiral flagellated, Gram-negative microaerophilic bacterium that is thought to have co-existed with humans for over 5000 years[6,7]. Since its discovery in 1984[8], *H. pylori* has been characterized as the causative agent for peptic ulceration and has been implicated in various autoimmune diseases[9]. *Helicobacter* are also excellent colonizers of the gastrointestinal surface for their microaerophilic metabolism, spiral shape, and peculiar motility. Considering the immune regulation, the capacity for colonization, and the nature of autoimmune-related damage in CD, it is theoretically plausible that *H. pylori* infection may take part in the pathogenesis of CD.

In immunodeficient rodents, *Helicobacter hepaticus* and *Helicobacter bilis* induce a persistent inflammation of the colon and cecum[10,11]. Nevertheless, the observations from human studies were confusing. *Helicobacter* was absent or only detected in the intestinal mucosa of a small subgroup of patients in both an English and an Australian study[12,13]. In contrast, Bohr *et al*[14] identified enterohepatic *Helicobacter* species in patients with inflammatory bowel disease (IBD). Furthermore, a meta-analysis suggested a protective role of *H. pylori* infection in CD pathogenesis but the heterogeneity among enrolled studies and the possibilities of publication bias limited the confidence of these results[15]. Therefore, we conducted a large-scale case control study to investigate the association between *H. pylori* infection and different severity and types of CD.

MATERIALS AND METHODS

Ethics statement

The protocol was approved by the institutional review board at Zhejiang University and conducted in accordance with the Declaration of Helsinki. We followed guidelines from the STROBE statement when designing the study and preparing the manuscript[16]. Written informed consent was collected from all patients.

Patients

Study subjects were selected from patients who were admitted for abdominal pain, hematochezia, diarrhea, and other GI symptoms between January 2008 and September 2012. Patients either underwent both a *H. pylori* test and endoscopy (gastroscopy, colonoscopy, or capsule) screen during admission or had recorded evidence of current *H. pylori* infection and CD. For the CD group, diagnosis was based on endoscopy manifestation and biopsy, as adopted by the Asia-Pacific consensus[17]. Exclusion criteria included previous acid inhibition or *H. pylori* eradication, 5-aminosalicylic administration and differential diagnosis with intestinal tuberculosis, ulcerative colitis (UC), Behçet's disease, or ischemic colitis. The control group was comprised of patients who underwent the initial screening but were subsequently excluded by negative results for CD and other known GI diseases.

CD patients were further categorized into subgroups according to the severity and extent of disease following the Chinese IBD guidelines[18]. For convenience, we adopted the Harvey-Bradshaw index (HBI)[19], a simplified version of the Crohn's disease activity index (CDAI), to evaluate CD severity. This index comprises general conditions, degree of abdominal pain, frequency of diarrhea, existence of abdominal mass, and complications such as arthritis, nodular erythema, gangrenous pyoderma, aphthous stomatitis, fistula, and abscess. Scores of 0-4 were appointed to each parameter according to disease severity. The HBI score was the summary of scores from each parameter, where $\leqslant$ 4 was remission, 5-8 was moderate, and $\geqslant$ 9 was severe. In addition, according to the extent revealed from radiology and endoscopy, CD was further divided into small intestinal CD, colonic CD, ileocolonic CD, and extensive CD, where the involved scale was over 100 cm.

Analysis of demographic, anthropometric and serologic data

Demographic and anthropometric data were retrieved

from the medical records of enrolled patients and included age, sex, smoking history, alcohol intake history (with positive designation made according to the Chinese guideline of alcoholic liver disease[20]), hypertension (defined as a patient on antihypertensive drug for blood pressure over 140/90 mmHg), body mass index (BMI; calculated as weight in kilograms divided by height in meters squared), and diabetes mellitus (DM; defined as fasting glucose $\geqslant$ 7.0 mmol/L or with past history of diagnosed DM). Patient blood samples were routinely gathered and tested for general condition and inflammation, including C-reactive protein (CRP) and complete blood cell counts. The main complications of CD were also recorded, including fistula and obstruction.

^{14}C-urea blood test and biopsy sample culture

H. pylori infection was detected by a ^{14}C-urea blood test (UBT) and biopsy sample culture. Serum *H. pylori*-IgG was not accepted as a diagnostic tool, as it does not reflect the current infection status. First described in 1989[21], ^{14}C-UBT is considered a rapid diagnostic procedure for *H. pylori* detection for its ability to convert urea to ammonia and carbon dioxide. UBT is also recommended in leading society guidelines as a preferred non-invasive choice for *H. pylori* detection[22]. According to the manufacturer's instruction (Headway, Shenzhen, China), patients were given a tablet of urea labeled with an uncommon isotope of radioactive carbon-14. In the following 30 min, the amount of isotope labeled carbon dioxide was measured in exhaled breath by scintillation. A positive result indicated the existence of *H. pylori*. For biopsy sample culture, samples from the gastric antrum obtained by gastroscopy were cultured for *H. pylori* as previously described[23].

Statistical analysis

Data were assessed for normality, and log-transformed as needed. Quantitative variants were expressed as mean ± SD, and analyzed by Student's *t* test or Mann-Whiney *U* test. For qualitative variants, percentages or frequencies were calculated and a χ^2 test was used for comparison. SPSS 17.0 software (Chicago, IL, United States) was used for all statistical analyses, and a P value < 0.05 was considered statistically significant.

RESULTS

Characteristics of study subjects

Following careful review of medical records, we identified 1563 patients that had been admitted to our ward for abdominal pain, hematochezia, diarrhea, and other GI symptoms over the past five years. Among these patients, 1231 were selected according to their having undergone both endoscopy and *H. pylori* test. The endoscopic diagnosis of CD was given to 921 patients by colonoscopy, 603 by gastroscopy, and 105 by capsule endoscopy. Furthermore, many of the patients had received more than one type of endoscopy. For the *H. pylori* infection test, 603 patients were investigated by biopsy sample culturing and 628 patients were investigated by ^{14}C-UBT. Among these 1231 subjects, 287 were diagnosed as CD by endoscopy and/or biopsy confirmation, with 18 of these patients having already received 5-aminosalicylic therapy, 11 being under proton pump inhibitor (PPI) therapy for reflux symptoms, and 21 having achieved *H. pylori* eradication. Among the remaining patients, 8 were further excluded due to positive serum *H. pylori*-IgG but negative ^{14}C-UBT or biopsy sample culture results. Finally, a total of 229 patients were enrolled into the CD group. Among them, 132 and 97 patients had ^{14}C-UBT and biopsy sample culture, respectively.

Among 1231 subjects, 251 were diagnosed with UC, 279 with different degrees of hemorrhoids, 41 with ischemic colitis, 7 with antibiotic-associated colitis, 5 with radiation enterocolitis, 11 with intestinal tuberculosis, 71 with sigmoiditis and proctitis, and 2 with Behçet's disease. The remaining 277 patients had GI symptoms but normal endoscopy and biopsy results. Nevertheless, there were still 14 patients under anti-acid therapy (10 with PPI and 4 with H_2 receptor antagonist) and 15 patients in which *H. pylori* had been eradicated. Finally, there were 248 patients enrolled into control group. Among them, 147 and 101 patients had ^{14}C-UBT and biopsy sample cultures, respectively.

Demographic, anthropometric and serologic data of enrolled patients

The average age and sex distribution of patients were balanced between two groups (Table 1). Differences in the rates of hypertension and DM between the two groups were not significant. However, BMI was significantly lower in the CD group than that in the control group, while the rate of smoking history was approximately twice that of the CD group ($P < 0.01$), reinforcing a correlation between smoking history and CD pathogenesis. In addition, percentage of alcohol intake was also significantly higher in the CD group. Finally, the two inflammation-associated markers, CRP and white blood cell (WBC) count, were significantly higher in the CD group, supporting the potential involvement of inflammation in CD.

Association between CD and H. pylori infection

Total *H. pylori* infection rate in the CD group was 27.1%, significantly lower than that of 47.9% in the control group. In the CD group, there were 45 patients with colonic CD, 28 with small intestine CD, 112 with ileocolonic CD, and 34 with extensive CD. In a subgroup analysis, all of the above-mentioned CD subgroups had significantly lower *H. pylori* infection rate than that in the control group, but the differences among these subgroups did not reach statistical significance (Table 2). We further divided the CD group into three subgroups according to severity determined by endoscopic appearance. Briefly, there were 32, 88 and 109 patients

Table 1 Demographic, anthropometric and serologic data of enrolled patients

Group	CD (n = 229)	Control (n = 248)	P value
Age (yr)	46.2 ± 10.2	46.8 ± 9.4	0.79
Sex (male/female)	133/96	141/107	0.07
Smoking history	34.90%	18.10%	< 0.01
Alcohol intake	17.40%	8.10%	< 0.01
BMI (kg/m^2)	24.5 ± 2.0	26.0 ± 2.2	< 0.01
Hypertension	14.80%	16.10%	0.09
Diabetes,	7.90%	6.90%	0.13
CRP (mg/L)	36.3 ± 20.8	5.5 ± 2.3	< 0.01
WBC (× 10^9/L)	9.7 ± 2.9	4.3 ± 0.9	< 0.01

BMI: Body mass index; CRP: C-reactive protein; WBC: White blood cell.

Table 2 *Helicobacter pylori* infection rate between different Crohn's disease types n (%)

Group	*H. pylori*-positive	*H. pylori*-negative	P value
CD	62 (27.1)	167 (72.9)	< 0.01
Colonic CD	14 (31.1)	31 (68.9)	< 0.01
Small intestine CD	11 (28.9)	27 (71.1)	< 0.01
Ileocolonic CD	30 (26.8)	82 (73.2)	< 0.01
Extensive CD	7 (25.9)	27 (74.1)	< 0.01
Control	119 (47.9)	129 (52.1)	

H. pylori: *Helicobacter pylori*; CD: Crohn's disease.

in the CD subgroups of remission, moderate CD, and severe CD, with corresponding *H. pylori*-positive rates of 34.3%, 30.7% and 22.0% respectively (Table 3). All three subgroups had significantly lower *H. pylori* infection rates than the control group. Nevertheless, though there was a decrease trend in *H. pylori* infection rate from the CD remission group to the severe CD group, there was no significant difference among these three groups.

DISCUSSION

Crohn's disease can affect the entire digestive system from mouth to anus, but it is most commonly seen in the final segment of the small bowel and the first part of the colon. The frequency of CD has significantly increased in the last century, becoming a heavy economic burden[24]. The etiology of CD is still not completely understood[25], although *NOD2/CARD15* was the first CD susceptibility gene to be discovered[26]. Oxidative stress, autophagy, endoplasmic reticulum stress and other molecular pathways have been correlated with CD prevalence[27,28]. Moreover, an infectious organism might be the initiating factor for CD pathogenesis. This hypothesis was supported by evidence from animal models showing that spontaneous colitis did not develop in a germ-free environment[29]. Nevertheless, none of suggested bacterial causes have been conclusively proven[5].

The role of *H. pylori* in IBD pathogenesis has been enticing. Generally, *H. pylori* had two main subgroups: gastric *Helicobacter* that preferentially colonize the stomach, and enterohepatic *Helicobacter* that infect the intestinal or hepatobiliary system[30]. Accumulating evidence from gene knockout rodents indicate that the presence of enterohepatic helicobacter worsens the severity or hastens the development of colitis[31,32]. A more causative role was suggested by the observation that *Helicobacter muridarum* can provoke CD in severe combined immunodeficiency mice upon receipt of T cells[33]. In addition, lower *H. pylori* infection rate in CD patients was not influenced by antibiotic use[34]. However, the results from human studies are confusing. The largest study examining the association between *H. pylori* infection and CD was conducted in the Netherlands by Wagtmans *et al*[35], which enrolled 386 CD patients and 277 controls. Though their results supported the positive association between *H. pylori* infection and CD prevalence, the credibility of these findings was weakened by the use of serum *H. pylori*-IgG as the diagnostic tool. A meta-analysis was conducted that supported the involvement of *H. pylori* infection in CD, but study heterogeneity and publication bias decreased its credibility[15]. In contrast, two independent studies found higher *H. pylori* infection in CD patients than in controls (12% *vs* 4% and 14.0% *vs* 1.4%, respectively)[14,36]. Furthermore, other studies investigating *Helicobacter* species in human colon also failed to find any correlation with CD[37,38].

To tackle this discordancy, we retrospectively investigated the association between *H. pylori* infection and CD in a large case control study of Chinese patients. The initial results showed a significantly lower *H. pylori* infection rate in the CD group, which is in accordance with the previous meta-analysis[15]. The ^{14}C-UBT and biopsy sample culture had higher sensitivity and specificity than the serum *H. pylori*-IgG test, increasing the credibility of these findings. The significantly lower BMI in CD patients may be due to malnutrition caused by diarrhea and other GI symptoms. Based on subgroup analysis (Tables 2 and 3), we found significantly lower *H. pylori* infection in each CD subgroup and a trend of decreased *H. pylori* infection paralleling with increased CD severity, increasing the correlation between *H. pylori* infection and CD. Theoretically, it is possible for a protective role of *H. pylori* infection in CD[39]. In detail, *H. pylori* is able to decrease immune-mediated intestinal injury by triggering Th1 dominated cell defense[40] and inhibit other bacterially-induced mucosal damage by inducing antibacterial peptide production[41].

Several limitations of this study should be acknowledged. First, *H. pylori* infection in colon biopsy was not detected, which may decrease disease occurrence rate. Furthermore, other members of the *Helicobacter* family are associated with the development of gut inflammation and some are found more commonly in people with IBD when compared to healthy controls. However, these members colonize the lower gut, rather than having a location limited to the stomach. Therefore, while these data are convincing, it would be helpful to detect them in another independent experiment. Second, it is better to use ^{13}C-UBT instead of ^{14}C-UBT, since the for-

Table 3 *Helicobacter pylori* infection between different severity of Crohn's disease and control *n* (%)

Group	*H. pylori*-positive	*H. pylori*-negative	*P* value
CD	11 (34.3)	21 (65.7)	< 0.01[1]
Colonic CD	27 (30.7)	61 (69.3)	< 0.01[1]
Small intestine CD	24 (22.0)	85 (88.0)	< 0.01[1]
Control	119	129	

[1]*vs* control group. *H. pylori*: *Helicobacter pylori*; CD: Crohn's disease.

mer has no radiation and is safer for patients[42]. We have plans to implement this technique in our laboratory in the future. Third, wireless capsule endoscopy was used to detect small intestine CD. Though the effect of capsule endoscopy in small intestine CD diagnosis has been recognized[43], the lack of biopsy results may decrease its credibility. It would be helpful to include double balloon small bowel endoscopy. Fourth, the trend of decreased *H. pylori* infection paralleling with increased CD severity should be repeated in a larger clinical trial for statistical significance. Fifth, it is better to report *H. pylori* infection rate in IBD, where *H. pylori* in UC should be reported. We found a 30.5% *H. pylori* infection rate in UC patients and are currently preparing these data for submission. Finally, the causative effect of *H. pylori* infection in CD cannot be established through case control studies and further prospective clinical trial data are necessary.

In conclusion, our results provide evidence for the involvement of *H. pylori* in CD prevalence. These findings should serve as an important reminder to clinicians when considering *H. pylori* eradication in CD patients.

COMMENTS

Background

Crohn's disease (CD) is a chronic and relapsing inflammatory disease affecting any part of the intestine, with distal ileal, ileocecal and colonic regions most commonly involved. CD is more common in Northern Europe and America than in Southern Europe and developing countries, with increased incidence since the mid-1970s and unknown etiologies.

Research frontiers

Increasing evidence supports the combinational involvement of genetic predisposition, immune response, and environment, especially gut bacteria and antigens. Though some pathogens have been considered as infectious agents in CD, conclusive data is still lacking. Considering the immune regulation and colonization capacity of *Helicobacter pylori* (*H. pylori*) and the nature of autoimmune-related damage in CD, it is plausible that *H. pylori* infection may take part in the etiology of CD. However, results in humans remain unclear and contradictory. While *Helicobacter* was absent or only detected in the intestinal mucosa of a few patients in English and Australian studies, another study identified enterohepatic *Helicobacter* species in patients with inflammatory bowel disease. Furthermore, a meta-analysis suggested a protective role of *H. pylori* infection in CD pathogenesis. However, the heterogeneity among enrolled studies and the possibilities of publication bias limited the confidence of those results.

Innovations and breakthroughs

The authors conducted a large-scale case control study to investigate the association between *H. pylori* infection and different severity and type of CD. These findings are the first example of a significant correlation between *H. pylori* infection rate in CD patients and different subtypes.

Applications

Lower *H. pylori* infection in CD patients provides evidence for bacterial involvement in the pathogenesis of CD and reminds clinicians remain cautious when considering *H. pylori* eradication in CD patients.

Peer review

Their results supported the potential involvement of *H. pylori* infection in CD pathogenesis and raised concern for the necessity of *H. pylori* eradication in CD patients. This manuscript provides some key information, and builds on the published literature. The authors should be able to enhance this by extensive revisions.

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BRIEF ARTICLE

Antinociceptive effect of berberine on visceral hypersensitivity in rats

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Abstract

AIM: To assess the protective effect of berberine administration and the role of nitric oxide (NO) in visceral hypersensitivity.

METHODS: Fifty male Sprague-Dawley rats were randomly assigned to five groups. An inflammatory bowel disease model was induced in rats by intracolonic instillation of 1 mL 4% acetic acid at 8 cm proximal to the anus for 30 s and restraint stress. After subsidence of inflammation on day 7 of the experiment, the rats were subjected to rectal distension, performed by a balloon (6-Fr, 2 mm external diameter, disposable silicon balloon-urethral catheter for pediatric use) which was rapidly inflated with increasing volumes of prewarmed (37 ℃) water (0.1, 0.2, 0.3, 0.4, 0.6, 0.8 and 1 mL) for 30 s at four-minute intervals, and then the abdominal withdrawal reflex (AWR) and the level of fecal output were measured, respectively. AWR scores either 0, 1, 2, 3 or 4 were obtained by blinded observers. Rats had been pretreated with berberine or aminoguanidine (NO synthetase inhibitor) or berberine + aminoguanidine before measurement.

RESULTS: The rats in the placebo group showed a hypersensitive response to rectal distension (2.69 ± 0.08 *vs* 1.52 ± 0.08, P = 0.000) and defecated more frequently than those in the control group (5.0 ± 0.16 *vs* 0.44 ± 0.16, P = 0.000). Comparing the berberine with placebo group, the AWR scores were reduced for all distension volumes and were significant at 0.2-1 mL (1.90 ± 0.08 *vs* 2.69 ± 0.08, P = 0.000), while the numbers of hard pellets, soft pellets, formless stools, and total fecal output in the placebo group were significantly larger than in the berberine group (5.0 ± 0.16 *vs* 2.56 ± 0.16, P = 0.000). Administration of aminoguanidine or berberine + aminoguanidine before VH score measurement reversed the antinociceptive effect of berberine (2.52 ± 0.08 *vs* 1.90 ± 0.08, P = 0.000; 2.50 ± 0.08 *vs* 1.90 ± 0.08, P = 0.000). The numbers of hard pellets, soft pellets, formless stool, and total of fecal output in aminoguanidine group were significantly larger than the corresponding values in control group, berberine group, and berberine + aminoguanidine group (4.81 ± 0.16 *vs* 0.44 ± 0.16, P = 0.000; 4.81 ± 0.16 *vs* 2.56 ± 0.16, P = 0.000; 4.81 ± 0.16 *vs* 3.75 ± 0.16, P = 0.000). The berberine and berberine + aminoguanidine groups showed reduced defecation, but aminoguanidine alone did not reduce defecation (2.56 ± 0.16 *vs* 4.81 ± 0.16, P = 0.000; 3.75 ± 0.16 *vs* 4.81 ± 0.16, P = 0.000).

CONCLUSION: Berberine had an antinociceptive effect on visceral hypersensitivity, and NO might play a role in this effect.

Key words: Berberine; Irritable bowel syndrome; Visceral hypersensitivity; Nitric oxide

Core tip: Berberine had an antinociceptive effect on visceral hypersensitivity. This effect was reduced by nitric oxide (NO) synthetase inhibitor, thus NO might play a role in the effect of berberine.

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INTRODUCTION

It is believed that chronic visceral hypersensitivity (VH), abnormal gastrointestinal motility and altered central processing may be major pathophysiological mechanisms of irritable bowel syndrome (IBS)[1]. Gut hypersensitivity may lead to alterations in gut motility by disturbing regulatory reflex pathways and secretory function[2]. These abnormalities typically reflect the symptom pattern of IBS, which is characterized by abdominal pain or discomfort, and is associated with alterations in defecation frequency, stool passage, and stool form[3,4].

According to the recent Rome Ⅲ Criteria[5], IBS can be diagnosed based on at least 3 mo, with onset at least 6 mo, of recurrent abdominal pain or discomfort associated with two or more of the followings: (1) improvement with defecation; (2) onset associated with a change in stool frequency; and (3) onset associated with a change in stool form (appearance). Additionally, IBS patients are further subdivided into IBS with diarrhea, IBS with constipation, mixed IBS with diarrhea and unspecified IBS.

VH is a consistent finding in a large proportion of patients with IBS and provides a physiological basis for the development of IBS symptoms[2,6]. However, the exact mechanism of its action is still unclear. Recently, it has been documented that increasing the nitric oxide (NO) level in the extracellular space of the target tissue is one of the major mechanisms involved and this has been clarified in recent molecular studies[7]. There is growing evidence from previous studies that NO plays an important role in pain transmission and the antinociceptive action on VH or peritoneal pain[2,6,8-10].

It has been discovered that some drugs can be used to attenuate VH in IBS patients[11]. However, many drug treatments have not been satisfactory, with intractable adverse effects. Therefore, it is necessary to seek an effective and low-cost treatment for IBS. Berberine (Coptis chinensis Franch, var. asperma Don, family Ranunculaceae) is a botanical alkaloid isolated from the root and bark of *Rhizoma coptidis*, an ancient Chinese herb that has been used to treat gastroenteritis for many years, which is preferred for its inexpensiveness and low incidence of adverse effects[12]. It has been demonstrated that berberine has multiple pharmacological activities including anti-inflammatory[13], antimicrobial[14], anticancer[15,16], antidiabetic[17], antiarrhythmic[18], and antiseptic[19] effects. According to former studies, berberine has a significant effect in the treatment of experimental colitis[20-22]. Further evidence has shown that, in relation to the NO pathway, berberine has a significant effect on ethanol-induced gastric ulcers[23], endothelial progenitor cell mobilization and function[24], hyperglycemia-induced cellular injury and endothelial dysfunction[25], the early phase of hepatocarcinogenesis[26], and a rat model of Alzheimer's disease[27]. To establish whether berberine has a beneficial effect in IBS patients through reversal of VH, we examined the effects of berberine in a validated rodent model in which acute inflammation of the colon was associated with VH. The aim of this study was to evaluate whether berberine treatment prevents progression of VH to colorectal distension (CRD), and the involvement of NO in these effects.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, weighing 270-300 g, were obtained from the Animal Facility of Southeast Hospital, Zhangzhou, China. The rats were housed individually in an access-restricted room with controlled conditions (22 ± 1 ℃ and 65%-70% humidity) with free access to standard laboratory food and water. All the experimental protocols in this study were reviewed and approved by the Animal Studies Ethics Committee of Southeast Hospital.

Experiment model

The rats were lightly anesthetized with ether after an overnight fast and colitis was induced by intracolonic instillation of 1 mL 4% acetic acid at 8 cm proximal to the anus for 30 s. Then, 1 mL PBS was instilled to dilute the acetic acid and flush the colon. The control animals were handled identically except that 1 mL saline was instilled instead of 4% acetic acid. Rats were left to recover from colitis for 6 d, and were used for the experiments 7 d after induction of colitis.

Histological examination of inflammation

To examine the extent of colonic inflammation, histological samples were collected at the selected time points (2 and 7 d post-enema in two rats of each group). Sections with a thickness of 5 μm were cut and processed for hematoxylin and eosin staining. The coded slides were analyzed by a pathologist blinded with regard to the treatment group and the time points.

Rectal distension procedure

At 7 d post-enema, eight rats in each group were used for studying progression of VH to CRD. A 6-Fr (2 mm external diameter) disposable silicon balloon-urethral catheter for pediatric use was used. The maximal inflation volume for the balloon was 1 mL and the length of the maximally inflated balloon was 1.2 cm. After an overnight fast, the animals were lightly anesthetized with

ether, and the balloon was carefully inserted into the rectum until the premarked line on the catheter (2 cm distal from the end of the balloon) was positioned at the anus. The catheter was taped to the base of the tail to prevent displacement. After this procedure, the rats were placed in a transparent cubicle (20 cm × 8 cm × 8 cm) on a mirror-based, elevated platform while still sedated, and were allowed to recover and adjust for a minimum of 30 min before testing. The catheter was connected to a pressure transducer *via* a three-way connector. The signals from pressure transducer were processed and recorded on an IBM-compatible computer.

After the animals were fully awake and adjusted to the environment, ascending-limit phasic distension (0.1, 0.2, 0.3, 0.4, 0.6, 0.8 and 1.0 mL) was applied for 30 s every 4 min to induce CRD. The balloon was distended with prewarmed (37 ℃) water. We chose this protocol because hypersensitivity was reported to be best elicited by rapid phasic distension. The abdominal withdrawal reflex (AWR) was semiquantitatively scored as previously described[4]. The AWR score was assigned as follows: 0 = no behavioral response to distension; 1 = brief head movements followed by immobility; 2 = contraction of abdominal muscle without lifting of the abdomen; 3 = lifting of the abdomen; and 4 = body arching and lifting of pelvic structure.

After the experiments, the balloon was withdrawn and immersed in 37 ℃ water. The compliance of balloon was not infinite, therefore, we measured intraballoon pressure at each distension volume in 37 ℃ water, and digitally subtracted the value from that recorded during the CRD experiment to calculate the intrarectal pressure.

Restraint stress procedure

The rats were housed individually with no restrictions on food intake before testing. At 7 d post-enema, eight rats from each group were placed in restraint cages (5 cm × 5 cm × 20 cm), which could limit their body movement, but not restrict breathing. The rats were in the restraint cages for 3 h at room temperature. The feces excreted during restraint stress were divided into three types: hard pellet, soft pellet, and formless, and counted separately.

Experimental protocol

Ten healthy rats without treatment served as controls. In the placebo group, IBS was induced as described above and eight rats were treated once with physiological saline 1 d after enema. In the berberine group, IBS was induced as described above and eight rats were treated once daily with berberine (50 mg/kg) 1 d after enema. In the aminoguanidine group, eight rats were treated once daily with aminoguanidine (100 mg/kg) *via* intraperitoneal injection 1 d after enema. In the berberine + aminoguanidine group, eight rats were treated once daily with berberine (50 mg/kg) 1 d post-enema, and then were treated once daily with aminoguanidine (100 mg/kg) *via* intraperitoneal injection.

Statistical analysis

Data were expressed as mean ± SD. Significant differences between the three groups (AWR score) at each distension volume were statistically analyzed using ANOVA. The relationship between the intraballoon volume and intrarectal pressure was determined by linear regression analysis, and the estimated slope coefficients and intercepts were compared between groups using ANOVA. The level of fecal output was compared using ANOVA and further analyzed using Bonferroni's or Tamhane's *T2* test. Differences with $P < 0.05$ were considered to be significant. Multiple comparisons between the groups were corrected by SPSS version 13.0 software.

RESULTS

Histology of colonic tissue

Figure 1 shows the histology of the distal colon at days 2 and 7 after acetic acid instillation in the control group, placebo group, berberine group, aminoguanidine group, and berberine + aminoguanidine group. Mucosal hemorrhage with an inflammatory infiltrate in the lamina propria and the edematous submucosa were observed in the IBS model group, berberine group, aminoguanidine group and berberine + aminoguanidine group 2 d after acetic acid instillation. At day 7 after induction of colitis, all signs of inflammation had disappeared. No remarkable inflammatory features were detected at 7 d after acetic acid instillation in each group.

Detection of VH

The AWR scores were recorded in at least eight rats in each group after CRD was induced. The changes in AWR score paralleled the balloon volume in CRD, which confirmed that the AWR score reflected the intensity of distension. Comparing the berberine with placebo group, the AWR scores were reduced for all distension volumes and were significant at 0.2-1.0 mL (1.90 ± 0.08 *vs* 2.69 ± 0.08, $P = 0.000$). These data indicated that progression of VH to CRD was attenuated by berberine (Figure 2A). As shown in Figure 2A, rats in the placebo group showed a hypersensitive response to the ascending-limit phasic rectal distension, and berberine effectively reduced VH (2.69 ± 0.08 *vs* 1.90 ± 0.08, $P = 0.000$). However, VH was not effectively reduced in the aminoguanidine and berberine + aminoguanidine groups. The pain threshold (minimal volume to induce AWR 2) was measured in rats that underwent CRD. As shown in Figure 2, berberine significantly increased the nociceptive threshold in rats. Administration of aminoguanidine or berberine + aminoguanidine before VH score measurement reversed the antinociceptive effect of berberine (2.52 ± 0.08 *vs* 1.90 ± 0.08, $P = 0.000$; 2.50 ± 0.08 *vs* 1.90 ± 0.08, $P = 0.000$). In order to examine whether VH in rats was related to changes in rectal compliance, we compared the intraballoon volume-intrarectal pressure relationship in the five groups. A distension volume of 0.2-1.0 mL and

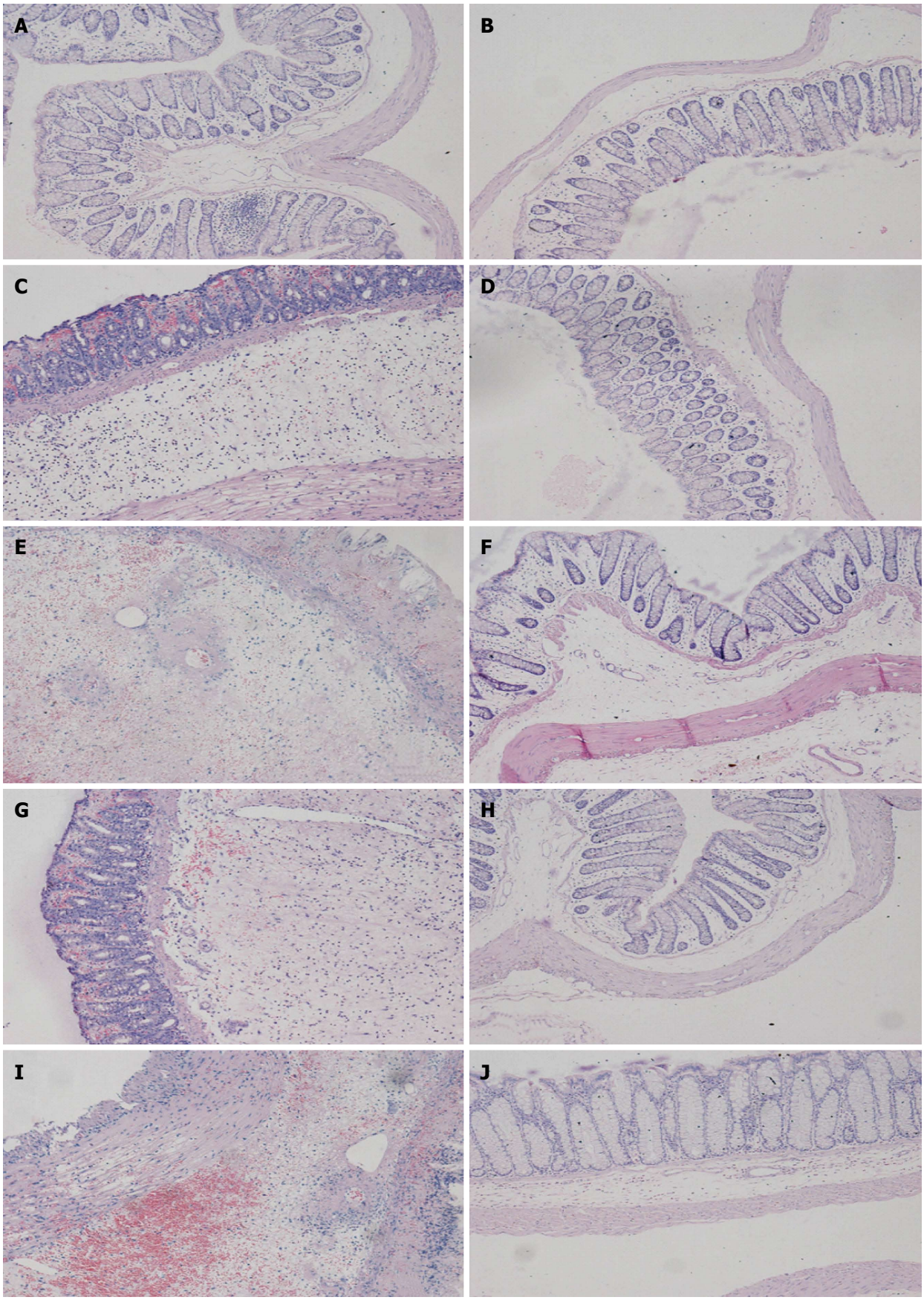

Figure 1 Photomicrographs (hematoxylin and eosin stain, × 100) of distal colon at 2 and 7 d, respectively. A, B: Control group; C, D: Placebo group; E, F: Berberine group; G, H: Aminoguanidine group; I, J: Berberine + aminoguanidine group. At 2 d, histological inflammatory features including mucosal hemorrhage, submucosal edema, and inflammatory infiltration in the lamina propria and the submucosa were observed in the placebo, berberine, aminoguanidine, and berberine + aminoguanidine groups (A, C, E, G, I). At 7 d, there was no marked inflammatory feature compared with the control group (B, D, F, H, J).

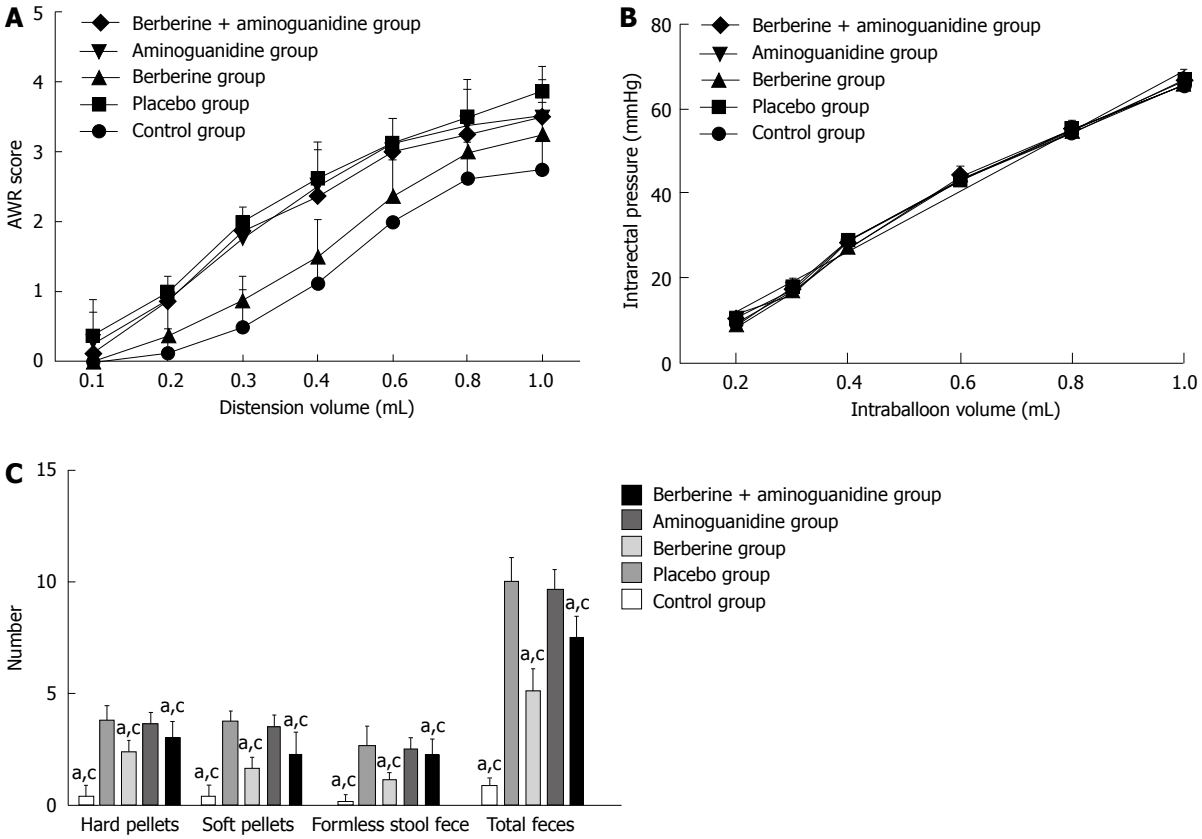

Figure 2 Summarized plots. A: Colorectal distension-induced abdominal withdrawal reflex (AWR) in each group. Comparing the berberine and placebo groups, the AWR scores were reduced at all distension volumes and were significant at 0.2-1.0 mL. The AWR score in the berberine group was significantly lower than in the aminoguanidine and berberine + aminoguanidine groups; B: The relationship between intraballoon volume and intrarectal pressure in each group. The distension volume from 0.2 to 1.0 mL and the corresponding intrarectal pressure were plotted for regression analysis. Intrarectal pressure was linearly increased as the balloon was inflated. The fitted functions of the five groups were not significantly different; C: Restraint-stress-induced defecation in each group. Defecation in the placebo and aminoguanidine groups was significantly more frequent than in the control, berberine and berberine + aminoguanidine groups ($^{a}P < 0.05$ *vs* placebo group; $^{c}P < 0.05$ *vs* aminoguanidine group). The berberine and berberine + aminoguanidine groups showed reduced defecation, but aminoguanidine group alone did not effectively reduce defecation.

the corresponding intrarectal pressure were plotted for regression analysis. Intrarectal pressure increased linearly as the balloon was inflated ($r = 0.9758$, $P < 0.001$, in the control group; $r = 0.9842$, $P < 0.001$, in the placebo group; $r = 0.9822$, $P < 0.001$, in the berberine group; $r = 0.9773$, $P < 0.001$ in the aminoguanidine group; and $r = 0.9827$, $P < 0.001$ in the berberine + aminoguanidine group). The fitted functions of the five groups were not significantly different (Figure 2B).

Restraint-stress-induced defecation

As demonstrated in Figure 2C, the numbers of hard pellets, soft pellets, formless stools, and total fecal output in the placebo were significantly larger than in the berberine groups (5.0 ± 0.16 *vs* 2.56 ± 0.16, $P = 0.000$). As shown in Figure 2C, the number of hard pellets, soft pellets, formless stool,and total of fecal output in aminoguanidine group were significantly larger than the corresponding values in control group, berberine group, and berberine + aminoguanidine group (4.81 ± 0.16 *vs* 0.44 ± 0.16, $P = 0.000$; 4.81 ± 0.16 *vs* 2.56 ± 0.16, $P = 0.000$; 4.81 ± 0.16 *vs* 3.75 ± 0.16, $P = 0.000$). The berberine and berberine + aminoguanidine groups showed reduced defecation, but aminoguanidine alone did not reduce defecation (2.56 ± 0.16 *vs* 4.81 ± 0.16, $P = 0.000$; 3.75 ± 0.16 *vs* 4.81 ± 0.16, $P = 0.000$).

DISCUSSION

This study was performed in order to clarify the effects of berberine administration on VH in a rat model of IBS. Berberine effectively attenuated the heightened visceral nociceptive response, that is, an increase in AWR score to CRD, in rats recovering from experimental colitis. In the placebo group, 7 d after instillation of acetic acid when there was no sign of inflammation in the colon, these rats still had VH and a high frequency of defecation in response to restraint stress. Stool form in the placebo group was softer and more shapeless than in the control group. These findings are in accordance with the clinical symptoms in IBS patients, but there is a great dilemma in using this model to investigate the effect of drugs on IBS.

To avoid the ambiguity that any sign of improvement can be interpreted as a drug effect on colitis, histopathological parameters of inflammation in each group, 2 and 7 d after acetic acid instillation, were evaluated and there

was no difference. Therefore, we concluded that berberine administration, at least at the dose used in our study, had neither a positive nor negative effect on the histopathological parameters of inflammation in the colon, and did not impair establishment of the postinflammatory model. Considering all these factors, we elucidated the effects of berberine administration at a dose that showed a beneficial effect on the inflammatory response. This indicated that berberine significantly reduced VH and stool frequency and increased stool consistency. We investigated the role of NO in the protective effects of berberine using an experimental model with the NO synthetase (NOS) inhibitor aminoguanidine. We demonstrated that aminoguanidine significantly reduced the effect of berberine on VH, which suggests this effect of berberine is at least partly mediated through NOS.

Berberine has been used in the treatment of gastroenteritis and infectious diarrhea in Chinese traditional medicine for thousands of years. Some recent studies have indicated that it has various pharmacological effects, including anti-inflammatory[20] and antimicrobial[28] effects; each of which may contribute to the antidiarrheal effect. The fact that berberine has low bioavailability and shows poor absorption through the colon wall (< 5%)[29] support the thesis that it may exert its antidiarrheal effect on the intestinal epithelial cells before absorption. However, until now the effect of berberine on HV has not been confirmed.

NO is a key neurotransmitter in both short- and long-acting inhibitory motor neurons[7] and plays a critical part in mediating gastrointestinal motility. Some studies have revealed that NOS neuronal activity considerably changes after inflammation and is responsible for some acute postinflammatory consequences in bowel-like ileus[30,31]. Apart from its major role in the peripheral nervous system, such as in the enteric inhibitory nerves of the myenteric plexus, NO is believed to be an intracellular messenger or neurotransmitter in the central nervous system (CNS). It has been verified that due to its free diffusibility, NO acts as a retrograde transmitter in the CNS, mediating some nervous paradigms, for instance, long-term potentiation, and is the key neurotransmitter in descending inhibitory neurons modulating nociception at the spinal level[32]. Furthermore, it proves that NO is involved in the modulation of visceral perception, for example, intraperitoneal injection of acetic acid in rats increases nitrergic neurons in specific regions of the brain, and NOS immunoreactivity has been confirmed in lumbosacral afferents and preganglionic neurons innervating the pelvic viscera[33]. Therefore, some hypotheses can be performed based on our results, which interpret the protective effects of berberine through NO on VH in IBS at the myenteric plexus level, CNS, and smooth muscles. Our results could be interpreted at the myenteric plexus level.

Our findings were similar to a recent study that identified no significant difference in NO-containing neurons of the colonic myenteric plexus between IBS rats and controls[34]. Thus, we hypothesize that basal NO synthesis is not significantly decreased in IBS, and it might be that the positive effects of berberine decrease VH through increasing NO levels. Therefore, we speculate that the positive effects of berberine on VH in the postinflammatory rat model are at least partly exerted through NO synthesis potentiation.

We also examined the effect of aminoguanidine (NOS inhibitor) on stool form (hard pellets, soft pellets, and shapeless) in IBS rats under restraint stress. Aminoguanidine had no noticeable effect on stool form, but it did diminish the protective effects of berberine on stool form. NO plays a major role in mediating gastrointestinal motility, which is critical for stool formation. As a result of the short time between acute drug administration and defecation measurement, aminoguanidine did not produce any change in the stool consistency pattern of rats. The effects of aminoguanidine on stool frequency were more sophisticated and hard to explain. The protective effects of berberine on stool frequency were diminished by aminoguanidine, but the specific mechanism was not established. Further study will be needed to explore the specific mechanism. However, it should be noted that this study investigated only the antinociceptive effect of berberine on VH. One of the limitations of this study is the lack of measurement of NOS inhibitors.

In conclusion, we indicated that berberine administration prevented progression of VH to CRD. It is possible that NO released by berberine may affect colonic hypersensitivity. All of our data suggest that berberine may be of interest in the treatment of visceral hyperalgesia, particularly in IBS.

COMMENTS

Background

It is believed that chronic visceral hypersensitivity (VH), abnormal gastrointestinal motility, and altered central processing may be major pathophysiological mechanisms of irritable bowel syndrome (IBS). Berberine is an ancient Chinese herb that has been used to treat gastroenteritis for many years, which is preferred for its low cost and low incidence of adverse effects. Recently, berberine has been shown to have a considerable effect in the treatment of experimental colitis. However, the mechanism remains unknown.

Research frontiers

Berberine was used to treat rats with VH induced by 4% acetic acid. Berberine administration significantly increased the nociceptive threshold in rats, whereas the administration of aminoguanidine or berberine + aminoguanidine before VH measurement reversed the antinociceptive effect of berberine. The mechanism underlying the effect of berberine on VH of rats appears to be partly mediated by nitric oxide (NO).

Innovations and breakthroughs

Recently, it has been demonstrated that berberine has multiple pharmacological activities. In this study, authors indicated that berberine may be of interest in the treatment of VH, particularly in IBS.

Applications

The present study demonstrated that berberine administration prevented progression of VH to colorectal distension in a rat model of IBS. The effect of berberine is mediated by NO pathways, thus providing evidence for the treatment of VH in IBS.

Terminology

IBS is a common gastrointestinal disorder characterized by chronic visceral pain and bloating in association with altered gut movements. Berberine is an ancient Chinese herb that might play a role in the treatment of IBS.

Peer review

This paper describes positive effects of berberine on VH in rats with IBS. It is reported that berberine administration significantly increased the nociceptive threshold in rats, whereas administration of aminoguanidine or berberine + aminoguanidine before VH measurement reversed the antinociceptive effect of berberine. The results presented are crucial for clinicians and for the fundamental scientific community as well.

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BRIEF ARTICLE

Increased expression of matrix metalloproteinase-9 associated with gastric ulcer recurrence

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Abstract

AIM: To compare matrix metalloproteinase (MMP)-9 and tissue inhibitor of metalloproteinase (TIMP)-1 in gastric ulcer (GU) and chronic superficial gastritis (CSG).

METHODS: This study enrolled 63 patients with GU and 25 patients with CSG. During upper gastroduodenal endoscopy, we took samples of gastric mucosa from the antrum and ulcer site from patients with GU, and samples of antral mucosa from patients with CSG. Mucosal biopsy tissues were cultured for 24 h, and the culture supernatant was measured for levels of MMP-9 and TIMP-1. After receiving eradication therapy for *Helicobacter pylori* (*H. pylori*) and 8 wk proton-pump inhibitor therapy for GU, follow-up endoscopy examination was performed after 6 mo and whenever severe symptoms occurred.

RESULTS: Levels of MMP-9 and TIMP-1 at the ulcer site or in the antrum were significantly higher in GU than CSG patients. MMP-9 levels at the ulcer site were significantly higher than in the antrum in GU patients, and had a significantly positive correlation with TIMP-1. MMP-9 levels were significantly higher in *H. pylori*-positive than *H. pylori*-negative GU and CSG patients. Levels of MMP-9 or TIMP-1 at the ulcer site were associated with the histological severity of activity and inflammation. About 57 GU patients were followed up, and seven had GU recurrence. *H. pylori*-infection and MMP-9 levels were risk factors for the recurrence of GU adjusted for age and sex by multiple logistic regression analysis.

CONCLUSION: MMP-9 may perform an important function in gastric ulcer formation and recurrence.

Key words: Gastric ulcer; Matrix metalloproteinase-9; Tissue inhibitor of metalloproteinase-1; *Helicobacter pylori*

Core tip: Gastric ulcer is a multifaceted process including acid secretion, reactive oxygen species generation, prostaglandin inhibition, and extracellular matrix degradation. Gastric mucosal damage is directly associated with extracellular matrix degradation in which matrix metalloproteinases (MMPs) play a crucial role. In this study, the authors compared MMP-9 and tissue inhibitor of metalloproteinase-1 levels in patients with gastric ulcer or chronic superficial gastritis.

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INTRODUCTION

Gastric ulcer (GU) is a multifaceted process including acid secretion, reactive oxygen species generation, prostaglandin inhibition, and extracellular matrix (ECM) degradation[1]. Gastric mucosal damage is directly associated with extracellular matrix degradation in which matrix metalloproteinases (MMPs) play a crucial role[2]. MMPs are endopeptidases that perform important functions in ECM remodeling, cell proliferation, and inflammatory processes. Recent studies have indicated that gastric ulceration is associated with cleaving and remodeling of the ECM by MMPs[3,4]. In several animal studies of GU, attention has focused on the role of MMP-1, MMP-2, MMP-3, MMP-9 and MMP-13[4-6]. In particular, MMP-9 is important in the early phase of chronic GU[7]. However, these data are mostly derived from animal studies, and human clinical data remains rare, especially in assessing MMPs expression in GU formation and recurrence. Here, we compared MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-1 in patients with GU or chronic superficial gastritis (CSG), and how they correlated with GU recurrence.

MATERIALS AND METHODS

Patient selection

We examined 63 consecutive patients with GU and 25 with CSG who were diagnosed during upper gastroduodenal endoscopic examination at Liaocheng People' s Hospital between January and December 2010. The patients were enrolled in the study if they met the following criteria: (1) age 18-75 years; (2) no nonsteroidal anti-inflammatory drugs (NSAIDs), antibiotics, or bismuth compounds in the 2 wk prior to the study; and (3) acute phase GU. Patients were excluded as follows: (1) a history of gastric or duodenal surgery; (2) allergy to the study drugs; (3) required long-term treatment with NSAIDs, corticosteroids, aspirin, or anticoagulant agents; (4) pregnant women; and (5) active cancer, acute serious medical illness, or terminal illness. The study protocol was approved by the Ethics Committee of our institution. All patients gave written informed consent before participating in the study.

Endoscopic examination

During endoscopic examination, three antral specimens were taken from all patients, including one for rapid urease test (Triwizard, Fujian, China), one for histological examination, and one for *in vitro* culture for measurement of levels of MMP-9 and TIMP-1. Two additional specimens were taken from the margin of the ulcer in GU patients; one for histological examination and one for *in vitro* cultures for MMP-9 and TIMP-1.

Helicobacter pylori infection detection

Helicobacter pylori (*H. pylori*) infection was confirmed by positive results for at least two of three diagnostic tests, namely rapid urease test, ^{13}C-urea breath test, or identification of the organism on tissue sections by Giemsa stain. Absence of infection was defined by a negative result in all three tests. Cases satisfying at least two test results were defined as positive for infection.

Tissue cultures and MMP-9, TIMP-1 assay

Mucosal biopsy tissues were weighed and then cultured in a 5% CO_2 incubator for 24 h in a culture bottle (Xiangya Gene Technology, Changsha, China) containing 5 mL RPMI 1640 medium with 5% heat-inactivated fetal calf serum, 15 mmol/L HEPES buffer, 100 U/mL penicillin-G, 100 mg/mL streptomycin and 10 mg/mL phytohemagglutinin-P. At the end of the culture period, the supernatant was drawn off and stored at -70 ℃ until measured by enzyme-linked immunosorbent assay for MMP-9 and TIMP-1 (Boster, Wuhan, China). A modified version of the Lowry method was used to assay total protein in biopsy homogenates (Boster). The amount of MMP-9 and TIMP-1 was expressed relative to protein content in the biopsy tissue homogenate (per milligram of biopsy protein).

Histology

Tissue sections stained with hematoxylin-eosin were used to assess activity, inflammation, glandular atrophy, and intestinal metaplasia. Grading was done on a four-item scale of 0, 1, 2 and 3, corresponding to none, mild, moderate and severe, respectively, in accordance with the updated Sydney system[8].

Follow up

H. pylori-positive GU patients received eradication treatment with triple therapy using lansoprazole (30 mg, *bid*), amoxicillin (1000 mg, *bid*), and clarithromycin (500 mg, *bid*) for 1 wk, and subsequently received lansoprazole (30 mg, *qd*) for 8 wk, whereas *H. pylori*-negative GU patients received lansoprazole (30 mg, *qd*) for 8 wk. After that, the presence of the ulcer scar was confirmed by endoscopy, and six patients who still had active ulcer were excluded from follow-up. An additional ^{13}C urea breath test or rapid urease test was conducted to assess the final *H. pylori* status after 6 mo for all GU patients. Follow-up endoscopy examination was performed at the end of the 6 mo and whenever severe symptoms occurred. Ulcer recurrence was defined as a lesion of white coat with a distinct depressed area and a diameter of $\geqslant$ 5 mm.

Statistical analysis

All data were expressed as mean ± SD. Frequency variables were compared using the χ^2 test. Quantitative variables were analyzed using Student's *t* test. Correlation was analyzed by Pearson's correlation or Spearman's rank correlation. Logistic analysis was used for risk factors for GU recurrence. SPSS version 17.0 (Chicago, IL, United States) was used, and $P < 0.05$ was regarded as significant.

RESULTS

A total of 88 patients were enrolled. The 63 GU patients

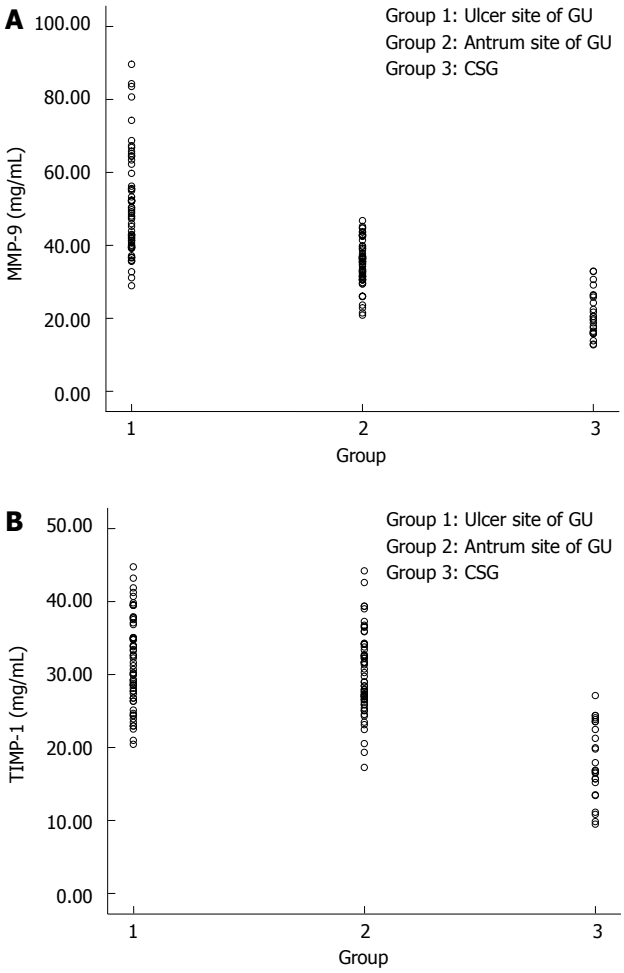

Figure 1 Production of matrix metalloproteinase-9 or tissue inhibitor of metalloproteinase-1 by gastric mucosa in patients with gastric ulcer (*n* = 63) or chronic superficial gastritis (*n* = 25). A: Tissue culture was performed for 24 h. Matrix metalloproteinase (MMP)-9 concentration in tissue culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA); B: Tissue inhibitor of metalloproteinase (TIMP)-1 concentration in tissue culture supernatants was measured by ELISA. GU: Gastric ulcer; CSG: Chronic superficial gastritis.

Table 1 Clinical characteristics of the patients enrolled in the study *n* (%)

Characteristics	GU group (*n* = 63)	CSG group (*n* = 25)
Sex		
Male	31 (49.2)	15 (60.0)
Female	32 (50.8)	10 (40.0)
Age, yr (mean ± SD)	47.8 ± 12.9	51.3 ± 8.5
H. pylori infection		
Positive	54 (85.7)	10 (40.0)
Negative	9 (14.3)	15 (60.0)
Position of ulcer		
Corpus	18 (28.6)	-
Antrum	45 (71.4)	-

GU: Gastric ulcer; CSG: Chronic superficial gastritis; *H. pylori*: *Helicobacter pylori*.

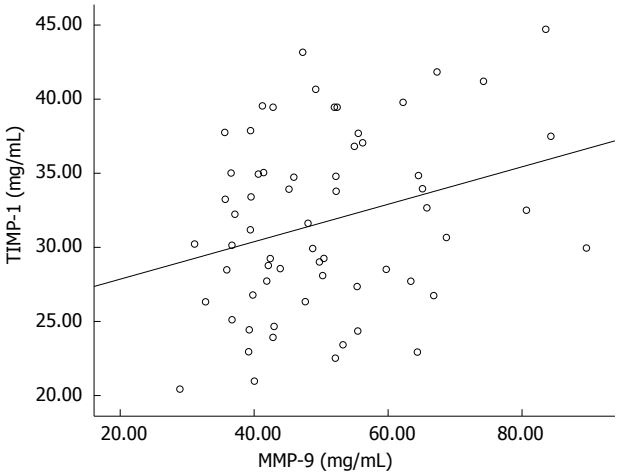

Figure 2 Correlation between matrix metalloproteinase-9 or tissue inhibitor of metalloproteinase-1 production by gastric mucosa in gastric ulcer patients (*n* = 63). Linear regression analysis showed a significant correlation between the two mediators ($r = 0.29$, $P = 0.021$). A significant positive correlation was observed between levels of matrix metalloproteinase (MMP)-9 and tissue inhibitor of metalloproteinase (TIMP)-1 at the ulcer site.

included 31 males and 32 females, with an average age of 47.8 years (range, 24-71 years). The 25 CSG patients included 15 males and 10 females, with an average age of 51.3 years (range, 29-68 years). Fifty-four GU patients were positive and nine were negative for *H. pylori*, and 10 CSG patients were positive and 15 were negative for *H. pylori* (Table 1). There were no significant difference between the GU and CSG patients in age and sex, except in *H. pylori* infection ($\chi^2 = 18.86$, $P < 0.01$).

In all patients, MMP-9 levels (Figure 1A) were significantly higher at the margin of the ulcer (50.50 ± 13.72 mg/mL, $t = 13.96$, $P < 0.01$) or in the antrum (35.08 ± 6.07 mg/mL, $t = 9.78$, $P < 0.01$) of the GU patients than the CSG patients (21.06 ± 6.04 mg/mL). In the GU patients, MMP-9 levels were significantly higher ($t = 8.16$, $P < 0.01$) at the margin of the ulcer (50.50 ± 13.72 mg/mL) than in the antrum (35.08 ± 6.07 mg/mL).

With regard to TIMP-1 levels (Figure 1B), a significant difference was seen between at the margin of the ulcer (18.17 ± 5.14 mg/mL *vs* 31.71 ± 5.97 mg/mL, $t = 9.96$, $P < 0.01$) or in the antrum (18.17 ± 5.14 mg/mL *vs* 30.07 ± 5.42 mg/mL, $t = 9.42$, $P < 0.01$) of the GU and CSG patients. There was no significant difference between the antrum and the margin of the ulcer (30.07 ± 5.42 mg/mL *vs* 31.71 ± 5.97 mg/mL, $t = 1.62$, $P = 0.108$) of the GU patients. A significant positive correlation was observed between levels of MMP-9 and TIMP-1 (50.50 ± 13.72 mg/mL *vs* 31.71 ± 5.97 mg/mL, $r = 0.29$, $P = 0.021$) at the margin of the ulcer in the GU patients (Figure 2).

For the GU patients, ulcers were classified according to their anatomical location, that is, 18 patients had corpus or fundus ulcers, and 45 had antral or prepyloric ulcers. Both MMP-9 (47.45 ± 11.92 mg/mL *vs* 51.72 ± 14.32 mg/mL, $t = -1.12$, $P = 0.267$) and TIMP-1 (30.85 ± 5.93 mg/mL *vs* 32.05 ± 6.02 mg/mL, $t = -0.72$, $P = 0.476$) levels were not significantly different at the margin of the ulcer between corpus or fundus ulcers and antral or prepyloric ulcers.

In the GU patients, levels of MMP-9 (Figure 3A) at

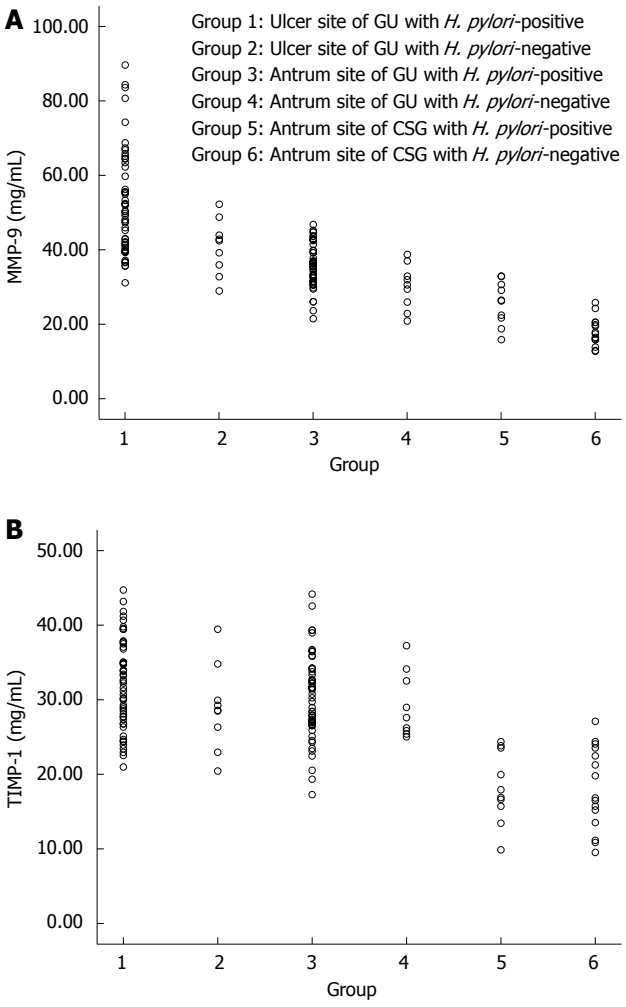

Figure 3 Production of matrix metalloproteinase-9 or tissue inhibitor of metalloproteinase-1 by gastric mucosa with negative or positive H. pylori-infection in patients with gastric ulcer (*n* = 63) or chronic superficial gastritis (*n* = 25). A: Tissue culture was performed for 24 h. Matrix metalloproteinase (MMP)-9 concentration in tissue culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA); B: Tissue culture was performed for 24 h. Tissue inhibitor of metalloproteinase (TIMP)-1 concentration in tissue culture supernatants was measured by ELISA. GU: Gastric ulcer; CSG: Chronic superficial gastritis; *H. pylori*: *Helicobacter pylori*.

the margin of the ulcer or in the antrum in the *H. pylori*-positive patients were significantly higher than in the *H. pylori*-negative patients (52.12 ± 13.90 mg/mL *vs* 40.77 ± 7.43 mg/mL, $t = 2.38$, $P = 0.020$; 35.92 ± 5.72 mg/mL *vs* 30.03 ± 6.01 mg/mL, $t = 2.84$, $P = 0.006$, respectively). In the CSG patients, levels of MMP-9 in the *H. pylori*-positive patients were significantly higher than in the *H. pylori*-negative patients (25.70 ± 5.89 mg/mL *vs* 17.96 ± 3.82 mg/mL, $t = 4.00$, $P = 0.001$).

In the GU patients, levels of TIMP-1(Figure 3B) at the margin of the ulcer or in the antrum in the *H. pylori*-positive patients did not differ significantly from those in the *H. pylori*-negative patients (32.18 ± 5.94 mg/mL *vs* 28.91 ± 5.71 mg/mL, $t = 1.53$, $P = 0.130$; 30.21 ± 5.60 mg/mL *vs* 29.22 ± 4.40 mg/mL, $t = 0.50$, $P = 0.617$, respectively). In the CSG patients, levels of TIMP-1 in the *H. pylori*-positive patients also did not differ significantly from those in the *H. pylori*-negative patients (18.22 ± 4.76 mg/mL *vs* 18.13 ± 5.55 mg/mL, $t = 0.04$, $P = 0.967$). Levels of MMP-9 (35.92 ± 5.72 mg/mL) and TIMP-1 (30.21 ± 5.60 mg/mL) in the antrum in the *H. pylori*-positive GU patients were significantly higher ($t = 5.17$, $P < 0.01$; $t = 6.35$, $P < 0.01$, respectively) than in the *H. pylori*-positive CSG patients (25.70 ± 5.89 mg/mL and 18.22 ± 4.76 mg/mL, respectively).

Table 2 Association between levels of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 and the histological degree at the margin of the gastric ulcer

Variables		Activity	Inflammation	Atrophy	Metaplasia
MMP-9	*r*	0.280	0.310	0.180	-0.030
	P	0.026[1]	0.014[1]	0.163	0.842
TIMP-1	*r*	0.270	0.280	0.120	0.050
	P	0.030[1]	0.025[1]	0.371	0.687

[1]By Spearman correlation analysis. MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinase.

For the GU patients, we compared levels of MMP-9 and TIMP-1 *in vitro* with the severity of histological gastritis (activity, inflammation, atrophy, and metaplasia) at the margin of the ulcer. A significant association was identified between levels of MMP-9 or TIMP-1 and the histological degree of activity and inflammation, but not with the degree of glandular atrophy or intestinal metaplasia (Table 2).

Of the 63 GU patients, six were excluded because they still had active ulcer after 8 wk PPI treatment. Among 57 follow-up patients, seven (12.3%) had recurrence at the time of or before endoscopy examination at the end of 6 mo. There were nine patients with *H. pylori* infection. A multivariate logistic regression analysis adjusted for age and sex demonstrated that *H. pylori* infection (OR = 17.705, 95%CI: 2.091-149.929, $P = 0.008$) and MMP-9 levels (OR = 1.078, 95%CI: 1.007-1.154, $P = 0.031$) were GU recurrence risk factors.

DISCUSSION

We found that MMP-9 production was increased in the gastric mucosa at the margin of the ulcer in GU patients. This increase had a significant positive correlation with production of TIMP-1, an MMP-9 inhibitor. Several studies have investigated the association between MMPs and GU. Indomethacin-induced ulcerated gastric tissues exhibited about 12-fold higher pro-MMP-9 activity as compared to control tissues. Similarly, ethanol induced about 22-fold higher pro-MMP-9 activities in rat gastric tissues[5]. One study showed that significant up-regulation of MMP-9 expression in indomethacin-induced GU in mice was correlated with increased activity of activator protein-1, and oxidative stress was preceded by chronic inflammation that enhanced expression of MMP-9[9].

MMPs have recently been shown to be up-regulated in gastric epithelial cells infected with *H. pylori*, and might contribute to the pathogenesis of peptic ulcer. Our study

showed that MMP-9 levels were associated with *H. pylori* infection. Significantly elevated serum levels of MMP-9 and reduced serum levels of TIMP-1 have been demonstrated in patients with *H. pylori* gastritis as compared to *H. pylori*-negative controls[10]. *H. pylori*-infected GUs had even higher MMP-9 and TIMP-1 expression in epithelial cells than in NSAID-related GU[11]. One study showed that there were no significant differences in serum levels of MMP-9 between *H. pylori*-positive and *H. pylori*-negative children[12].

We showed that levels of MMP-9 correlated with the histological degree of activity and inflammation at the margin of the ulcer. In BALB/c mice, NSAIDs caused dose-dependent induction in MMP-9 activity and expression in ulcerated gastric tissues, along with significant infiltration of inflammatory cells and disruption of the gastric mucosal layer[13]. GU is associated with infiltration of the gastric mucosa by neutrophils, lymphocytes, monocytes, and plasma cells. Inflammatory cells secrete an array of pro-inflammatory cytokines and growth factors (epidermal growth factor, platelet-derived growth factor, transforming growth factor-β, vascular endothelial growth factor, angiopoietins). MMPs can be induced by the activity of pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin (IL)-1, IL-6 and IL-8[1,9,14]. Oxidative stress is preceded by chronic inflammation that enhances the expression of MMP-9. By decreased synthesis and secretion of MMP-9, as well as infiltration of inflammatory cells and oxidative damage in gastric tissues, we may block or heal acute GU[3,13].

The C/C genotype of MMP-9-1562 C/T gene polymorphism might be associated with *H. pylori* infection[15]. *H. pylori* infection increases the secretion of MMPs in the gastric mucosa, leading to severe mucosal damage. Genetic variations in the *MMP-9* gene may be part of a complex genetic risk profile to develop GU in chronic *H. pylori* infection[16]. MMP-9 levels decrease consistently and significantly after successful *H. pylori* eradication, whereas the elevated levels remain unchanged when treatment fails[17].

We found that patients with high levels of MMP-9 and *H. pylori* infection were the risk factors for GU recurrence. *H. pylori* infection associated with GU recurrence has been verified[18]. Some studies found that severity of GU was strongly correlated with increased secretion of proMMP-9 in ethanol-induced acute gastric ulceration in rats[19,20]. Higher levels of MMP-9 in chronic wound fluid correlate with a clinically worse wound[21]. Measurements of MMP-9 and TIMP-1 may help to identify diabetic foot ulcers at risk of poor healing[22]. These findings suggest that MMP-9 may be indicative of inflammation and poor wound healing, and that we can reduce GU recurrence by inhibition of MMP-9 activity.

In conclusion, we observed increased expression of MMP-9 and TIMP-1 in GU patients and found a significantly positive correlation between MMP-9 and TIMP-1 production at the margin of the ulcer. Increased production of MMP-9 was significantly correlated with increased GU recurrence. These results suggest that MMP-9 may play an important role in the occurrence of GU. A clearer understanding of the significance and implications of these findings may provide insights into ulcer healing. Further study is needed to clarify the roles of MMP-9 and elucidate any potential clinical implications in the healing of GU.

COMMENTS

Background

Gastric ulcer (GU) is a multifaceted process including acid secretion, reactive oxygen species generation, prostaglandin inhibition, and extracellular matrix (ECM) degradation. Gastric mucosal damage is directly associated with ECM degradation in which matrix metalloproteinases (MMPs) play a crucial role. In several animal studies of GU, attention has focused on the role of MMP-1, MMP-2, MMP-3, MMP-9 and MMP-13. However, these data are mostly derived from animal studies, and human clinical data remains rare, especially in assessing MMPs expression in GU formation and recurrence.

Research frontiers

In this study, the authors compared levels of MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-1 in GU patients, and how they correlated with GU recurrence.

Innovations and breakthroughs

This study enrolled 63 patients with GU and 25 patients with superficial gastritis (CSG). Samples of gastric mucosa from the antrum and the ulcer site were harvested from GU patients and samples of antral mucosa were taken from CSG patients during upper gastroduodenal endoscopy. Levels of MMP-9 and TIMP-1 at the ulcer site or in the antrum were significantly higher in GU than CSG patients. MMP-9 levels at the ulcer site were significantly higher than in the antrum in GU patients, and had a significantly positive correlation with TIMP-1. MMP-9 levels were significantly higher in *Helicobacter-pylori*-positive than -negative GU and CSG patients. Levels of MMP-9 or TIMP-1 at the ulcer site were associated with the histological severity of activity and inflammation.

Applications

The authors found that the MMP-9 may perform an important function in gastric ulcer formation and recurrence.

Peer review

This study compared MMP-9 and TIMP-1 levels in GU and CSG patients. The authors measured the levels of MMP-9 and TIMP-1 from the tissues. The levels of MMP-9 and TIMP-1 at the ulcer site or in the antrum were significantly higher in GU than CSG patients. MMP-9 levels at the ulcer site were significantly higher than in the antrum in GU patients, and had a significantly positive correlation with TIMP-1. They concluded that MMP-9 may perform an important function in gastric ulcer formation and recurrence.

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META-ANALYSIS

Impact of being overweight on the surgical outcomes of patients with gastric cancer: A meta-analysis

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Abstract

AIM: To investigate the effect of being overweight on the surgical results of patients with gastric cancer.

METHODS: Comprehensive electronic searches of the PubMed, Web of Science, and Cochrane Library databases were conducted. Studies were identified that included patients with surgical complications from gastric cancer who were classified as normal weight [body mass index (BMI) < 25 kg/m^2] or overweight (BMI ⩾ 25 kg/m^2). The operative time, retrieved lymph nodes, blood loss, and long-term survival were analyzed. A subgroup analysis was conducted based on whether patients received laparoscopic or open gastrectomy procedures. All statistical tests were performed using ReviewerManager 5.1.2 software.

RESULTS: This meta-analysis included 23 studies with 20678 patients (15781 with BMI < 25 kg/m^2; 4897 with BMI ⩾ 25 kg/m^2). Overweight patients had significantly increased operation times [MD: -29.14; 95%CI: -38.14-(-20.21); P < 0.00001], blood loss [MD: -194.58; 95%CI: -314.21-(-74.95); P = 0.001], complications (RR: 0.75; 95%CI: 0.66-0.85; P < 0.00001), anastomosis leakages (RR: 0.59; 95%CI: 0.42-0.82; P = 0.002), and pancreatic fistulas (RR: 0.486; 95%CI: 0.34-0.63; P < 0.00001), whereas lymph node retrieval was decreased significantly in the overweight group (MD: 1.69; 95%CI: 0.75-2.62; P < 0.0001). In addition, overweight patients had poorer long-term survival (RR: 1.14; 95%CI: 1.07-1.20; P < 0.0001). No significant difference was detected for the mortality and length of hospital stay.

CONCLUSION: This meta-analysis demonstrates that a high BMI not only increases the surgical difficulty and complications but also impairs the long-term survival of patients with gastric cancer.

Key words: Overweight; Body mass index; Gastric cancer; Gastrectomy

Core tip: Surgical and postoperative complications are believed to be greater for overweight patients with gastric cancer, but this is controversial due to conflicting results from previous studies. This meta-analysis identified 23 studies with a total of 20678 patients, and the results indicate that overweight patients had significantly increased operation times, blood loss, complications, anastomosis leakages, and pancreatic fistulas, whereas lymph node retrieval was decreased significantly in the overweight group. In addition, overweight patients had poorer long-term survival. Therefore, being overweight not only increased the surgical difficulty and complications but also impaired the long-term survival of patients with gastric cancer.

Wu XS, Wu WG, Li ML, Yang JH, Ding QC, Zhang L, Mu JS, Gu J, Dong *P*, Lu JH, Liu YB. Impact of being overweight on the surgical outcomes of patients with gastric cancer: A meta-analysis. *World J Gastroenterol* 2013; 19(28): 4596-4606 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i27/4596.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i27.4596

INTRODUCTION

The increasing global prevalence of overweight and obese individuals is problematic[1,2] for Western countries[3] and is also a concern for Eastern countries such as China[4] and South Korea[5]. Consequently, abdominal surgeries are increasingly more difficult because increasing numbers of surgeries are performed on overweight and obese individuals. In particular, gastric cancer studies[6,7] reported that excess body weight is associated with unfavorable surgical results, including longer operating times, decreased lymph node retrieval, increased postoperative complications, and decreased survival rates. Radical gastrectomy with D2 node dissection is the recommended surgical approach for patients with resectable (curable) gastric cancer[8]. However, the results of postoperative morbidity, mortality, and long-term survival after D2 node dissection differed significantly between different studies from Asia and Europe[8-14]. This discrepancy may be due to the variable prevalence of overweight patients in Western and Eastern countries. Excess visceral fat in overweight patients theoretically complicates manipulation of the omentum and impedes lymph node dissection during radical gastrectomy due to decreased visualization of the branches of the arteria celiaca, which could increase surgical and postoperative complications and mortality. However, a number of studies[15-18] reported conflicting results about the effect of being overweight on both the short-term and long-term surgical outcomes for patients with gastric cancer. To more comprehensively understand this issue, we conducted a meta-analysis.

MATERIALS AND METHODS

Search strategy

Two authors (Wu XS and Wu WG) independently conducted comprehensive electronic searches of the PubMed, Web of Science, and Cochrane Library databases for all dates prior to January 2013. The search strategy was unrestricted for English-language journals and used combinations of MeSH and text words for overweight, body mass index (BMI), gastric cancer, and gastrectomy, *e.g.*, the string "Body Mass Index" (Mesh) or "overweight" (MeSH Terms) or overweight (Text Word) and "gastrectomy" (MeSH Terms) or gastrectomy (Text Word) or "stomach neoplasms" (MeSH Terms) or gastric cancer (Text Word). In addition, reference lists of all retrieved articles were manually searched for additional studies that were missed by the electronic search.

Inclusion and exclusion criteria

The inclusion criteria for the meta-analysis were studies that examined the influence of body weight on gastric cancer surgical outcomes (morbidity, anastomotic leakage, pancreatic fistula, postoperative mortality, operative time, lymph node retrieval, blood loss, postoperative hospital stay, and long-term survival). In the studies we chose, there were patients with normal-weight and overweight presurgical BMIs based on World Health Organization definitions (overweight BMI $\geqslant$ 25 kg/m^2; healthy-weight BMI < 25 kg/m^2)[19,20]. Reviews, case reports, and series reports were excluded. When data were presented in more than one publication, publications with smaller data sets were excluded. Disagreements regarding a study's eligibility were resolved based on a consensus of reviews from two additional authors (Li ML and Yang JH).

Outcome measures analyzed

Three outcome variables, including the operation time, number of retrieved lymph nodes, and blood loss, were analyzed as indices of the surgical difficulty. We estimated the influence of a high BMI on surgical safety, morbidity, anastomotic leakage, pancreatic fistula, postoperative mortality, and postoperative hospital stay. The long-term survival of overweight and healthy-weight patients was also compared as an index of successful clinical resolution.

Data extraction and risk of bias assessment

Data were extracted from each study by two independent reviewers (Ding QC and Zhang L), who also rated the overall quality of each outcome according to the recommendation of the Cochrane Handbook for Systematic Reviews of Interventions[21]. The criteria to assess nonrandomized studies were taken from the Grading of Recommendations Assessment, Development, and Evaluation Working Group[22]. By combining the aforementioned recommendations, the following aspects of each included study were evaluated: the application of an internal control, adequate control of confounding factors, adequate reporting of outcomes, and the absence of a variable definition. Agreement for ratings was achieved *via* author consensus, as needed.

Statistical analysis

The statistical analysis was performed using Reviewer-Manager (Version 5.1.2, 2011, The Nordic Cochrane Centre, Cochrane Collaboration, www.cochrane-handbook.org). Statistical methods were based on the *Cochrane Handbook for Systematic Reviews of Interventions*[21]. Heterogeneity was checked using χ^2 tests, and $P < 0.1$ was the cutoff for statistical significance. A random effects model was applied for the meta-analysis using a more conservative perspective. Data from different trials reporting the same or similar outcomes were combined. The results were expressed using the RR for binary variables and the MD for continuous variables. Methods for relevant data extraction were based on Tierney *et al*[23]. The cutoff for

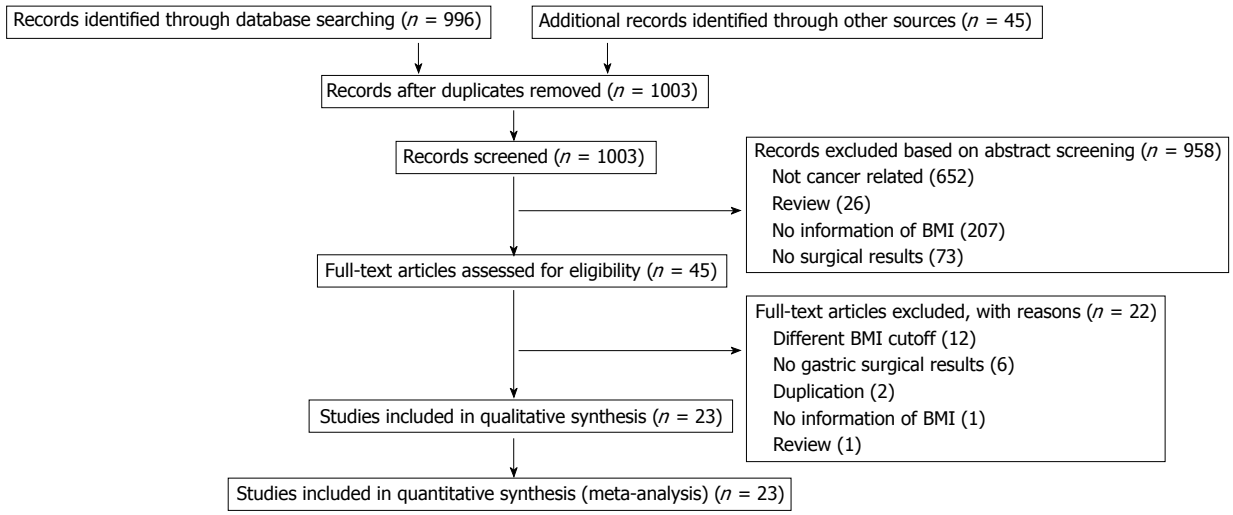


Figure 1 PRISMA flow chart showing study selection process. BMI: Body mass index.

statistical significance was $P < 0.05$, and the 95%CI was presented for each effect measure. Subgroup analysis was conducted based on whether patients received a laparoscopic gastrectomy or a total gastrectomy. Whenever possible, all analyses were based on the intention-to-treat principle. Publication bias exploration using a funnel plot and Egger's regression method[24] was performed if at least 10 trials were included in an outcome variable. Publication bias was considered to exist for $P < 0.05$.

RESULTS

Description of the included trials

We retrieved 996 records from the PubMed search and 45 records from the manual search. Twenty-three trials[15-18,25-43], which included multiple study types, procedures, percentages of patients with early gastric cancer, therapeutic modalities, and BMI cutoffs, met the eligibility criteria and were included in the meta-analysis (Table 1). Excluded reports largely had irrelevant topics. Twelve studies were excluded because they did not define overweight patients using the 25 kg/m^2 criteria. Figure 1 shows the flow chart for the selection of articles based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses[44]. This meta-analysis identified patients with healthy-weights (BMI < 25 kg/m^2) (n = 15781) and patients who were overweight (BMI ⩾ 25 kg/m^2) (n = 4897). For five included studies[27,28,30,33,35] that classified patients using more than one BMI cutoff point, binary variables were successfully combined, but it was not possible to pool these studies' continuous variables. Only four studies[16,17,32,35] were considered to have a low risk of bias, and all others were considered to have high risk of bias. Most of them were considered high risk because of the selective reporting or absence of variables definition.

Surgical results for all patients

Overweight patients had significantly longer operation times [MD: -29.14; 95%CI: -38.14-(-20.21); $P < 0.00001$, Figure 2A], greater blood loss [MD: -194.58; 95%CI: -314.21-(-74.95); $P = 0.001$], reduced lymph node retrieval (MD: 1.69; 95%CI: 0.75-2.62; $P < 0.0001$) (Table 2), and more postoperative complications (RR: 0.75; 95%CI: 0.66-0.85; $P < 0.00001$, Figure 2B). Specifically, anastomotic leakage (RR: 0.59; 95%CI: 0.42-0.82; $P = 0.002$, Figure 2C) and pancreatic fistula (RR: 0.486; 95%CI: 0.34-0.63; $P < 0.00001$, Figure 2D) were significantly greater in the overweight cohort. There was no significant difference between the two cohorts for the postoperative mortality or postoperative hospital stay. Patients in the normal-weight cohort had higher cancer-specific survivorship (RR: 1.14; 95%CI: 1.07-1.20; $P < 0.0001$, Figure 2E).

There was significant heterogeneity in the operation time, morbidity, anastomotic leakage, blood loss, long-term survival, and postoperative hospital stay results. No heterogeneity was detected for any of the other assessed outcomes. No publication bias was detected for the morbidity outcomes ($P = 0.05$), anastomotic leakage ($P = 0.291$), or mortality ($P = 0.272$).

Surgical results for patients receiving open gastrectomy

Overweight patients who received open gastrectomy had longer operation times [MD: -25.24; 95%CI: -33.53-(-16.95); $P < 0.00001$], greater intraoperative blood loss [MD: -212.93; 95%CI: -301.04-(-124.82); $P < 0.00001$], increased postoperative complications (RR: 0.78; 95%CI: 0.66-0.94; $P = 0.007$), more anastomotic leakage (RR: 0.58; 95%CI: 0.38-0.89; $P = 0.01$), and increased pancreatic fistulas (RR: 0.46; 95%CI: 0.38-0.67; $P < 0.0001$) compared with patients with healthy weights (Table 2). There were no significant differences between the cohorts for mortality, postoperative hospital stay, or number of retrieved lymph nodes. Non-overweight patients had better overall survival results than overweight ones (RR: 1.14; 95%CI: 1.07-1.20; $P < 0.0001$).

There was significant heterogeneity in the morbidity, anastomotic leakage, operative time, number of retrieved

Table 1 Basic data of included studies

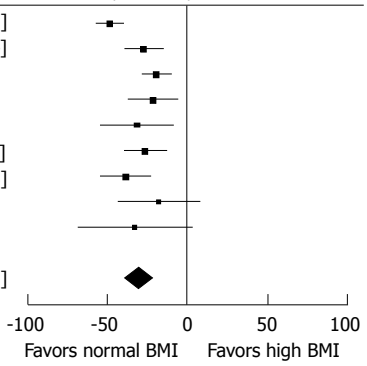
Study	Country	Inclusion period	Sample size	Study type	Follow up period	Percentage of T1 (normal weight *vs* overweight)	Percentage of N0 (normal weight *vs* overweight)	Percentage of stage 1/2 (normal weight *vs* overweight)	Percentage of differentiated (normal weight *vs* overweight)	Type of gastrectomy	Node dissection	Laparoscopic	Chemotherapy/ radiotherapy	BMI cutoff point	Risk of internal control	Risk of confounding factors	Risk of slective report	Risk of variables definition
Gretschel *et al*[27]	Germany	1992-2001	199	Retrospective	-	15.8% *vs* 31.6%	26.8% *vs* 45.3%	Not stated	Not stated	Total	D2	Not stated	Not stated	25, 30	Low	High	High	High
Yamada *et al*[17]	Japan	1999-2005	248	Retrospective	8-118 mo	53.2% *vs* 78.3%	Not stated	89.9% *vs* 93.3%	Not stated	Distal	D2	141 LADG	Not stated	25	Low	Low	Low	Low
Oh *et al*[29]	South Korea	2009-2009	61	Prospective	-	Not stated	Not stated	Not stated	Not stated	Total	D2	Not stated	Not stated	25	Low	High	Low	Low
Nobuoka *et al*[40]	Japan	1992-2008	644	Retrospective	-	Not stated	Not stated	53.8% *vs* 56.6%	Not stated	Total	D2	Not stated	Chemotherapy for advanced cancer and recurrence	25	Low	High	Low	Low
Tsujinaka *et al*[32]	Japan	1995-2001	523	Prospective	-	0.0% *vs* 0.0%	Not stated	Not stated	Not stated	Not stated	D2; D3	No	Not stated	25	Low	Low	Low	Low
Ohno *et al*[37]	Japan	2004-2009	197	Retrospective	-	73.9% *vs* 77.5%	Not stated	87.2% *vs* 95.0%	57.3% *vs* 57.5%	LADG; ODG	D1a; D1b; D2	120 LADG	Not stated	25	Low	Low	Low	High
Pacelli *et al*[33]	Italy	2000-2006	145	Retrospective	-	Not stated	Not stated	Not stated	Not stated	Distal; total	D2 + D3	No	No preoperative chemotherapy	18.5, 25, 30	Low	High	Low	Low
Yoshikawa *et al*[39]	Japan	2007-2009	66	Retrospective	-	Not stated	Not stated	92.4% *vs* 100.0%	Not stated	LADG; LATG[c]	D1ab + D2	56 LADG; 10 LATG	Not stated	25	Low	Low	Low	High
Murphy *et al*[35]	United Kingdom	1997-2002	50	Prospective	-	Not stated	Not stated	29.6% *vs* 65.2%	Not stated	Not stated	D2	No	No preoperative chemotherapy	20, 25, 30	Low	Low	Low	Low
Yasuda *et al*[34]	Japan	1994-2002	99	Retrospective	48 mo	100.0% *vs* 100.0%	93.8% *vs* 95.2%	Not stated	80.7% *vs* 87.5%	LADG	D1	LADG	Not stated	25	Low	Low	Low	High
Lee *et al*[38]	South Korea	-2005	1485	Retrospective	At least 3 mo	Not stated	Not stated	89.1% *vs* 89.0%	Not stated	LADG	D1a; D1b	LADG	Not stated	25	Low	High	Low	High
Kulig *et al*[18]	Poland	1986-1998	1992	Retrospective	104 mo	13.8% *vs* 10.6%	17.5% *vs* 15.0%	Not stated	Not stated	Distal; proximal; total	D1; D2; D2+	No	Not stated	25	Low	High	Low	High
Kim *et al*[30]	South Korea	2005-2010	1100	Prospective	-	Not stated	Not stated	Not stated	Not stated	LADG	D2	LADG	Not stated	25, 30	Low	High	Low	High
Inagawa *et al*[28]	Japan	1990-1997	293	Retrospective	10-104 mo	Not stated	Not stated	88.7% *vs* 94.0%	Not stated	Distal	D2	-	Not stated	20, 25	Low	Low	Low	High
Kawamura *et al*[36]	Japan	2003-2008	473	Retrospective	-	Not stated	Not stated	100.0% *vs* 100.0%	Not stated	Distal	Regional	249 LADG	Not stated	25	Low	Low	Low	High
Ojima *et al*[31]	Japan	1992-2002	689	Retrospective	At least 60 mo	55.3% *vs* 51.7%	66.1% *vs* 65.5%	Not stated	53.4% *vs* 56.9%	Distal; proximal; total	D1; D2; D2+	-	No preoperative chemotherapy	25	Low	High	Low	Low
Tokunaga *et al*[15]	Japan	1970-2004	7925	Retrospective	At least 60 mo	51.0% *vs* 60.0%	61.0% *vs* 68.0%	75.0% *vs* 83.0%	45.0% *vs* 40.0%	Distal; proximal; total	Not stated	-	Not stated	25	Low	High	High	Low
Oh *et al*[16]	South Korea	2000-2003	410	Retrospective	50 mo	15.7% *vs* 20.9%	41.8% *vs* 40.4%	47.6% *vs* 46.5%	32.5% *vs* 35.4%	Total	D2	-	Not stated	25	Low	Low	Low	Low
Tanaka *et al*[26]	Japan	2001-2007	191	Retrospective	-	Not stated	Not stated	Not stated	Not stated	Total	D1; D2	-	Not stated	25	Low	High	Low	High
Kunisaki *et al*[25]	Japan	2002-2008	152	Retrospective	-	Not stated	Not stated	Not stated	Not stated	LADG	D1a; D1b; D2	LADG	Not stated	25	Low	High	High	High
Lee *et al*[42]	South Korea	2006-2010	243	Retrospective	-	Not stated	Not stated	100.0% *vs* 100.0%	Not stated	Distal	Not stated	Not stated	Not stated	25	Low	Low	High	High
Bickenbach *et al*[43]	United States	1985-2007	1853	Retrospective	35 mo	24.6% *vs* 29.8%	44.5% *vs* 48.3%	58.6% *vs* 65.9%	Not stated	Not stated	D1; D2; D2+	Not stated	Not stated	25	Low	High	Low	Low
Deguchi *et al*[41]	Japan	1999-2008	1640	Retrospective	-	Not stated	Not stated	Not stated	Not stated	Proximal; total	D0; D1; D2; D2+	Not stated	Not stated	25	Low	High	Low	Low

LADG: Laparoscopic-assited distal gastrectomy; ODG: Opem distal gastrectomy; LATG: Laparoscopic-assited total gastrectomy.

A

Study or subgroup	Normal BMI Mean	SD	Total	High BMI Mean	SD	Total	Weight	Mean difference IV, random, 95%CI
Bickenbach *et al*[43]	272	84	728	320	102	1125	15.3%	-48.00 [-56.53, -39.47]
Kawamura *et al*[36]	236	60.7	349	262.4	58.2	124	13.4%	-26.40 [-38.46, -14.34]
Lee *et al*[38]	223.7	75.6	1053	242.5	84.6	432	15.0%	-18.80 [-27.99, -9.61]
Nobuoka *et al*[40]	245.1	69.3	545	265.9	74.1	99	11.5%	-20.80 [-36.51, -5.09]
Oh *et al*[5]	254.11	50.13	37	285.03	37.99	24	8.5%	-30.92 [-53.10, -8.74]
Oh *et al*[16]	173.7	47.6	311	199.2	61.8	99	12.8%	-25.50 [-38.77, -12.23]
Ojima *et al*[31]	277	85	573	315	75	116	11.7%	-38.00 [-53.32, -22.68]
Tanaka *et al*[26]	269	83	144	286	77	47	7.1%	-17.00 [-42.85, 8.85]
Yasuda *et al*[34]	239	48	83	271	69	16	4.7%	-32.00 [-67.35, 3.35]
Total (95%CI)			3823			2082	100.0%	-29.14 [-38.08, -20.21]

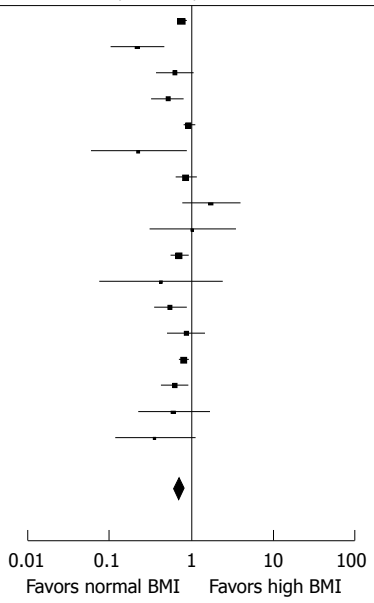
Heterogeneity: Tau^2 = 116.74; χ^2 = 26.79, df = 8 (P = 0.0008); I^2 = 70%
Test for overall effect: Z = 6.39 (P < 0.00001)



B

Study or subgroup	Normal BMI Events	Total	High BMI Events	Total	Weight	Risk ratio MH, random, 95%CI
Bickenbach *et al*[43]	279	728	551	1125	14.4%	0.78 [0.70, 0.87]
Inagawa *et al*[28]	12	239	12	54	2.5%	0.23 [0.11, 0.48]
Kawamura *et al*[36]	37	349	20	124	4.6%	0.66 [0.40, 1.09]
Kim *et al*[30]	45	791	33	309	5.7%	0.53 [0.35, 0.82]
Kulig *et al*[18]	533	1500	182	492	13.7%	0.96 [0.84, 1.10]
Kunisaki *et al*[25]	4	123	4	29	0.9%	0.24 [0.06, 0.89]
Lee *et al*[38]	147	1053	68	432	9.6%	0.89 [0.68, 1.16]
Lee *et al*[42]	23	157	7	86	2.2%	1.80 [0.81, 4.02]
Murphy *et al*[35]	5	27	4	23	1.1%	1.06 [0.32, 3.50]
Nobuoka *et al*[40]	192	545	48	99	10.5%	0.73 [0.58, 0.92]
Oh *et al*[5]	2	37	3	24	0.5%	0.43 [0.08, 2.40]
Oh *et al*[16]	43	311	24	99	5.5%	0.57 [0.37, 0.89]
Pacelli *et al*[33]	21	76	21	69	4.5%	0.91 [0.55, 1.51]
Tokunaga *et al*[15]	1276	6799	250	1126	14.1%	0.85 [0.75, 0.95]
Tsujinaka *et al*[32]	101	446	27	77	7.3%	0.65 [0.46, 0.92]
Yasuda *et al*[34]	13	83	4	16	1.5%	0.63 [0.23, 1.68]
Yoshikawa *et al*[39]	6	53	4	13	1.2%	0.37 [0.12, 1.12]
Total (95%CI)		13317		4197	100.0%	0.75 [0.66, 0.85]
Total events	2739		1262			

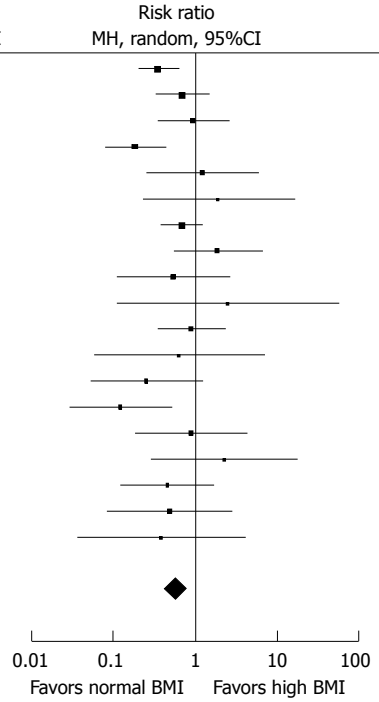
Heterogeneity: Tau^2 = 0.03; χ^2 = 38.26, df = 16 (P = 0.001); I^2 = 58%
Test for overall effect: Z = 4.48 (P < 0.00001)



C

Study or subgroup	Normal BMI Events	Total	High BMI Events	Total	Weight	Risk ratio MH, random, 95%CI
Bickenbach *et al*[43]	15	728	65	1125	12.3%	0.36 [0.21, 0.62]
Deguchi *et al*[41]	26	1320	9	320	9.7%	0.70 [0.33, 1.48]
Gretschel *et al*[27]	6	82	9	117	7.2%	0.95 [0.35, 2.57]
Inagawa *et al*[28]	9	239	11	54	8.8%	0.18 [0.08, 0.42]
Kawamura *et al*[36]	7	349	2	124	3.8%	1.24 [0.26, 5.91]
Kim *et al*[30]	5	791	1	309	2.2%	1.95 [0.23, 16.65]
Kulig *et al*[18]	36	1500	17	492	12.1%	0.69 [0.39, 1.23]
Lee *et al*[38]	14	1053	3	432	5.3%	1.91 [0.55, 6.63]
Lee *et al*[42]	3	157	3	86	3.7%	0.55 [0.11, 2.66]
Murphy *et al*[35]	1	27	0	23	1.1%	2.57 [0.11, 60.24]
Nobuoka *et al*[40]	25	545	5	99	7.7%	0.91 [0.36, 2.32]
Oh *et al*[16]	2	311	1	99	1.8%	0.64 [0.06, 6.95]
Ohno *et al*[37]	3	157	3	40	3.8%	0.25 [0.05, 1.22]
Ojima *et al*[31]	3	573	5	116	4.4%	0.12 [0.03, 0.50]
Pacelli *et al*[33]	3	76	3	69	3.8%	0.91 [0.19, 4.35]
Tanaka *et al*[26]	7	144	1	47	2.4%	2.28 [0.29, 18.09]
Tsujinaka *et al*[32]	8	446	3	77	5.0%	0.46 [0.12, 1.70]
Yamada *et al*[17]	3	106	2	35	3.1%	0.50 [0.09, 2.84]
Yasuda *et al*[34]	2	83	1	16	1.9%	0.39 [0.04, 4.00]
Total (95%CI)		8687		3680	100.0%	0.59 [0.42, 0.82]
Total events	178		144			

Heterogeneity: Tau^2 = 0.17; χ^2 = 27.27, df = 18 (P = 0.07); I^2 = 34%
Test for overall effect: Z = 3.07 (P = 0.002)



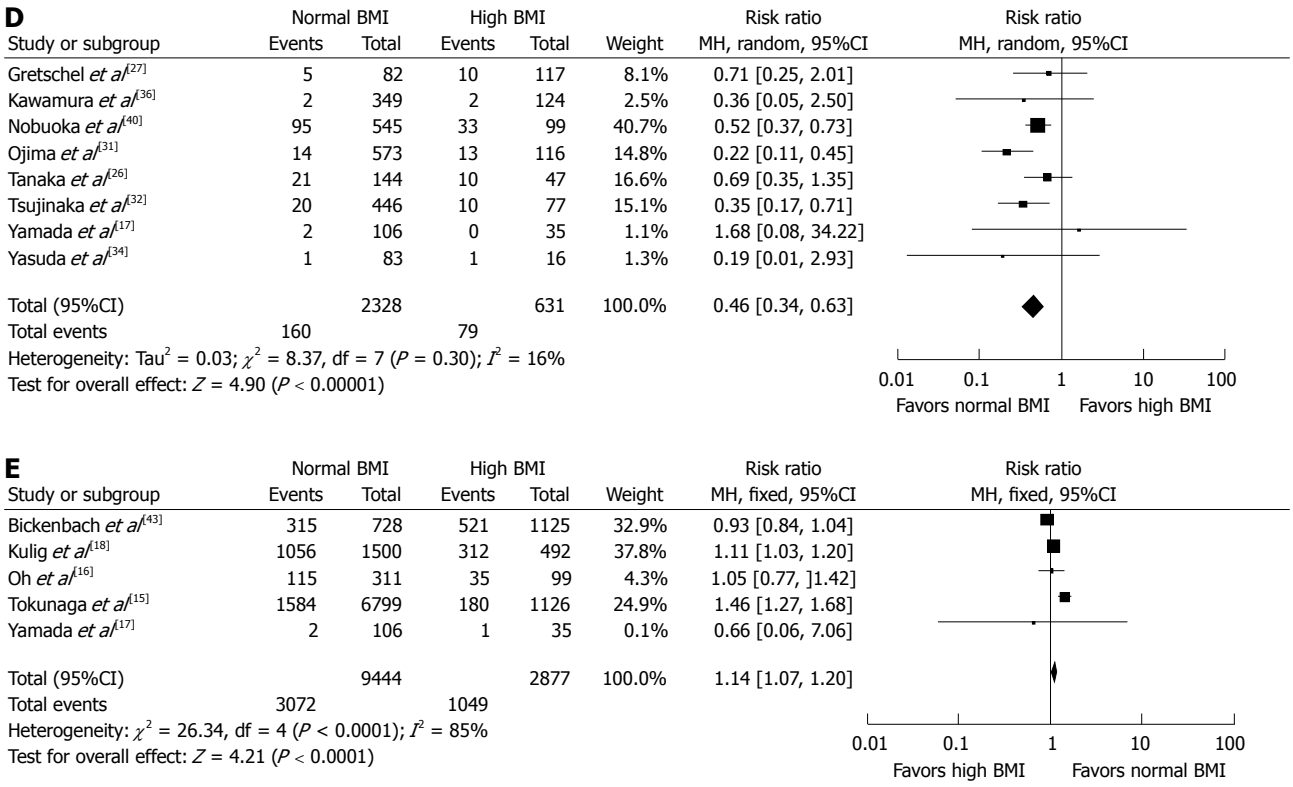


Figure 2 Forest plot. A: For operative time showing overweight in association with longer duration of operative time than non-overweight; B: For morbidity showing overweight in association with more postoperative complication than non-overweight; C: For anastomotic leak indicating that overweight correlates with higher rate of anastomotic leak; D: For pancreatic fistula showing overweight in association with more pancreatic fistula than non-overweight; E: For long-term survival favoring normal weight with better survival results. BMI: Body mass index.

lymph nodes, blood loss, and postoperative hospital stay. No heterogeneity was found in any of the other assessed outcomes. There was no evidence of publication bias ($P > 0.05$ for all 3 of the following outcomes: morbidity, anastomotic leakage, and mortality).

Surgical results for patients receiving laparoscopic gastrectomy

Overweight patients receiving laparoscopic gastrectomies had increased complications (RR: 0.48; 95%CI: 0.29-0.79; $P = 0.004$), longer operation times [MD: -15.06; 95%CI: -17.41-(-12.70); $P < 0.00001$], more blood loss [MD: -47.83; 95%CI: -68.12-(-27.53); $P < 0.00001$], and fewer retrieved lymph nodes (MD: 2.11; 95%CI: 1.35-2.88; $P < 0.00001$) than healthy-weight patients (Table 2). There were no significant differences in any of the other outcomes. Morbidity was a heterogeneous outcome with very low quality, whereas the other outcomes were rated as low quality. Egger's regression method was not applied in this subgroup analysis because none of the outcome variables included at least 10 trials.

Surgical results for patients receiving total gastrectomy

Overweight patients receiving total gastrectomies had increased complications (RR: 0.68; 95%CI: 0.56-0.84; $P = 0.0003$), more pancreatic fistulas (RR: 0.56; 95%CI: 0.42-0.74; $P < 0.0001$), longer operation times [MD: -23.94; 95%CI: -32.62-(-15.25); $P < 0.00001$], more blood loss [MD: -293.84; 95%CI: -401.80-(-185.87); $P < 0.00001$], and fewer retrieved lymph nodes (MD: 3.99; 95%CI: 1.14-6.83; $P = 0.006$) than healthy-weight patients. There were no significant differences in any of the other outcomes.

Surgical results for patients receiving subtotal gastrectomy

Overweight patients receiving subtotal gastrectomies had increased complications (RR: 0.61; 95%CI: 0.40-0.94; $P = 0.02$), longer operation times [MD: -22.02; 95%CI: -29.18-(-14.86); $P < 0.00001$], and more blood loss [MD: -58.36; 95%CI: -93.56-(-23.45); $P = 0.001$] than healthy-weight patients. There were no significant differences in any of the other outcomes, including pancreatic fistulas and the number of retrieved lymph nodes.

DISCUSSION

Theoretically, comorbidity risk factors[45] and surgical complications could cause prolonged surgical times, increased blood loss, more postoperative complications, and greater intraoperative mortality. However, the effects of comorbidity risk factors are uncertain because published papers[25-43] assessing the relationship between being overweight and poor surgical outcomes have reported conflicting results, especially for the outcome variables, such as morbidity, mortality, and long-term survival.

Table 2 Summary statistics of pooled data comparing normal body mass index *vs* high body mass index for overall patients, patients receiving open gastrectomy and laparoscopic gastrectomy

Outcome variables	Studies	Pooled patients	Pooled RR or MD or HR	95%CI	Test for overall effect		Test for heterogeneity	
					Z	*P* value	I^2	*P* value
Overall patients								
Operative time	9	5905	-29.14	-38.08, -20.21	6.39	< 0.00001	70%	0.0008
Retrieved lymph nodes	6	4612	1.69	0.75, 2.62	3.55	0.0004	9%	0.36
Blood loss	5	2096	-194.58	-314.21, -74.95	3.19	0.001	86%	< 0.00001
Morbidity	17	17514	0.75	0.66, 0.85	4.48	< 0.00001	58%	0.001
Anastomotic leak	19	12367	0.59	0.42, 0.82	3.07	0.002	34%	0.07
Pancreatic fistula	8	2959	0.46	0.34, 0.63	4.90	< 0.00001	16%	0.3
Mortality	13	16590	0.86	0.58, 1.29	0.71	0.48	0%	0.76
Postoperative hospital stay	6	4552	-5.83	-13.44, 1.78	1.5	0.19	98%	< 0.00001
Cancer-specific survival	5	12321	1.14	1.07, 1.20	4.21	< 0.0001	85%	< 0.0001
Patients receiving open gastrectomy								
Operative time	7	2179	-25.24	-33.53, -16.95	5.97	< 0.00001	53%	0.05
Retrieved lymph nodes	6	2838	3.81	-0.34, 7.96	1.8	0.07	91%	< 0.00001
Blood loss	5	1708	-212.93	-301.04, -124.82	4.74	< 0.00001	74%	0.004
Morbidity	11	12510	0.78	0.66, 0.94	2.68	0.007	64%	0.002
Anastomotic leak	14	7320	0.58	0.38, 0.89	2.51	0.01	37%	0.08
Pancreatic fistula	6	2470	0.46	0.38, 0.67	4.03	< 0.0001	37%	0.16
Mortality	10	12763	1.17	0.69, 2.01	0.58	0.56	0%	0.83
Postoperative hospital stay	4	1339	-2.04	-6.00, 1.91	1.01	0.31	80%	0.002
Cancer-specific survival	4	12180	1.14	1.07, 1.20	4.23	< 0.0001	89%	< 0.00001
Patients receiving laparoscopic gastrectomy								
Operative time	4	1845	-15.06	-17.41, -12.70	12.52	< 0.00001	0%	0.52
Retrieved lymph nodes	3	1746	2.11	1.35, 2.88	5.39	< 0.00001	0%	0.61
Blood loss	3	360	-47.83	-68.12,-27.53	4.62	< 0.00001	47%	0.15
Morbidity	6	3151	0.48	0.29, 0.79	2.91	0.004	67%	0.009
Anastomotic leak	6	3194	0.83	0.42, 1.65	0.53	0.6	32%	0.2
Pancreatic fistula	3	489	0.3	0.08, 1.20	1.7	0.09	10%	0.33
Mortality	3	1833	0.4	0.12, 1.30	1.53	0.13	0%	0.41
Postoperative hospital stay	3	1833	0.17	-0.80, 1.15	0.35	0.73	34%	0.22
Cancer-specific survival	1	141	1.65	0.13, 20.70	0.39	0.7	Not applicable	Not applicable

We evaluated the operation time, intraoperative blood loss, and number of retrieved lymph nodes as indices of the surgical difficulty. Both the operation time and blood loss for overweight patients with gastric cancer were significantly higher than for the normal-weight cohort, regardless of whether open gastrectomy or laparoscopic gastrectomy was performed. Being overweight was also correlated with significantly fewer retrieved lymph nodes. Two reasons may contribute to the lower number of retrieved lymph nodes[6]. First, the excess fat tissue in the abdomen could limit the node dissection for overweight patients. Second, pathologists would have difficulty obtaining lymph nodes from a large amount of adipose tissue.

The relationship between high BMI and surgical safety for patients with gastric cancer is controversial. In the 17 trials providing data about morbidity, ten studies[18,29,33-36,38,39,42,43] did not indicate that being overweight affected the overall postoperative complication rate, whereas the remaining 7[15,16,25,28,30,32,40] did. Our meta-analysis strongly suggests that overweight patients have more complications. More specifically, the rates of pancreatic fistula and anastomotic leakage were significantly higher in the overweight patients, which also was true in the subgroup analysis of patients receiving open gastrectomy. According to these results, it is clear that overweight patients have high risks of postoperative complications. However, it is still uncertain whether a high BMI has a direct influence on the postoperative morbidity. High BMIs directly affect the operation times for cholecystectomies, colectomies, and unilateral mastectomies but have no direct relationship with complications[46]. Increased operation times and blood loss secondary to high BMI are also responsible for high postoperative complication rates[28,31], which is likely because prolonged operative times prolong the duration of anesthesia and increase the risk of thromboembolic, cardiac, and respiratory complications. Our study found strong evidence (RR < 0.5) for an association between being overweight and high rates of pancreatic fistula, as suggested in earlier reports[13,32]. This effect on the occurrence of a pancreatic fistula could be because removal of overweight patients' pancreatic capsules is difficult; they have poor differentiation between the pancreas and excess pancreatic fat deposition[47,48]. This could also hamper peripancreatic node dissection and increase the potential for iatrogenic injury to pancreatic tissue. More interestingly, according to one included study[26], minimal damage to the pancreatic tissue, which would never cause a pancreatic fistula in patients with low visceral fat area (VFA), could result in pancreatic fistula in high-VFA patients. Visceral fat maybe play an important role in the pathogenesis from pancreatic injury to pancreatic fistula. Therefore, being overweight could have a direct influence on the postoperative complication rate, as is the case for pancreatic fistulas. Although overweight patients suffered

more complications, no difference was detected for mortality, which might be attributed to the advancement of perioperative management. Changes in perioperative management have dramatically decreased the death rate from serious postoperative complications such as pancreatic fistula and anastomotic leakage. Thus, it is safe to perform radical gastrectomy in overweight patients.

Relevant studies reported conflicting results on the relationship between being overweight and long-term survival[6,7,28,31,40,49,50]. Theoretically, excess visceral fat and being overweight could negatively affect survivorship by increasing the rates of coexisting disease and postoperative complications. In addition, according to Adachi *et al*[6], incomplete lymph node dissection in overweight patients could result in retention of metastatic nodes that are responsible for the worse survivorship. Increased long-term survival in normal-weight patients was found in the current review and is consistent with the hypothesis that excess accumulation of visceral fat could impair patient survival and promote tumor recurrence. Unfortunately, among the 23 analyzed studies, only five were included in the analysis of survivorship; thus, the survivorship results, with fewer data points, are less convincing. However, during the data extraction, we noticed that the percentage of patients with early gastric cancer was greater for the overweight cohort. Compared with advanced gastric cancer patients, patients with early gastric cancer have a significantly higher long-term survival rate[51]. Although the overweight cohort had more patients with early gastric cancer, who might have a more promising prognosis, the overall long-term survival was still significantly lower in this cohort. This is indirect evidence that being overweight can impair the long-term survival of gastric patients. In addition, we do not think that the decreased long-term survival was caused by the increasing comorbidity related to being overweight, such as diabetes and cardiovascular disease because we used cancer-specific survival as the indicator of long-term survival.

High BMIs increase the difficulty and decrease the safety of laparoscopic gastrectomy procedures, as is the case with open gastrectomy. These findings are consistent with some previous studies[25,30,36]. However, other studies[37-39] did not show significant differences in the morbidity between overweight and normal-weight cohorts for laparoscopic gastrectomy. Unlike open gastrectomy, laparoscopic gastrectomy can achieve excellent visibility even for overweight patients because the pneumoperitoneum creates sufficient extra space in the abdominal cavity. Although the laparoscopic procedure has these advantages, the results of our study still suggest that being overweight negatively affects the difficulty and safety of laparoscopic gastrectomy.

Moreover, being overweight increases the difficulty and impairs the safety of both total and subtotal gastrectomies. However, a subtotal gastrectomy seems to be safer than a total gastrectomy for overweight patients because subtotal gastrectomy did not increase the rate of pancreatic fistula occurrence in the overweight group, which is a severe complication after gastric surgery. In addition, after subtotal gastrectomy, the numbers of retrieved lymph nodes did not differ significantly between the two cohorts, while there was a difference in the number of lymph nodes retrieved after total gastrectomy.

Because of the relationship between being overweight and impaired surgical safety, surgeons should be more careful when performing radical gastrectomy in the future. In addition, for suitable cases, performing a subtotal gastrectomy might be safer than performing a total gastrectomy.

This meta-analysis has some limitations. First, most of the studies in this meta-analysis were rated as low or very low quality due to their retrospective study designs. All included studies are nonrandomized in nature and have a risk of bias. Although randomized trials are the gold standard for study design, random allocation of patients with different BMIs is hardly feasible. To overcome this limitation in the future, more rigorously designed studies with a good balance of other confounding factors, such as age and tumor-node-metastasis stage, are needed. Second, although BMI $\geqslant$ 25 kg/m^2 was used as a criterion for classifying patients as overweight, it may be not the best index because the distribution of fat tissue could differ greatly between individuals, even those with the same BMI[26,52]. Therefore, individuals with the same BMI could have different surgical outcomes due to their different fat distributions. Some studies[26,39,53] have indicated that the VFA is a better index than BMI. Third, the procedure type and extent of node dissection differed among the studies in our meta-analysis. Moreover, gastric cancer was more prevalent in Eastern countries than Western ones. As a result, surgeons from Eastern countries could have more experience in performing the surgeries and dealing with the postoperative complications. Additionally, the higher incidence of gastric cancer has led to earlier diagnosis in Asian countries. Therefore, the proportions of early gastric cancer cases differed between studies from the East and West in this review. All these factors could account for the heterogeneity of some results and jeopardize the reliability of the conclusions. The limitations in the previously published data could potentially affect the analysis of both groups. Publication bias was a possible source of bias during the meta-analysis because positive results are more likely to be published. Several methods have been proposed for detecting bias and, in this review, we detected publication bias by a funnel plot and Egger's regression method, which is reliable when the number of included trials is not less than 10. It turned out that our results did not show significant publication bias ($P > 0.05$) for the parameters in this review.

In conclusion, this meta-analysis indicates that overweight patients with gastric cancer have increased surgical complications and worse short-term operative outcomes than patients with healthy weights, and these results were consistent for patients who underwent either a laparoscopic gastrectomy or an open gastrectomy. Although no

evidence was detected to indicate that being overweight had higher postoperative morbidity, being overweight decreased the long-term survival.

COMMENTS

Background

The increasing global prevalence of overweight and obese individuals is problematic for Western countries and is also a concern for Eastern countries such as China and South Korea. Surgical results and postoperative complications are believed to be greater for overweight patients with gastric cancer, but this is controversial due to conflicting results from previous studies.

Research frontiers

The postoperative morbidity, mortality, and long-term survival after D2 node dissection differed between different studies from Asia and Europe. It is possible that this discrepancy is due to the differing prevalence of overweight patients in Western and Eastern countries. However, different studies have conflicting results for the effect of being overweight on both the short-term and long-term surgical outcomes for gastric cancer patients.

Innovations and breakthroughs

To the knowledge, this is the first meta-analysis studying the effect of being overweight on the surgical results of gastric cancer patients. The authors found that overweight patients with gastric cancer have increased surgical complications and worse short-term operative outcomes than patients with healthy weights, and these results were consistent for patients who underwent either a laparoscopic gastrectomy or an open gastrectomy.

Applications

This meta-analysis emphasizes the influence of being overweight on gastric cancer surgical results. Surgeons should pay particular attention when they perform radical gastric cancer surgery.

Peer review

Overall, this manuscript provides a detailed and comprehensive review of the influence of elevated patient body mass index on outcomes following gastrectomy as a treatment for cancer. This article includes information about the complications of gastric surgery and has potential clinical implications. Finally, this meta-analysis demonstrates that being overweight is significantly correlated with surgical difficulty, a high rate of postoperative complications, and poor survival in patients with gastric cancer.

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META-ANALYSIS

Prediction of the severity of acute pancreatitis on admission by urinary trypsinogen activation peptide: A meta-analysis

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Abstract

AIM: To undertake a meta-analysis on the value of urinary trypsinogen activation peptide (uTAP) in predicting severity of acute pancreatitis on admission.

METHODS: Major databases including Medline, Embase, Science Citation Index Expanded and the Cochrane Central Register of Controlled Trials in the Cochrane Library were searched to identify all relevant studies from January 1990 to January 2013. Pooled sensitivity, specificity and the diagnostic odds ratios (DORs) with 95%CI were calculated for each study and were compared to other systems/biomarkers if mentioned within the same study. Summary receiver-operating curves were conducted and the area under the curve (AUC) was evaluated.

RESULTS: In total, six studies of uTAP with a cut-off value of 35 nmol/L were included in this meta-analysis. Overall, the pooled sensitivity and specificity of uTAP for predicting severity of acute pancreatitis, at time of admission, was 71% and 75%, respectively (AUC = 0.83, DOR = 8.67, 95%CI: 3.70-20.33). When uTAP was compared with plasma C-reactive protein, the pooled sensitivity, specificity, AUC and DOR were 0.64 *vs* 0.67, 0.77 *vs* 0.75, 0.82 *vs* 0.79 and 6.27 *vs* 6.32, respectively. Similarly, the pooled sensitivity, specificity, AUC and DOR of uTAP *vs* Acute Physiology and Chronic Health Evaluation Ⅱ within the first 48 h of admission were found to be 0.64 *vs* 0.69, 0.77 *vs* 0.61, 0.82 *vs* 0.73 and 6.27 *vs* 4.61, respectively.

CONCLUSION: uTAP has the potential to act as a stratification marker on admission for differentiating disease severity of acute pancreatitis.

Key words: Acute pancreatitis; Urinary trypsinogen activation peptide; C-reactive protein; Acute Physiology and Chronic Health Evaluation Ⅱ score; Meta-analysis

Core tip: Currently, the assessment of acute pancreatitis severity on admission remains a challenge to clinicians. A single, rapid biochemical marker is the preferred choice than clinical and computed tomography scoring systems. In this study, the value of urinary

trypsinogen activation peptide (uTAP), on admission, in predicting severity of acute pancreatitis was assessed. It was found that the ability of uTAP to predict severity of acute pancreatitis on admission was comparable to C-reactive protein (at 48 h) and was potentially better than the Acute Physiology and Chronic Health Evaluation Ⅱ score (at 48 h), the most frequently used biochemical marker and clinical scoring system in acute pancreatitis, respectively.

Huang W, Altaf K, Jin T, Xiong JJ, Wen L, Javed MA, Johnstone M, Xue P, Halloran CM, Xia Q. Prediction of the severity of acute pancreatitis on admission by urinary trypsinogen activation peptide: A meta-analysis. *World J Gastroenterol* 2013; 19(28): 4607-4615 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i28/4607.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i28.4607

INTRODUCTION

Acute pancreatitis causes up to 210000 admissions in the United States annually and remains a diagnostic, prognostic and therapeutic dilemma for surgeons and physicians[1]. Although mild acute pancreatitis is associated with virtually no mortality, severe acute pancreatitis continues to be at the other end of the spectrum with mortality reaching up to 30%, mainly due to pancreatic necrosis and organ failure[2].

As severe acute pancreatitis may progress very quickly and is normally associated with a complicated clinical course and higher mortality, it is vital to identify these patients as early as possible to initiate appropriate supportive management, especially within the first 24 h after symptoms onset[3]. Therefore, in the last few decades many biomarkers[4], radiological[5] and clinical scoring systems[6,7] have been developed and validated to fulfil this role. These, however, have not been entirely successful. The Glasgow[8], Acute Physiology and Chronic Health Evaluation Ⅱ (APACHE Ⅱ)[9], and Ranson[10] scoring systems and plasma C-reactive protein (CRP)[11] are still the most widely used parameters and form part of many guidelines, however, their use does come with its own limitations.

There is enough evidence to establish trypsinogen activation as one of the earliest steps in the pathophysiology of the disease[12,13], and consequently, trypsinogen activation peptide (TAP) has been shown to be an excellent marker for severity stratification in different experimental acute pancreatitis models[14]. In human acute pancreatitis, TAP is rapidly excreted in urine and in urinary[15] and peritoneal fluid[16]. TAP concentrations correlate well with disease severity. Therefore, it is reasonable to hypothesize that pancreas-specific activation peptides would be elevated (in the urine) from the onset of disease and could potentially serve as early biomarkers. Urinary TAP (uTAP) is the most studied peptide for predicting severity of acute pancreatitis[17], but its diagnostic value in severe acute pancreatitis has not been systematically assessed. In this study, a meta-analysis was carried out to evaluate existing evidence of uTAP in predicting the severity of acute pancreatitis.

MATERIALS AND METHODS

Study selection

A comprehensive literature search of Medline, Embase, Science Citation Index Expanded and the Cochrane Central Register of Controlled Trials in The Cochrane Library was carried out to identify studies evaluating the prognostic efficacy of uTAP from January 1990 (the first human study)[15] to February 2013. The following medical subject headings (MeSH) and keywords were used: "trypsinogen activation peptide" or "activation peptide" and "acute pancreatitis" or "severe acute pancreatitis" or "post endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis". Equivalent free-text search terms were used in the search strategy. All abstract supplements from published literature or from relevant international meetings were searched manually. Relevant papers were also identified from the reference lists of previous papers. Only studies which were published in English as full-text articles were included. Final inclusion of articles was determined by consensus; when this failed, a third author adjudicated. Severe acute pancreatitis was defined as the development of organ failure and/or local complications[15,18].

Inclusion and exclusion criteria

Two authors independently identified and screened the search findings for potentially eligible studies.

Inclusion criteria: (1) English language studies published as full text articles in peer-reviewed journals; (2) Human studies; (3) Studies with available data; and (4) When similar studies were reported by the same institution, the best quality study was included.

Exclusion criteria: (1) Abstracts, letters, editorials, expert opinions, reviews and case reports; (2) Where only concentration or *P* value was reported; (3) Studies assessing the efficacy of serum/plasma TAP in predicting the severity of acute pancreatitis; and (4) Studies assessing the efficacy of uTAP in diagnosing acute pancreatitis.

Data extraction and quality assessment

Data were extracted by two independent observers using standardized forms. The recorded data included study design, demographics (age, gender, etiology and country of origin), severity of disease, duration from symptoms onset to admission, time point for the collection of samples and cut-off values. Diagnostic parameters including true positivity (TP), false positivity (FP), false negativity (FN) and true negativity (TN) were extracted directly or by calculating the sensitivity and specificity of uTAP for predicting the severity of acute pancreatitis. TP, FP, FN and

Table 1 Characteristics of included prospective studies for urinary trypsinogen activation peptide as a predictor of severity of acute pancreatitis

Ref.	Year	Country	Sampling time after admission	Cut-off value (nmol/L)	Total (*n*)	Male/ female (*n*)	Mild/ severe (*n*)	Mean age: male/ female (yr)	Etiology
Neoptolemos *et al*[32]	2000	Multicenter	On admission	35	172	87/85	137/35	52 (29-84)	Biliary 74, alcoholic 62, other 36
Liu *et al*[34]	2002	China	On admission	35	41	NA	29/12	NA	NA
Khan *et al*[33]	2002	United States	On admission	35	58	33/25	39/19	69 ± 19	Biliary 26, alcoholic 18, HTC 3, postoperative (including ERCP) 9, idiopathic 2
Lempinen *et al*[35]	2003	Finland	On admission	35	127	NA	98/29	NA	Biliary 24, alcoholic 74, other 29
Johnson *et al*[37]	2004	Multicenter	On admission	35	190	104/86	164/26	54 (42-70)	Biliary 70, alcohol 65, other 55
Huang *et al*[38]	2010	China	On admission	35	187	112/75	149/38	60.4 ± 6.7; 59.5 ± 8.1	Biliary 139, alcoholic 19, other 29

ERCP: Endoscopic retrograde cholangiopancreatography; HTC: Hypercholesterolemic; NA: Not available.

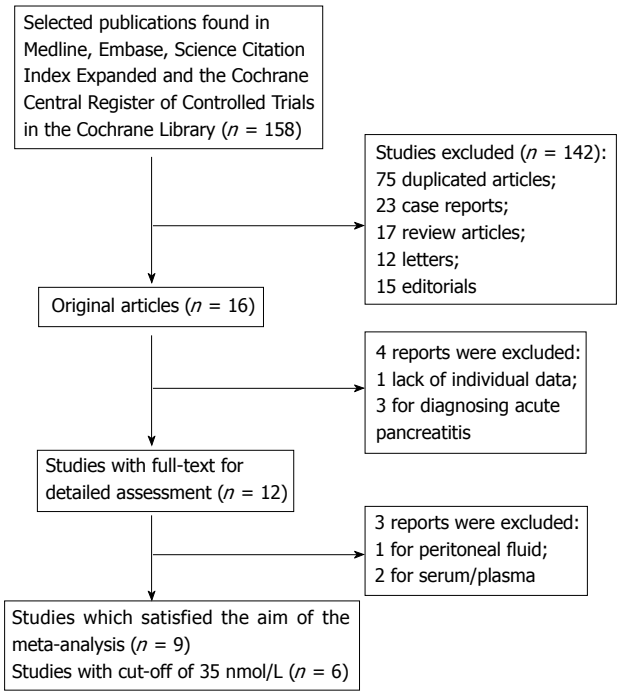

Figure 1 Flow diagram illustrating the process of identification of relevant studies.

TN were also extracted for serum CRP and APACHE Ⅱ score at the highest diagnostic values during the first 2 d after admission if these were reported in the included studies. The quality of the included studies was assessed independently by two reviewers using the Standards for Reporting of Diagnostic Accuracy (STARD) initiative guidelines[19]. Studies with a STARD score of ≥ 16 were considered as high quality studies.

Statistical analysis

The meta-analysis was performed with Meta-DiSc 1.4 software (Hospital Ramón y Cajal, Madrid, Spain). Pooled sensitivity, specificity, and diagnostic odds ratio (DOR) with diagnostic value Q were calculated. The mentioned parameters were pooled respectively with a corresponding 95%CI. Receiver operating characteristics were also generated and expressed by area under curve (AUC). The AUC represents the accuracy of diagnosis and DOR indicates its diagnostic capability for differentiating disease groups from negative groups[20]. Heterogeneity was evaluated using Cochran's Q test and a P value of 0.1 was considered significantly different. I^2 statistics were used to measure the percentage of total variation across the studies due to heterogeneity (I^2 of 50% or more indicating the presence of heterogeneity)[21]. The publication bias of included studies was assessed using a funnel plot of the effect of effective sample size weighted regression tests of asymmetry[22]. The meta-analysis was performed using a fixed-effect model if there was no heterogeneity among the studies, otherwise the random effects model was used[23]. The sensitivity analyses were undertaken by excluding each study from the analysis to ascertain its effect on the overall results. Subgroup analyses were dependent on the following items: high quality studies, sample size ≥ 50 in each study, single center studies and severity defined by the 1992 Atlanta Classification[18].

RESULTS

Description of included trials in the meta-analysis

Details of the literature research are shown in Figure 1 and 16 clinical studies were identified. Seven studies were excluded: one due to lack of data for analysis[24], one studied peritoneal fluid TAP[25], two studied serum/plasma TAP[26,27], three for diagnosing acute pancreatitis, but not for assessing the severity[28] or post-ERCP pancreatitis[29,30]. Of the 9 studies[15,31-38] that were potentially useful for analysis, only six had the cut-off of 35 nmol/L; it being variable in the remaining three and therefore, these six studies were included in the final analysis.

Study and patient characteristics

Table 1 describes the included studies and patient characteristics. All of the six included studies were prospectively designed and were of high quality (STARD score ≥ 16). There were 2 multicenter[32,37] and 4 single center[33-35,38] trials. Five studies[32-35,38] defined the severity of acute pancreatitis by the 1992 Atlanta Classification, in which severe cases included the moderate and the severe groups according to the revised Atlanta Classification[39]. One

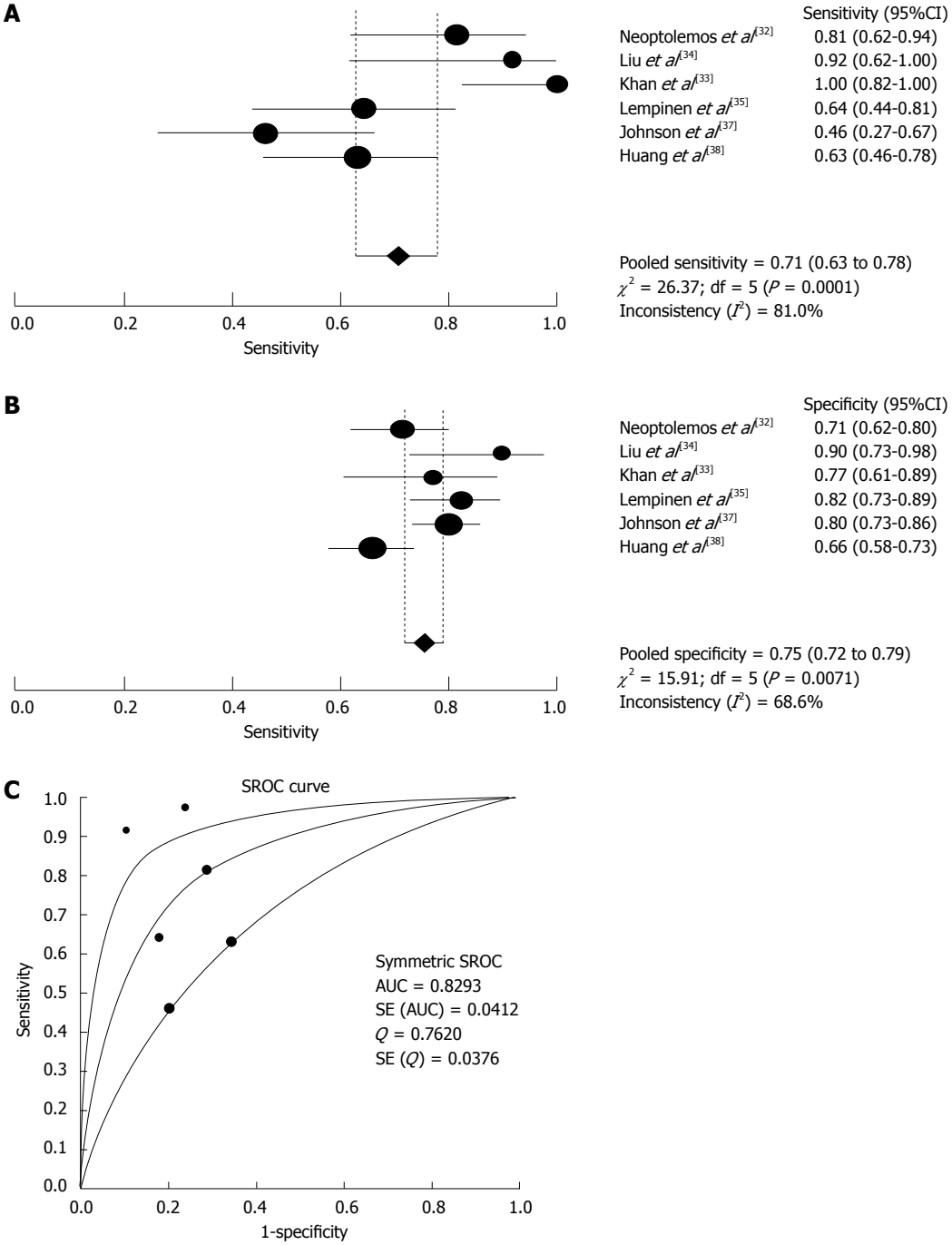

Figure 2 Forest plots of sensitivity (A), specificity (B), and summary receiver operating characteristic curve (C) for on admission urinary trypsinogen activation peptide in predicting severe acute pancreatitis. SROC: Summary receiver operating characteristic; AUC: Area under the curve.

Table 2 Diagnostic parameters of included studies

Ref.	Patients (*n*)	Patients analyzed (mild/severe)	TP	FP	FN	TN
Neoptolemos *et al*[32]	172	132 (105/27)	22	30	5	75
Liu *et al*[34]	41	41 (29/12)	11	3	1	26
Khan *et al*[33]	58	58 (39/19)	19	9	0	30
Lempinen *et al*[35]	127	118 (90/28)	18	16	10	74
Johnson *et al*[37]	190	190 (164/26)	12	33	14	131
Huang *et al*[38]	187	187 (149/38)	24	51	14	98

TP: True positive; FP: False positive; FN: False negative; TN: True negative.

study defined severe acute pancreatitis as the presence of local complications or the presence of persistent organ failure that was more than 48 h[37]. The predominant etiology in recruited patients was biliary in origin followed by alcoholic, ERCP and idiopathic.

Meta-analysis results

Results of the data extraction are shown in Table 2 and results of the meta-analysis are shown in Figures 2 and 3, and summarized in Table 3.

Table 3 Meta-analysis outcomes of included studies

	Trials (*n*)	Patients (*n*)	AUC	DOR (95%CI)	*Q*	*P* value	I^2
All studies	6	726	0.83	8.67 (3.70-20.33)	15.88	0.0072	68.50%
Study subgroups							
Sample size ⩾ 50	5	685	0.80	6.48 (3.05-13.74)	10.28	0.0360	61.10%
Single center	4	413	0.86	14.25 (3.39-59.80)	12.92	0.0048	76.80%
1992 Atlanta Classification	5	536	0.84	11.97 (4.17-34.36)	13.65	0.0085	70.70%

AUC: Area under the curve; DOR: Diagnostic odds ratios.

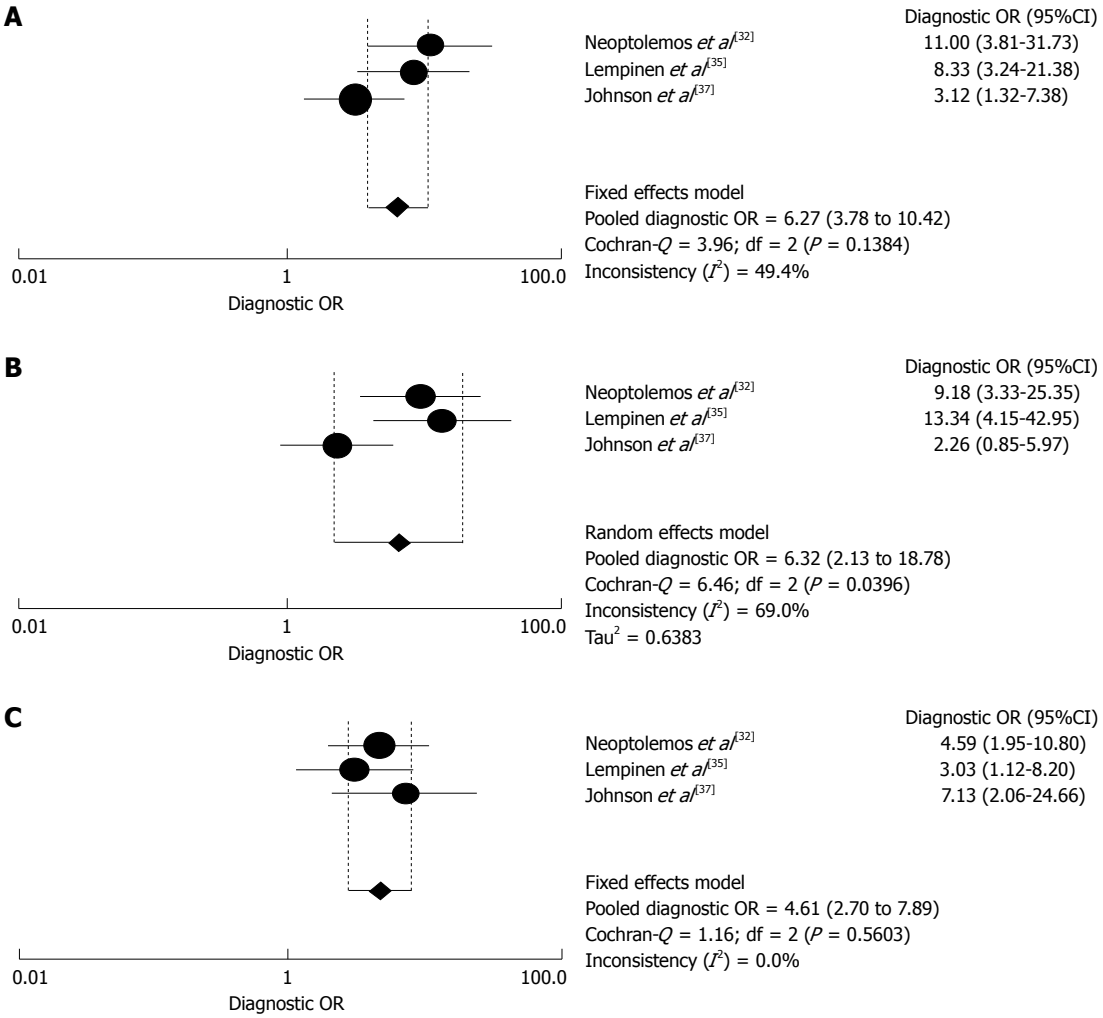

Figure 3 Forest plots of diagnostic OR for urinary trypsinogen activation peptide *vs* serum C-reactive protein and urinary trypsinogen activation peptide *vs* Acute Physiology and Chronic Health Evaluation Ⅱ score in predicting severe acute pancreatitis. The pooled diagnostic odds ratios of on-admission urinary trypsinogen activation peptide (A), plasma C-reactive protein at 48 h (B) and Acute Physiology and Chronic Health Evaluation Ⅱ score at 48 h (C).

On admission uTAP for predicting the severity of acute pancreatitis: Data from the six included studies (775 patients with 726 analyzed) revealed that the pooled sensitivity, specificity, AUC and DOR were 71% (95%CI: 63-78), 75% (95%CI: 72-79), 0.83 and 8.67 (95%CI: 3.70-20.33), respectively. Data are shown in Figure 2 and Table 3. These data suggest that uTAP has the potential to predict the severity of acute pancreatitis.

uTAP *vs* plasma CRP for severity stratification: There were 3 studies[32,35,37] that compared the prognostic value of on admission uTAP (440 patients analyzed) with plasma CRP (458 patients analyzed) within the first 48 h after admission in the severity stratification for acute pancreatitis. The pooled sensitivity, specificity, AUC value and DOR were 0.64 *vs* 0.67, 0.77 *vs* 0.75, 0.82 *vs* 0.79 and 6.27 *vs* 6.32, respectively (Figure 3A and B) for uTAP and CRP (best diagnostic values at 48 h). As suggested by the data, prognostic efficacy of the two markers was found to be similar.

uTAP *vs* APACHE-Ⅱ score for severity stratification: There were 4 studies[32,34,35,37] that compared the prognos-

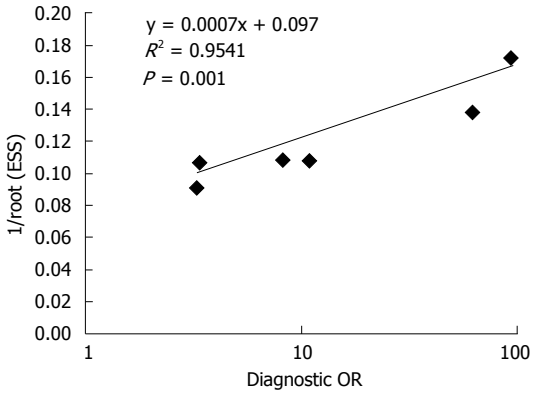

Figure 4 Funnel plot of the effect of effective sample size weighted regression tests of asymmetry for included studies. ESS: Effective sample size.

tic value of on admission uTAP with APACHE Ⅱ score within first 48 h after admission. Of these, 3 studies[32,35,37] used an APACHE Ⅱ score ⩾ 8 (422 patients analyzed) for defining severity and compared with uTAP (440 patients analyzed). The pooled sensitivity, specificity, AUC value and DOR were 0.64 *vs* 0.69, 0.77 *vs* 0.61, 0.82 *vs* 0.73 and 6.27 *vs* 4.61 for uTAP (values on admission) and APACHE Ⅱ score (best diagnostic values at 48 h), respectively (Figure 3A and C). These data suggest that uTAP may have a better prognostic value than the APACHE Ⅱ score in predicting the severity of acute pancreatitis.

Sensitivity and subgroup analysis: Outcomes for sensitivity and subgroup analysis are shown in Table 3. All six studies included were of high quality and sensitivity analysis demonstrated that significant heterogeneity still existed in these high quality studies (Q = 15.88, P = 0.0072, I^2 = 68.5%). Subgroup analysis showed that significant heterogeneity also existed in studies with sample size ⩾ 50 (Q = 10.28, P = 0.0360, I^2 = 61.1%), single center (Q = 12.92, P = 0.0048, I^2 = 76.8%), and severity defined by the 1992 Atlanta Classification (Q = 13.65, P = 0.0085, I^2 = 70.7%).

Publication bias: A funnel plot was created to demonstrate bias in the studies. The shape of the funnel plot showed asymmetry and this was confirmed by P = 0.001, showing that more significant results were present in smaller studies (Figure 4).

DISCUSSION

Upon admission, severity prediction of acute pancreatitis is crucial. This is still controversial, not universal and is mired by institutional differences. The current commonly used severity prediction systems include clinical assessment, biochemical markers, and both clinical and radiological scoring systems[40,41]. Clinical assessment provides a relatively high specificity (83%-98%) for ruling out mild acute pancreatitis, but has poor sensitivity (34%-64%) for the same[40]. When compared with clinical scoring systems, contrast-enhanced computed tomography (CECT) was not found to be superior in predicting outcomes of acute pancreatitis on admission[5].

Ideally, the best biomarker for predicting disease severity should be accurate, rapid, inexpensive and non-invasive. The pancreas-specific biomarkers are generally thought to be related to disease severity[14]. In 1988, a TAP assay with a detection limit of 10 picomolar concentration was developed by Hurley *et al*[42], enabling the detection of TAP in the body fluid to become more feasible. In a multicenter study conducted by Neoptolemos *et al*[32] that recruited 172 acute pancreatitis patients, uTAP concentration was found to be significantly different between mild and severe acute pancreatitis from 0-96 h after symptoms onset. Most importantly, uTAP values at both 24 and 48 h after admission provided the highest prognostic values for severe acute pancreatitis when compared to plasma CRP and clinical scoring systems (APACHE-Ⅱ, Glasgow and Ranson).

For the six studies included in this meta-analysis, the pooled results indicated that uTAP has potential for predicting the severity of acute pancreatitis upon hospital admission (AUC = 0.83, DOR = 8.67, 95%CI: 3.7-20.33). This is at least comparable with the current in-use biomarkers[6]. While most of the currently used biomarkers are non-specific in nature (specific for inflammation and other aspects), TAP is specific to the pancreas and is liberated within the first few hours after the onset of symptoms[15,32]. The prognostic value of uTAP (on admission) was similar to the APACHE-Ⅱ score obtained 24 h after admission in this meta-analysis. It is noteworthy that despite the APACHE-Ⅱ score being one of the most frequently used clinical scores to assess the severity of acute pancreatitis, it is cumbersome to use and has dubious use in certain settings; *i.e.,* in critical care environments where physiology has been corrected. Therefore, there is a need for simple and quick severity prediction techniques.

The prognostic value of an on-admission uTAP was also compared with plasma CRP (obtained 0-48 h after admission), currently the most widely used severity biomarker in acute pancreatitis and other acute inflammatory diseases[43]. uTAP had a relatively higher diagnostic value than plasma CRP, which suggested that uTAP might be a highly valuable biomarker for the quick assessment of acute pancreatitis severity on admission. It is unsurprising, therefore, that the revised Atlanta Classification[39] introduced the potential use of uTAP for severity stratification, although, as yet, it has not been widely adopted in the clinical arena.

To investigate the presence of heterogeneity, sensitivity and subgroup analyses were performed, based on sample size, study center and definition of severity. There was significant heterogeneity among studies with sample size ⩾ 50, single center and severity defined by the 1992 Atlanta Classification. It has been shown in many previous studies that multicenter studies tend to have better and more reliable results than single center studies. Similar to increasing sample size. Most of our studies used the Atlanta criteria for severity stratification, albeit one which represented a small proportion of the same cohort. From a clinical perspective, heterogeneity may also be caused by the defi-

nition of severity. All the severe acute pancreatitis cases included in this meta-analysis had two distinct entities: the moderate and the severe categories according to the revised Atlanta Classification. The proportion of moderate and severe cases may have had a significant impact on the results of uTAP. On the other hand, the proportion of patients who had pancreatic necrosis may also have an impact on the uTAP levels. Moreover, whether etiology plays a role in this regard remains unknown. Unsurprisingly, there was publication bias towards more significant effects reported in smaller sample sizes. This may have implications on the interpretation of the pooled sensitivity and specificity. These problems can only be overcome in the future by larger studies being performed.

The 1992 Atlanta Classification defines severe acute pancreatitis if organ failure/or local complications such as pancreatic necrosis, abscess, or pseudocyst are present[17]. Pancreatic necrosis, however, is only characterized by an area more than 30% necrosis non-enhanced on CECT, which might lead to false negative results of uTAP when pancreatic necrosis is less than 30%. The revised Atlanta Classification categorizes severity of acute pancreatitis into mild, moderate and severe classes[39]; the determinant-based classification stratifies severity of acute pancreatitis into mild, moderate, severe and critical categories[44]. These classifications consider pancreatic necrosis and persistent organ failure as the key determinants of outcome of acute pancreatitis. Compared to the 1992 Atlanta Classification, the new definition of pancreatic necrosis is described as the detection of any area of non-enhancement or every heterogeneous peri-pancreatic collection on CECT. These updated definitions and classifications might prove to be very useful in re-assessing the importance of an on-admission uTAP for the quick assessment of severity in acute pancreatitis. One might postulate that uTAP may have high prognostic accuracy in identifying patients with a disease course that is at least moderate to severe or for ruling out mild patients.

This review suffers from a relatively small sample size, publication bias in smaller studies and heterogeneity in some of the inclusion criteria. To the best of our knowledge, this is the most comprehensive meta-analysis on the subject to date. We have tried to summarize the existing data, identify problems in undertaking that, point out potential areas of improvement and suggest guidelines for future studies.

In summary, uTAP is a rapid assay for the assessment of acute pancreatitis severity on admission and provides good prognostic accuracy for severe acute pancreatitis based on the 1992 Atlanta Classification. New studies should assess its value in a larger patient cohort with uniform inclusion criteria and in line with the newly proposed classification systems.

COMMENTS

Background

Assessment of the severity of acute pancreatitis is crucial upon admission. Currently, clinical assessment, biochemical markers, and both clinical and computed tomography scoring systems are used individually or in combination to fulfil the need. However, a single, inexpensive and rapid biochemical marker is preferred due to practical and economic reasons. In this regard, urinary trypsinogen activation peptide (uTAP) has been developed and validated in many clinical studies, showing good diagnostic value in predicting severe acute pancreatitis. However, these results have not been systematically assessed.

Research frontiers

To conduct a meta-analysis on the value of uTAP in predicting the severity of acute pancreatitis on admission.

Innovations and breakthroughs

The Revised Atlanta Classification has introduced the potential use of uTAP in the prediction of severity stratification. However, current clinical studies regarding this topic have not been systemically analyzed to provide evidence on uTAP to ensure its wide adoption in the clinical arena. This is the first meta-analysis summarizing data obtained from six studies in which the uTAP cut-off concentration (35 nmol/L) was the same for severity stratification. The meta-analysis showed that the diagnostic value of uTAP (on admission) for the severity of acute pancreatitis was comparable to CRP (at 48 h after admission) and was potentially better than the APACHE-Ⅱ score (at 48 h after admission), the most frequently used biochemical marker and clinical scoring system in acute pancreatitis, respectively.

Applications

The results of the meta-analysis encourage the use of uTAP in routine clinical practice, although this needs to be established in further well designed studies with possible comparisons to the new severity classification systems.

Peer review

This is a well written study that provides useful data on the usefulness of uTAP in the diagnostic/staging algorithm for acute pancreatitis. It is a powerful study that essentially means that uTAP is unlikely to find a widespread place in acute pancreatitis prognostic scoring as there are other more widely used tests available that are equivalent.

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CASE REPORT

Neuroendocrine carcinoma of the extrahepatic bile duct: Case report and literature review

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Abstract

Neuroendocrine carcinoma (NEC) of the extrahepatic bile duct is rare, and only 22 cases have been reported. Only two of these were large-cell NEC (LCNEC); the vast majority were small-cell NEC. Here, we report a third case of LCNEC of the extrahepatic bile duct. A 76-year-old male presented to a local hospital with painless jaundice. Imaging studies revealed a tumor at the hepatic hilum. The patient underwent right hepatic lobectomy, bile duct resection, and cholecystectomy. The resection specimen showed a 5.0-cm invasive neoplasm involving the hilar bile ducts and surrounding soft tissue. Histologically, the tumor consisted of nests of medium to large cells with little intervening stroma. The tumor invaded a large portal vein branch. All four excised lymph nodes were positive for metastasis, and metastatic deposits were also present in the gallbladder wall. The tumor was diffusely positive for synaptophysin and focally positive for chromogranin A. Approximately 70%-80% of the tumor cells were positive for Ki-67, indicating strong proliferative activity. A diagnosis of LCNEC was made. A few bile ducts within and adjacent to the invasive tumor showed dysplasia of the intestinal phenotype and were focally positive for synaptophysin and chromogranin A, suggesting that the dysplastic intestinal-type epithelium played a precursor role in this case. A postoperative computer tomography scan revealed rapid enlargement of the abdominal and retroperitoneal lymph nodes. The patient died 21 d after the operation. NEC of the bile duct is an aggressive neoplasm, and its biological characteristics remain to be better defined.

Key words: Neuroendocrine neoplasm; Large cell neuroendocrine carcinoma; Small cell neuroendocrine carcinoma; Extrahepatic bile duct; Dysplasia

Core tip: The authors report a case of large-cell neuroendocrine carcinoma (LCNEC) of the hilar bile duct. Concurrent dysplasia with intestinal and neuroendocrine differentiation was suggested to be a precursor in this case. Neuroendocrine carcinoma (NEC) of the bile duct occurs more frequently in men (male:female ratio 1.9:1). The mid-portion of the common bile duct appears to be the commonest site of involvement. All three reported cases of LCNEC died within 12 mo and the prognosis of NEC of the bile duct appears to be equally poor in both small-cell NEC and LCNEC. Multimodal treatment may improve outcome in this highly aggressive cancer.

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INTRODUCTION

Neuroendocrine neoplasms of the extrahepatic bile ducts are rare, with carcinoid tumors representing the most

common type. Neuroendocrine carcinoma (NEC) is defined as a poorly differentiated, high-grade, malignant neuroendocrine neoplasm, which is classified as either small-cell NEC (SCNEC) or large-cell NEC (LCNEC)[1]. NEC of the extrahepatic bile ducts is exceedingly rare, and only 22 cases have been reported in the literature (Table 1)[2-23]. Of these, most cases are SCNEC, and only two cases of LCNEC have been reported to date. Since LCNEC was first described in the lung by Travis *et al*[24] in 1991, similar lesions have been described in extrapulmonary sites, including the gastrointestinal tract[25]. LCNEC shows immunohistochemical evidence of epithelial and neuroendocrine differentiation and is characterized by a diffuse growth pattern or neuroendocrine architecture (organoid, palisaded, rosettes, or trabeculae)[24,26]. Large cell size, low nuclear to cytoplasmic ratio, and frequent nucleoli are key cytologic features that help distinguish LCNEC from SCNEC. LCNEC of the biliary tract was first described in the gallbladder by Papotti *et al*[27] a little more than a decade ago; however, reported cases are still few, particularly in the extrahepatic bile ducts. Because of the paucity of cases, patient prognosis and responsiveness to anticancer treatments for LCNEC of the biliary tract largely remain to be elucidated. Most of the reported cases of LCNEC of the biliary tracts showed an aggressive course and short survival times; however, one case of LCNEC arising in the gallbladder achieved long survival (69 mo from the initial diagnosis) following multimodal treatment that included surgery, chemotherapy, and radiation therapy[28].

The origin of neuroendocrine neoplasms of the biliary tracts is unclear. They may arise from metaplastic epithelia, where there are a variety of epithelial cells (including neuroendocrine cells, goblet cells, and gastric-type epithelial cells) that are not found in the normal biliary epithelium. In fact, intestinal and/or gastric-type metaplasias are not uncommon in non-neoplastic mucosa adjacent to a variety of neuroendocrine neoplasms of the gallbladder, including carcinoid tumor, SCNEC, and LCNEC[27,29,30].

Here, we report a case of LCNEC of the hilar bile duct. The bile duct mucosa adjacent to the LCNEC showed high-grade dysplasia of the intestinal phenotype, which may have been the precursor in this case. While the presence of concurrent nearby dysplasia was described in 2 previously reported cases of SCNEC[4,16], neuroendocrine differentiation or metaplastic change has not been demonstrated in dysplastic epithelium. Therefore, we aimed to perform detailed immunophenotypic characterization of the dysplastic epithelium and demonstrated intestinal and neuroendocrine differentiation in the dysplastic epithelium. Another objective of this article is to provide a comprehensive literature review on NEC of the bile duct. Despite the paucity of cases, an attempt was made to compare the clinicopathologic characteristics and outcomes between SCNECs and LCNECs.

CASE REPORT

A 76-year-old male presented to a local hospital with a 2 wk history of increasing yellowish discoloration of the skin and dark-colored urine. The patient had no abdominal pain, nausea, or vomiting. He had a history of hypertension, rheumatoid arthritis, peptic ulcer disease, status post-stent placement for ischemic heart disease, and status post-right inguinal hernia repair. The patient denied any family history of cancer. His medication included metoprolol and benazepril. The patient was admitted to the local hospital for 7 d, during which time an abdominal computed tomography (CT) scan revealed a tumor involving the hepatic hilum. Endoscopic retrograde cholangiopancreatography demonstrated a stricture at the common hepatic duct, and two biliary stents were placed. The imaging studies suggested a malignant biliary stricture, but this could not be confirmed with biopsy. After the stent placement, the patient's serum bilirubin, which was initially reported to be 10 mg/dL, decreased to approximately 5 mg/dL and the yellowish discoloration of his skin normalized. The patient was transferred to the department of Surgery at the University of Pittsburgh Medical Center for further work-up and treatment. On admission, the patient's vital signs were within normal limits. The patient was obese with a body mass index of 34 kg/m^2. The sclerae were slightly icteric. The patient had normal cardiac and respiratory examinations and did not have cervical, axillary, or supraclavicular lymphadenopathy. Examination of the abdomen showed mild epigastric tenderness to deep palpation with no rebound tenderness or guarding. The right inguinal area had a well-healed scar from an inguinal hernia repair in the 1950s. The patient's complete blood count and serum biochemistry data on admission were as follows: white blood cells, 11300/mm^3; hemoglobin, 13.4 g/dL; hematocrit, 39.5%; platelets, 377000/mm^3; blood glucose, 122 mg/dL; total bilirubin, 3.5 mg/dL; aspartate aminotransferase, 106 IU/L; alanine aminotransferase, 88 IU/L; and gamma-glutamyl transpeptidase, 80 IU/L. Carbohydrate antigen 19-9 was 32.9 U/mL. Chest X-ray was normal. The patient underwent an operation in October 2011 with the presumed diagnosis of hilar cholangiocarcinoma (Klatskin tumor). The tumor of the hepatic hilum was resected with a right hepatic lobectomy, bile duct resection, and cholecystectomy. The patient had markedly enlarged lymph nodes in the hepatic hilum and retropancreatic area, which were also resected. There was no evidence of extrahepatic disease other than lymphadenopathy. Reconstruction was performed with a Roux-en-Y hepaticojejunostomy. In the resection specimen, the neoplasm diffusely involved the perihilar bile ducts and the surrounding portal connective tissue and measured 5.0 cm at its greatest dimension (Figure 1). The tumor had also invaded a large, right portal vein branch and formed an intraluminal mass. Histologically, the tumor cells were poorly differentiated with no evidence of glandular or squamous differentiation. The tumor cells were arranged in cellular nests and sheets with a small amount of intervening fibrovascular stroma (Figure 2A). The tumor cells had medium to large hyperchromatic nuclei with fine to coarse granular chromatin and occasional small nucleoli. The tumor cells had a small to

Table 1 Neuroendocrine carcinoma of the extrahepatic bile ducts-review of the literature

No.	Ref.	Age (yr)	Sex	Histology	Location	Maximal dimension (cm)	Treatment	Follow-up information	Other findings
1	Sabanathan *et al*[2]	67	M	SCNEC	Bm	5	Palliative bypass and chemotherapy	Alive 6 mo	
2	Miyashita *et al*[3]	85	F	SCNEC	Bi	3	Palliative bypass	DOD 5 mo after surgery	
3	Kuraoka *et al*[4]	75	M	SCNEC	Bi	4.5	Resection	Alive 5 mo after surgery	Dysplasia
4	Hazama *et al*[5]	60	M	SCNEC	CBD	0.3	Neoadjuvant chemotherapy and resection	DOD 12 mo after surgery	
5	Arakura *et al*[6]	70	F	SCNEC	Bm	3	Resection and chemotherapy	DOD 14 mo after surgery	
6	Park *et al*[7]	60	F	SCNEC	Bs-Bm	3	Resection	DOD 5 mo after surgery	
7	Thomas *et al*[8]	54	M	SCNEC	Bh-CBD	NA	Resection	Alive With Metastasis, 6 mo	Clonorchis sinensis infestation
8	Viana Miguel *et al*[9]	76	M	SCNEC	Bm	NA	Resection, chemotherapy and irradiation	Alive 5 mo after surgery	Gallstone
9	Jeon *et al*[10]	65	M	SCNEC	Bs-Bm	2	Presection and chemotherapy	DOD 12 mo after surgery	
10	Nakai *et al*[11]	32	M	SCNEC	CBD	NA	NA, diagnosed by autopsy	NA	
11	Arakura *et al*[12]	75	M	SCNEC	Bh, Bs, Bm, Bi	6.5	Chemotherapy and irradiation	DOD 10 mo after therapy	
12	Hosonuma *et al*[13]	69	F	SCNEC	Bs-Bm	3	Biliary drainage	Alive 2 mo after biliary drainage	
13	Okamura *et al*[14]	62	M	SCNEC	Bm	3	Preoperative chemotherapy, resection and irradiation	DOD 20 mo after surgery	
14	Yamaguchi *et al*[15]	77	F	NEC	Bi	NA	Resection and chemotherapy	Alive 27 mo	
15	van der Wal *et al*[16]	55	M	SCNEC + atypical carcinoid + AD	Bm	4	Resection	NA	Dysplasia/ carcinoma *in situ*
16	Nishihara *et al*[17]	64	M	SCNEC + AD	Bh-Bs	1.9	Resection	Alive 8 mo after surgery	
17	Yamamoto *et al*[18]	71	F	SCNEC + AD	Bh	6	Resection	DOD 7 mo after surgery	Common bile duct stones
18	Kim *et al*[19]	64	M	SCNEC + AD	Bm	3	Resection	Alive 30 d after surgery	Clonorchis sinensis infestation
19	Edakuni *et al*[20]	82	F	SCNEC + AD	Bm	6	Resection	Alive 45 mo after surgery	
20	Kaiho *et al*[21]	66	F	SCNEC + AD	Bm	3.5	Resection and chemotherapy	DOD 8 mo after surgery	
21	Sato *et al*[22]	68	M	LCNEC + AD	Bi	2	Resection and chemotherapy	DOD 3 mo after surgery	
22	Demoreuil *et al*[23]	73	M	LCNEC + AD	Bh-Bs	3	Resection and chemotherapy	DOD 12 mo after surgery	
23	Current report, 2013	76	M	LCNEC	Bh-Bs	5	Resection	DOD 21 d after surgery	Dysplasia, intestinal type

SCNEC: Small cell neuroendocrine carcinoma; NEC: Nneuroendocrine carcinoma, not otherwise specified; AD: Adenocarcinoma; LCNEC: Large cell neuroendocrine carcinoma; Bm: Mid portion of common bile duct; Bi: Inferior or distal common bile duct; CBD: Common bile duct, not otherwise specified; Bs; Superior or proximal common bile/hepatic duct; Bh: Hilar bile duct; DOD: Died of disease. N/A: Information not available; F: Female; M: Male.

moderate amount of amphophilic cytoplasm (Figure 2B). They showed brisk apoptotic activity and frequent mitotic figures (15-18 mitoses per 10 high-power fields). The tumor showed perineural and angiolymphatic invasion. All four lymph nodes were positive for metastatic tumor. The resected gallbladder had microscopic metastatic deposits in the perimuscular layer. No gallstones were present. The tumor cells were negative for mucicarmine stain. Immunohistochemical stains revealed that the tumor cells were strongly positive for synaptophysin (Figure 2C). They were also focally positive for chromogranin A, pancytokeratin, cytokeratin (CK) 7, CK 19, and MOC-31. Fewer than 1% of the tumor cells were weakly positive for thyroid transcription factor-1. They were negative for napsin-A, surfactant apoprotein A, alpha-fetoprotein, vimentin, CK5/6, p63, leukocyte common antigen, and S100. Approximately 70%-80% of the tumor cells were positive for Ki-67 (Figure 2D). The histologic findings and the immunoprofile of the neoplasm were consistent with LCNEC. A few foci of intermediate- to high-grade dysplasia were also identified

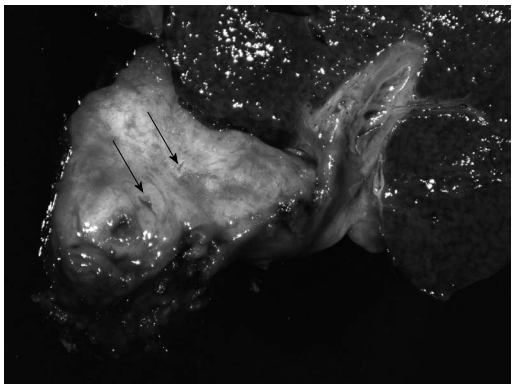

Figure 1 Gross appearance of the hilar neoplasm. The resected specimen showed a firm, tan-grey tumor measuring 5.0-cm in greatest dimension with extensive involvement of the perihilar bile ducts and surrounding soft tissue. Some of the small ducts and vessels within the lesion showed severe luminal narrowing (arrows).

in the perihilar bile ducts located within and adjacent to the invasive neoplasm (Figure 3A). The dysplastic epithelium contained some goblet cells. The dysplastic epithelial cells were immunoreactive for CK19, CK20, and CDX2 (Figure 3B and C). The goblet cells were positive for mucin (MUC)2 and negative for CK7, MUC1, MUC5AC, and MUC6. The immunoprofile of the dysplastic epithelium, together with the presence of goblet cells expressing MUC2 was consistent with an intestinal phenotype. The epithelial cells of this lesion were also positive for synaptophysin (Figure 3D) and chromogranin A but with less extensive and intense immunoreactivity compared to the invasive neoplasm. The findings of the in situ component were suggestive of a premalignant or preinvasive lesion rather than intraepithelial spread from the invasive neoplasm. The patient's immediate postoperative course was uncomplicated; however, a CT scan of the abdomen eight days after the operation revealed extensive enlargement of his abdominal and retroperitoneal lymph nodes, which was suggestive of rapid metastasis. The patient had poor oral intake, hypovolemia, and significant back pain. Postoperative chemotherapy or radiation therapy was not performed because of the patient's poor general condition. The patient died 21 d after operation. An autopsy was not performed.

DISCUSSION

According to the most recent World Health Organization (WHO) classification, neuroendocrine neoplasms of the digestive system are classified into three general categories based on histologic features and proliferation fraction[1,31]. These include NET, NEC, and mixed adenoneuroendocrine carcinoma (MANEC). A NET is defined as well-differentiated neuroendocrine neoplasm with mild to moderate nuclear atypia and a low proliferation fraction (≤ 20 mitoses per 10 high-power fields or ≤ 20% Ki67 index). This category encompasses neoplasms termed carcinoid tumors. A NEC is a poorly differentiated, high-grade malignant neuroendocrine neoplasm composed of either small or intermediate to large cells with marked nuclear atypia and a high proliferation fraction (> 20 mitoses per 10 high-power fields or > 20% Ki67 index). This category includes SCNEC and LCNEC, but a dichotomous subclassification of small cell *vs* non-small cell may also apply[25]. MANEC has a phenotype that is morphologically recognizable as both gland-forming epithelial and neuroendocrine carcinomas. Arbitrarily, at least 30% of either component should be identified to qualify for this definition.

Neuroendocrine neoplasms of the extrahepatic bile ducts are rare, and the majority of the reported cases are NET/carcinoid, which represent 0.1%-0.2% of all gastrointestinal carcinoids/NET[32]. According to the data obtained from the Surveillance, Epidemiology, and End Results program of the National Cancer Institute, there were 31 cases of carcinoid, 17 of SCNECs, and 10 of NECs of not-otherwise-specified type of the gallbladder and extrahepatic bile ducts between 1973 and 2005[33]. Thus, NEC of the extrahepatic bile duct is exceedingly rare and is probably less frequent than NET/carcinoid. According to the literature, 23 cases of NEC of the extrahepatic bile ducts, including the case described here, have been reported. The most common histologic subtype of NEC of the extrahepatic bile ducts is SCNEC (19 of 23 cases; Table 1). Only two cases of LCNEC of the common bile duct were previously reported (Table 1, cases 21 and 22); therefore, this is the third reported case of LCNEC arising in the extrahepatic bile duct. The reported cases of NEC include 15 males and 8 females, with a male to female ratio of 1.9: 1. Patient age ranged from 32-85 years with a mean age of 67.2 years. The mean age of LCNEC was higher than that of SCNEC (72.3 years *vs* 65.9 years), but this difference was not statistically significant. NEC can occur anywhere in the extrahepatic bile duct, but the mid portion of the common bile duct appears to be the most common site of involvement. Two cases had biliary stones (Table 1, cases 8 and 17). Concurrent *Clonorchis sinensis* infestation was seen in two cases (Table 1, cases 7 and 18). The presence of concurrent nearby dysplasia was described in three of 23 cases, including ours (Table 1, cases 3, 15 and 23). Eight cases were composite neuroendocrine and adenocarcinoma (Table 1, cases 15-22). Some of these composite cases may be classified as MANEC rather than NEC according to the current WHO classification system, depending on the proportion of the adenocarcinoma component. When NEC coexisted with adenocarcinoma, a gradual transition between areas of NEC and adenocarcinoma was observed in six of eight cases (Table 1, cases 15-17 and 19-21). In three of eight cases (Table 1, cases 18, 19 and 21), the adenocarcinoma component was located in the superficial portion of the tumor, and the NEC component was located mainly in the deeper portion of the tumor. No other particular spatial relationships between NEC and adenocarcinoma components have been described.

NEC of the gastrointestinal tract can show a spectrum of morphologic features ranging from classic SCNEC to LCNEC, and some cases have features between

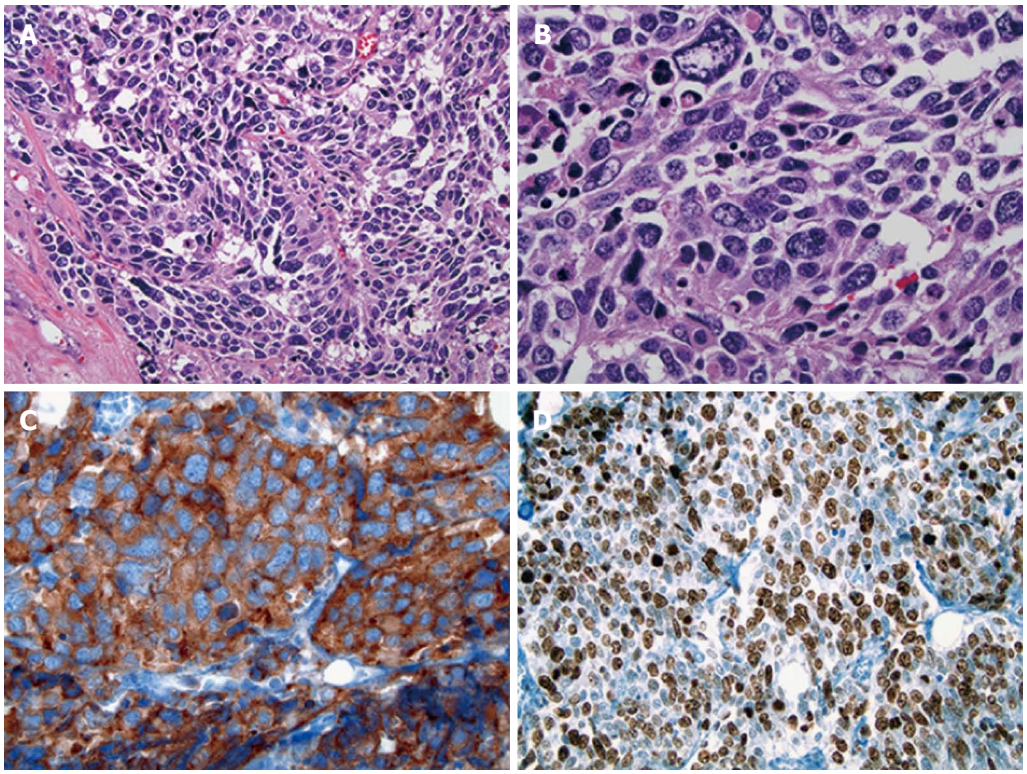

Figure 2 Histologic features of the hilar neoplasm. A: The tumor cells were arranged in cellular nests and sheets with little intervening fibrovascular stroma [Hematoxylin and eosin (HE), × 200]; B: The tumor cells had a small to moderate amount of amphophilic cytoplasm and medium to large hyperchromatic nuclei with fine to coarse granular chromatin and occasional small nucleoli (HE, × 400); C: The tumor cells were strongly positive for synaptophysin (× 400); D: 70%-80% of the tumor cells were positive for Ki-67 (× 200).

these two types. As Shia *et al*[25] previously pointed out, there were no criteria for classifying NEC of the GI tract with non-small cell morphology before the most recent WHO classification. Therefore, NEC of the extrahepatic bile ducts and gallbladder with non-small cell morphology may have been diagnosed inconsistently. In fact, histologic features of some of the previously reported cases of SCNEC of the extrahepatic bile ducts are not morphologically typical of SCNEC. For example, the NEC tumor component reported as a part of composite adenocarcinoma and SCNEC of the hilar bile duct by Yamamoto *et al*[18] (Figure 2B in their manuscript) showed prominent nucleoli and moderately abundant cytoplasm, which is not typical of SCNEC (Table 1, case 17). Thus, some of the non-small cell type NECs or LCNEC may have been diagnosed or reported as SCNEC because of the previous lack of a distinct diagnostic category.

LCNEC of the extrahepatic bile ducts is extremely rare. Both of the previously reported cases contained a minor component of adenocarcinoma (10%-20% of the entire tumor). One tumor was located in an intra-pancreatic portion of the common bile duct (Table 1, case 21), and another was located in the perihilar bile duct (Table 1, case 22). The adenocarcinoma component of one case (Table 1, case 21) showed focal expression of a neuroendocrine marker (chromogranin A), and the other case did not express neuroendocrine markers (Table 1, case 22). The coexistence of adenocarcinoma in these cases, as well as in the aforementioned six cases of composite adenocarcinoma and SCNEC suggests that NEC of the common bile duct may arise from pluripotent progenitor cells. This idea is further supported by the observation of transitional zones between NEC and adenocarcinoma components in the majority of cases. The invasive tumor described here was composed entirely of LCNEC. Although no adenocarcinoma component was identified, a few perihilar bile ducts located within and adjacent to the LCNEC showed dysplasia of the intestinal phenotype with focal endocrine differentiation. Although data on the histogenesis of neuroendocrine neoplasm of the biliary tracts in the literature are limited, neuroendocrine neoplasm of the bile duct and gallbladder may arise from neuroendocrine cells in intestinal or gastric metaplasia, where there may be progenitor cells with a greater ability to differentiate into neuroendocrine cells[29,34]. The presence of intestinal type dysplasia in our case suggests that LCNEC may have arisen from metaplastic epithelium, although we cannot exclude the possibility that the metaplastic/neuroendocrine phenotype may have been acquired at the time of or subsequent to the dysplasia. Regardless, the progression from dysplasia with an intestinal/neuroendocrine phenotype to an aggressive NEC may have occurred in the case described.

The prognosis of NEC of the bile duct appears to be poor. Among the 21 cases with follow-up data, 57% (12/21) of the patients died of disease 3 to 20 mo after surgery, and only two patients have been reported to survive more than 2 years (Table 1, cases 14 and 19). Among

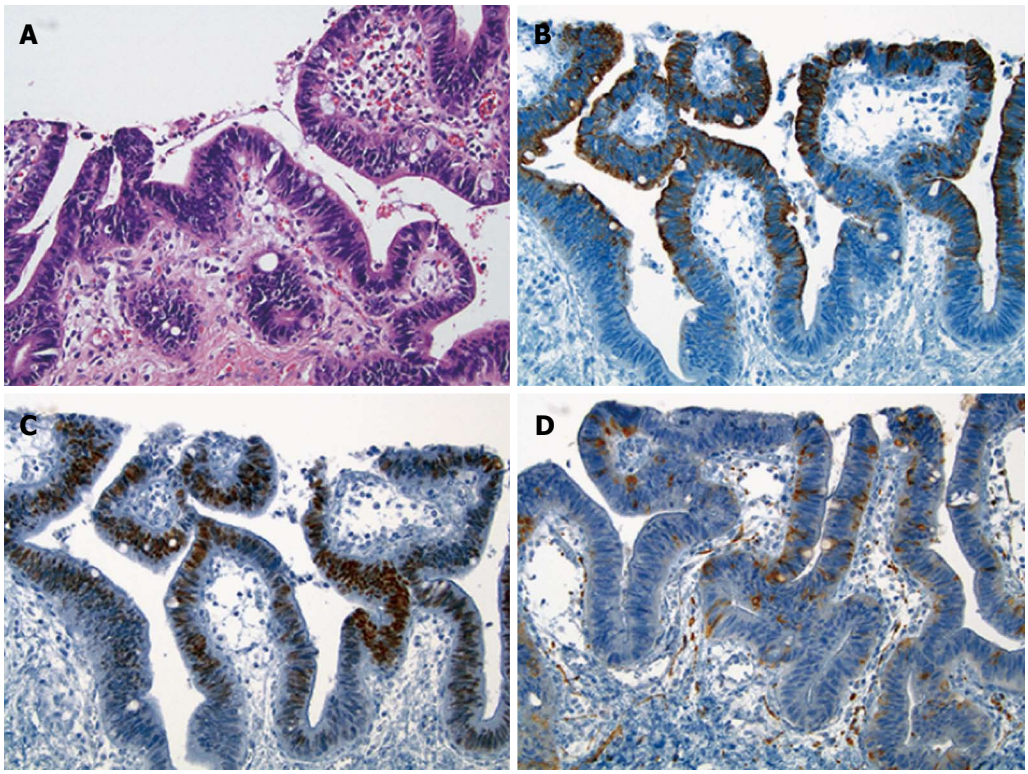

Figure 3 Histologic features of the dysplastic epithelium found in a medium-sized perihilar bile duct located within the invasive neoplasm. A: The dysplastic epithelium contained some goblet cells [Hematoxylin and eosin (HE), × 200]; B, C: The dysplastic epithelial cells were immunoreactive for CK20 (B, × 200) and CDX2 (C, × 200); D: Some of the dysplastic epithelial cells were positive for synaptophysin with less extensive and weak immunoreactivity compared to the invasive neoplasm (× 200).

the seven patients who survived at least 12 mo, five were treated with multidisciplinary treatment, including surgical resection, adjuvant or neoadjuvant chemotherapy, and radiation. The longest survival was 45 mo in a patient who was treated with surgical treatment alone (Table 1, Case 19). According to Edakuni *et al*[20], that tumor was a composite adenocarcinoma (-40%) and SCNEC (-60%). They speculated that the reason for the long survival may have been the low proliferative fraction (9.6% Ki-67-positive tumor cells) of the SCNEC. Recently, most neuroendocrine neoplasm grading systems rely extensively on the proliferation rate, which has been shown to provide significant prognostic information[31]. Based on the current WHO grading system, the NEC component of the case reported by Edakuni *et al*[20] may be best classified as intermediate-grade NET rather than SCNEC, and this appears to at least in part explain the long survival time. According to Iype *et al*[35], LCNECs appear to have a worse prognosis than SCNEC in the gallbladder. Although only two cases of LCNEC of the bile duct have been previously reported, both patients died within 12 mo of surgery despite postoperative chemotherapy (Table 1, cases 21 and 22). In our case, the patient died 21 d after the operation with radiographic evidence of rapid progression of metastatic disease. Thus, the survival for patients with LCNEC of the extrahepatic bile duct appears to be equally poor as for those with SCNEC. Multidisciplinary management appears to be effective and provide longer survival time for SCNEC of the bile duct (Table 1, cases 13 and 14). Recently, a long surviving case of LCNEC of the gallbladder was reported by Shimono *et al*[28]. That patient received multimodal treatment consisting of chemotherapy, radiation therapy, surgical resection, and γ-knife irradiation for brain metastases, which resulted in 69 mo of survival. This case suggests that multimodal treatment is potentially effective in treating LCNEC patients. The effectiveness of chemotherapy and radiotherapy in both SCNEC and LCNEC of the biliary tract needs to be further investigated in a larger number of cases to confirm this observation.

In summary, we reported a case of high-grade neuroendocrine neoplasm arising in the perihilar bile ducts that was best classified as LCNEC. The coexistent dysplasia with intestinal and neuroendocrine differentiation may represent a LCNEC precursor. We feel that histologic subtyping of NECs into SCNEC and LCNEC (or even non-small cell NEC), rather than grouping all types of high-grade neuroendocrine neoplasms together as NECs, is necessary because biologic characteristics of each subtype need to be more clearly defined for better prognostication and selection of therapy.

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CASE REPORT

Retroperitoneal cavernous hemangioma resected by a pylorus preserving pancreaticoduodenectomy

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Abstract

A retroperitoneal hemangioma is a rare disease. We report on the diagnosis and treatment of a retroperitoneal hemangioma which had uncommonly invaded into both the pancreas and duodenum, thus requiring a pylorus preserving pancreaticoduodenectomy (PpPD). A 36-year-old man presented to our hospital with abdominal pain. An enhanced computed tomography scan without contrast enhancement revealed a 12 cm × 9 cm mass between the pancreas head and right kidney. Given the high rate of malignancy associated with retroperitoneal tumors, surgical resection was performed. Intraoperatively, the tumor was inseparable from both the duodenum and pancreas and PpPD was performed due to the invasive behavior. Although malignancy was suspected, pathological diagnosis identified the tumor as a retroperitoneal cavernous hemangioma for which surgical resection was the proper diagnostic and therapeutic procedure. Reteoperitoneal cavernous hemangioma is unique in that it is typically separated from the surrounding organs. However, clinicians need to be aware of the possibility of a case, such as this, which has invaded into the surrounding organs despite its benign etiology. From this case, we recommend that combined resection of inseparable organs should be performed if the mass has invaded into other tissues due to the hazardous nature of local recurrence. In summary, this report is the first to describe a case of retroperitoneal hemangioma that had uniquely invaded into surrounding organs and was treated with PpPD.

Key words: Retroperitoneal tumor; Retroperitoneal cavernous hemangioma; Cavernous hemangioma; Pancreaticoduodenectomy; Pylorus preserving pancreaticoduodenectomy

Core tip: A retroperitoneal cavernous hemangioma is a rare disease. This case of retroperitoneal hemangioma had uniquely invaded into the duodenum and pancreas head, and thus required treatment with pylorus preserving pancreaticoduodenectomy. Although hemangiomas are typically benign, clinicians should be aware of the possibility of invasion into the surrounding organs such as with this case. In the event of invasion, we recommend a combined resection of both the tumor and affected organs to reduce the chance of local recurrence that may be associated with inadequate resection.

Hanaoka M, Hashimoto M, Sasaki K, Matsuda M, Fujii T, Ohashi K, Watanabe G. Retroperitoneal cavernous hemangioma resected by a pylorus preserving pancreaticoduodenectomy. *World J Gastroenterol* 2013; 19(28): 4624-4629 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i28/4624.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i28.4624

INTRODUCTION

A retroperitoneal hemangioma is an uncommon disease in adulthood[1-3]. Only 23 cases of adult retroperitoneal hemangioma have been reported in the literature since 1950. Among those, five cases, including the case detailed in this report, needed combined resection of surrounding organs because of adhesion of the tumor. In this report, we describe the diagnosis and treatment of a retroperitoneal hemangioma that had uniquely invaded into the pancreas and duodenum and required a pylorus preserving pancreaticoduodenectomy (PpPD).

CASE REPORT

A 36-year-old man came to a local hospital with right upper quadrant pain since the day before admission. He had no specific medical history or family history. A screening-enhanced computed tomography (CT) scan revealed a bulky tumor between the dorsal side of the pancreatic head and the right kidney. The tumor was diagnosed as a retroperitoneal sarcoma and the patient was referred to Toranomon Hospital for the operation. On physical examination, the patient was found to have no palpable mass. The results of urine, blood, and adrenal cortex functional tests were within normal limits. An abdominal enhanced CT scan showed a 12 cm × 9 cm tumor without marked contrast enhancement, pushing the pancreas to ventral side (Figure 1A). The mass was distinct from the surrounding organs, including the duodenum, pancreas, kidney and retroperitoneal spaces as observed from the CT scan and therefore appeared to be resectable. An abdominal ultrasound showed an uneven echoic lesion in the same area as observed by CT. T1-weighted image of magnetic resonance imaging (MRI) showed low and a few part of relatively high intensity area inside the tumor (Figure 1B). On fat suppression examination of the MRI image, the tumor was not suppressed to any degree. T2-weighted image also showed heterogeneous finding; there were high intensity area with a few part of intermediate signal intensity area. However, there were no typical findings which suggest the type of retroperitoneal tumor on diagnostic images (Figure 1C). Based on the qualitative assessments, the tumor was diagnosed as a retroperitoneal mesenchymal tumor. Because of the high rate of malignancy associated with retroperitoneal tumors, surgical resection was performed.

Intraoperatively, the mass measured 12 cm × 9 cm in diameter. We initially tried to perform a tumor excision without combined resection of surrounding organs. However, the intraoperative findings revealed that the mass encroached on the head of the pancreas and duodenum (Figure 2). As such, we performed Kocher's mobilization of the duodenum and tried to separate the tumor from the duodenum and pancreas; however, the tumor could not be successfully separated from these organs. After the tumor was lifted from the retroperitoneal space, we tried again to separate the tumor from the pancreas head and duodenum. The tumor adhered strongly to both tissues and could only be removed completely by PpPD. The duration of surgery was 4 h and 16 min and the total blood loss was 561 mL. The patient was discharged from the hospital on postoperative day 24 and was in good health without recurrence over two and a half years after the operation.

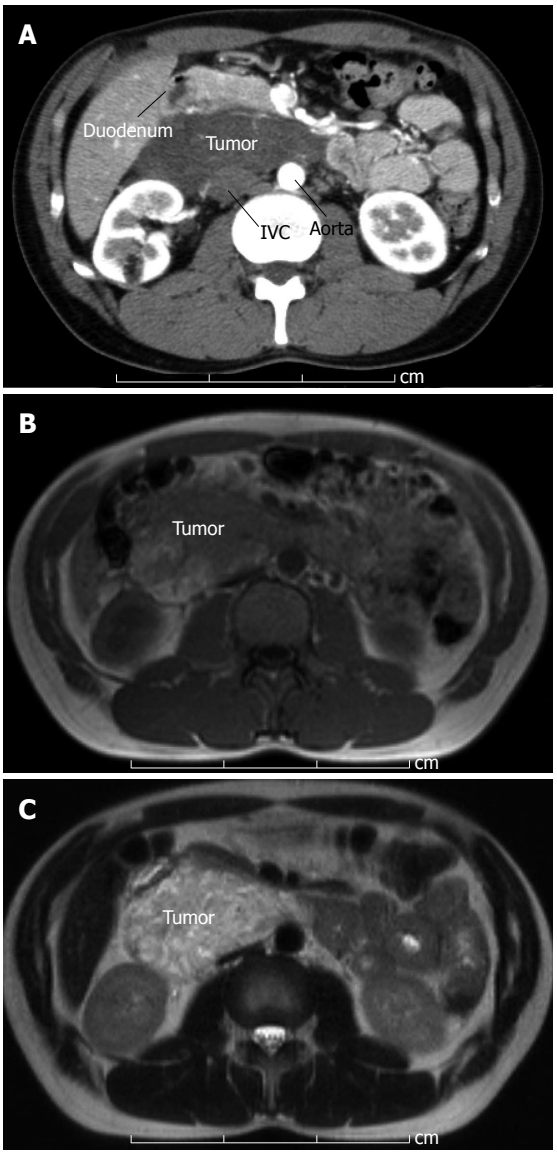

Figure 1 12 cm × 9 cm tumor was detected by computed tomography and magnetic resonance imaging. A: Abdominal enhanced computed tomography in early phase showed tumor without marked contrast. The tumor had pushed the pancreas to the ventral side; B: T1-weighted image of magnetic resonance imaging showed low and relatively high intensity area inside the tumor; C: T2-weighted image showed high intensity area with a few part of intermediate signal intensity area. IVC: Inferior vena cava.

Gross pathologic examination revealed a 120 mm × 95 mm × 50 mm hemangioma composed of multioculated cysts containing intra-cystic hemorrhages (Figure 3). The diagnosis on pathological examination was a cavernous hemangioma with a few focal areas of venous hemangioma (Figure 4A). As defined by the pathologist, the lesion had invaded into the muscle layer of the

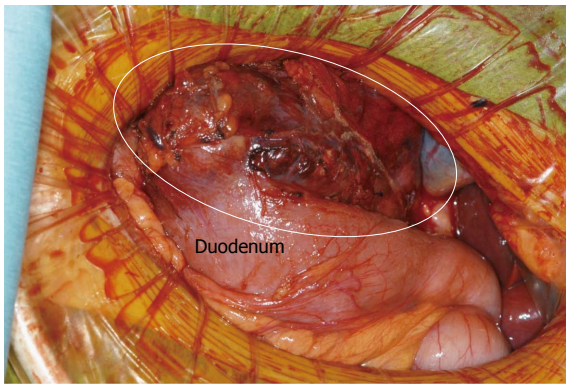

Figure 2 Macroscopic view of intraoperative findings. The mass was attached to both the duodenum and the pancreas head and required surgical resection by pylorus preserving pancreaticoduodenectomy. The circle denotes the tumor.

duodenum and the pancreas head (Figure 4B-D). Immunohistochemical analysis of tissue sections revealed that the lumina were positive for CD31 and CD34, markers of endothelial cells (Figure 4E), and partially weak positive for podoplanin/D2-40 (Figure 4F), a marker of lymphatic endothelial cells. Less angiogenic invasion was observed toward the retroperitoneal side of the tumor than toward the pancreas and duodenum. Interestingly, both macroscopically and microscopically, the tumor extended into both the pancreas and duodenum and not into the retroperitoneal space.

DISCUSSION

In this report, we describe a patient with retroperitoneal hemangioma that required PpPD. A retroperitoneal tumor is a very rare tumor that accounts for less than 0.2% of all tumor types[1]. Among malignant tumors located in the retroperitoneal space, liposarcomas and leiomyosarcomas are the most frequent, while teratomas, cysts and neurinomas are common benign masses. In the current case, the tumor was diagnosed initially as a retroperitoneal sarcoma; retroperitoneal liposarcomas are the most common malignant tumors of the retroperitoneal soft tissue[2]. Indeed, accurate diagnosis of a retroperitoneal hemangioma is classically difficult preoperatively and prior to pathological examination of the tissue.

An analysis of other retroperitoneal tumor types reveals subtle, yet distinct, differences in symptom presentation, localization, and radiographic features. Among retroperitoneal tumors, imaging studies of leiomyosarcomas demonstrate a non-specific mass but are helpful in delineating the relationship to adjacent structures[3]. Angiosarcomas classically present with cutaneous involvement of the head and neck region in elderly patients[4]. The solid growth pattern and epithelioid cytology can be easily confused with poorly differentiated carcinoma[5]. Most patients with lymphanginoleiomyomatosis (LAM), characterized by proliferation of smooth muscle cells, present with pulmonary symptoms, whereas extrapulmonary LAM is rare and typically presents in premenopausal females[6]. Cystic lymphangioma is a well-known benign tumor and its cystic abnormalities of the lymphatic vessels are predominantly congenital. By CT scan, the tumor is typically well-circumscribed and polycystic with thin septa similar in appearance to the cystadenomas[7]. Kaposiform hemangioendothelioma mainly occurs during childhood. MRI of the affected region is accepted as the

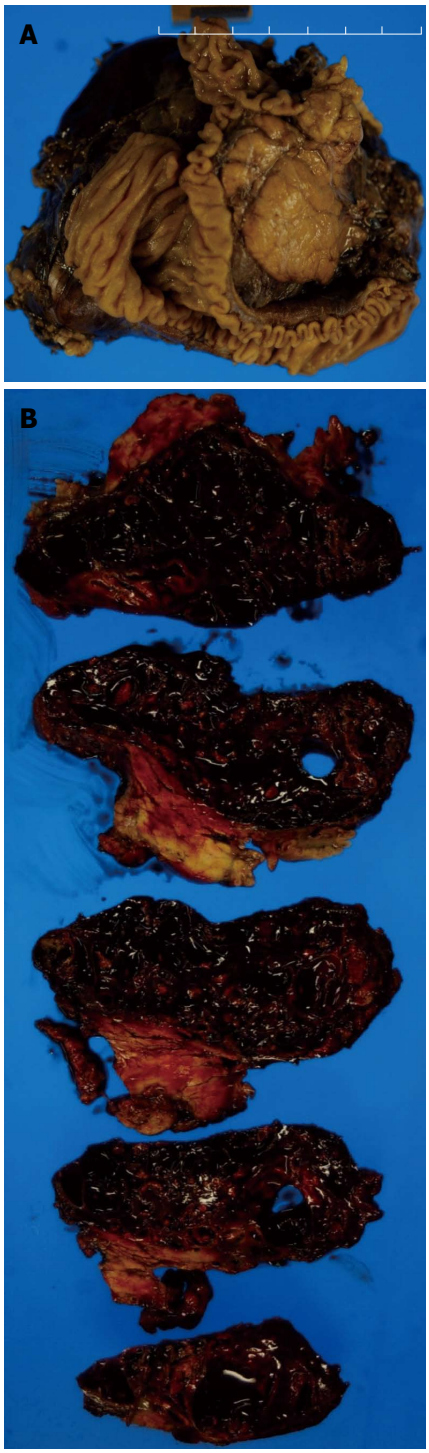

Figure 3 Macroscopic findings of the resected specimen. A: A 120 mm × 95 mm × 50 mm tumor was resected. Scale bar, 70 mm; B: The tumor contained multi-oculated cysts containing intra-cystic hemorrhages.

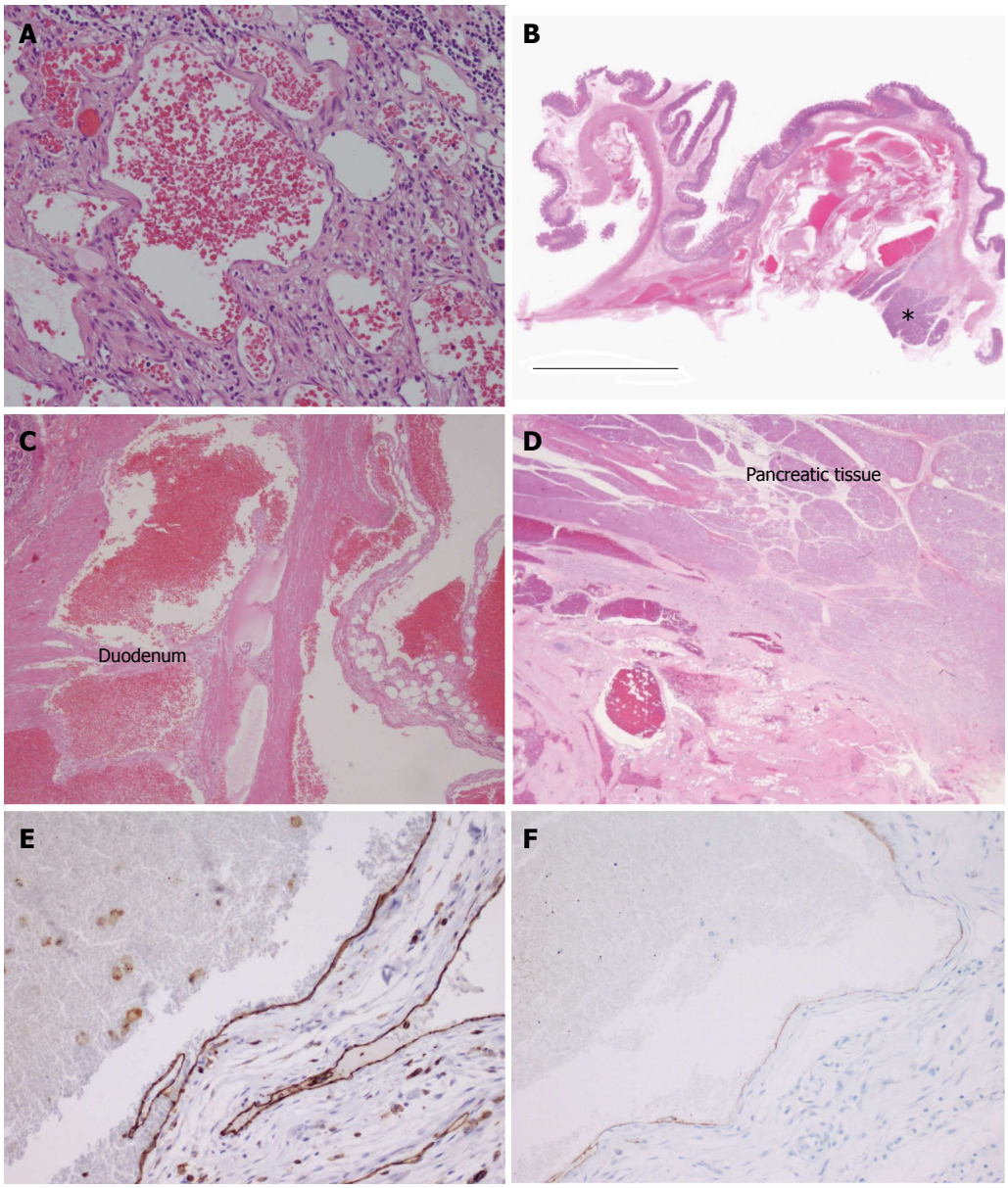

Figure 4 Pathological analysis by hematoxylin and eosin staining and immunohistochemistry. A: A representative tissue section from the main region of the cavernous hemangioma [hematoxylin and eosin (HE), magnification × 200]; B: The loupe view demonstrates a portion of cavernous hemangioma infiltrating the pancreatic head (asterisk) and duodenal wall (HE). The scale represents 10 mm; C: Tumor invasion into the muscle layer of the duodenum (HE, magnification × 4); D: Tumor invasion into the pancreas head (HE, magnification × 1); E: Positive immunostaining for CD31 supports the diagnosis of hemangioma (magnification × 20); F: The lumen showed partial and weakly positive staining for podoplanin/D2-40 (magnification × 20).

diagnostic imaging technique of choice[8]. Concerning hemangiomas arising from other tissues, pancreatic and mesenteric hemangiomas have been reported[9,10], which are extremely rare. MRI shows a common characteristic of pancreatic hemangiomas[9]. Mesenteric hemangiomas shows heterogeneous enhancement by enhanced CT and changes its shape during intestinal peristalsis[10].

As for pathological findings, gross pathological findings showed the tumor included multi-oculated cysts filled with blood. This cyst is suggested to be a pseudocyst which is a result of repeats of hemorrhage[11]. D2-40, reliable podoplanin antibody clone, has been described in a variety of lymphovascular neoplasms including lymphangioma, Kaposi sarcoma, and hemangioendothelioma[12]. Because lymphatic endothelial cells express high levels of podoplanin[13], we suggest that the weakly positive findings of D2-40 in the current case do not support a diagnosis of lymphangima. In addition to macroscopic and immunohystological findings, microscopic finding of almost all of lumen are filled with red blood cells supported the diagnosis as hemangioma.

Retroperitoneal hemangioma in the adult is extremely rare and confirmed in only 1%-3% of all retroperitoneal tumors[14]. Only 23 cases of adult retroperitoneal hemangioma have been reported in literature since 1950.

Retroperitoneal tumors are difficult to diagnose preoperatively[15] because there are usually no initial symptoms until tumors have grown large enough to produce

Table 1 Reported cases of retroperitoneal hemangioma with combined resection of surrounding organs

Ref.	Age (yr)	Sex	Tumor size (cm^3)	Hyper-vascularity	Curative resection	Pathological diagnosis	Organ(s) of combined resection
Ogura *et al*[21]	73	M	23 × 14 × 9	-	+	Cavernous	Left kidney
Takaha *et al*[20]	55	M	10 × 9 × 9	+	+	Cavernous	Spleen, diaphragm, chest wall
Syo *et al*[22]	72	F	9 × 7 × 7	-	+	Cavernous	Right ovarian artery
Tseng *et al*[18]	61	F	NA	+	-	Venous	NA
Hanaoka *et al*, 2013	36	M	12 × 19 × 5	-	+	Cavernous and Venous	Duodenum, pancreas head

Five cases, including this, needed combined resection of surrounding organs. In four cases, a complete combined resection of surrounding organs was performed, while in one case a subtotal resection was performed (Tseng *et al*[18]). NA: Not available.

patient discomfort[11]. Furthermore, retroperitoneal cavernous hemangiomas have features similar to ischemic tumors, but differ from hemangiomas arising from other tissues such as the skin or liver[11].

As for imaging studies of retroperitoneal cavernous hemangiomas, because they are usually only discovered when large enough to develop thrombi and organization at the center[16], these tumors often show slight to no enhancement in normal enhanced CT[11,14,15]. In the present case, the CT scan revealed a cystic mass with minor contrast enhancement, similar to cases reported previously[17,18]. In addition, retroperitoneal cavernous hemangiomas typically lack the complete fill-in or cotton-wool appearance in enhanced CT or high echoic areas with the same density as the abdominal echo, which is usually only seen in cavernous hemangiomas of the liver[11]. On T1-weighted image of MRI, relatively high intensity area inside the tumor is suggested to be hemorrhage and hyalinization of the tissue. A part of high intensity area of T2-weighted image suggests blood contain and relatively high signal intensity area shows hyalinization and fibrillization. However, these findings were suggested to be secondary change of structure, which don't indicate any typical tumors. With few clues for diagnosis, very few cases of retroperitoneal hemangiomas have been diagnosed preoperatively. Therefore, surgical resection is a choice for both diagnostic and therapeutic procedures.

One feature of a cavernous hemangioma is that it may be locally destructive by virtue of the pressure exerted on neighboring tissues[19]. In the present case, the pressure of the tumor affected the duodenum and pancreas, leading to invasion and destruction of these organs. Among the 23 reported retroperitoneal hemangiomas, five cases, including ours, needed combined resection of surrounding organs because of an adhesion (Table 1)[18,20-22]. Among the five cases, four cases performed complete combined resection of surrounding organs. In one case, subtotal resection was performed due to technical difficulties caused by firm adherence to the adjacent organs and the major blood vessels[22]. Like this case, which did not demonstrate findings typical of hemangioma from the contrast-enhanced CT, three cases including ours showed hypovascularity, while two cases showed hypervascularity. Pathologically, four cases, including this, were diagnosed as cavernous hemangiomas and one was diagnosed as a venous hemangioma. Hence, we conclude that vascularity from CT analysis and pathological diagnosis are not always directly correlated with adhesion to other organs.

The recommended treatment for retroperitoneal hemangioma has been surgical resection[11,15,23]. Hemangiomas are non-malignant, but patients run the risk of rupture and bleeding[24]. Therefore, surgical treatment is recommended for high-risk tumors, such as with those of large masses[25]. Although hemangiomas are benign, local recurrence has been reported with inadequate resection[26]. In this case, the decision was made to combine the tumor resection with resection of the surrounding organs to avoid local recurrence in the pancreas and duodenum from residual tumor tissue. Therefore, the PpPD procedure for this case was appropriate. From a treatment standpoint, we support a combined resection of both the tumor and the compromised organs if the tumor is invasive and cannot be removed cleanly because of adhesion.

In conclusion, a retroperitoneal cavernous hemangioma is an uncommon disease. Clinicians need to be aware of the possibility of a case that has invaded into surrounding organs despite its benign pathology. This case of a retroperitoneal hemangioma had uniquely invaded into the duodenum and pancreas and this is the first report of treatment using PpPD.

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CASE REPORT

Unusual upper gastrointestinal bleeding: Ruptured superior mesenteric artery aneurysm in rheumatoid arthritis

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Abstract

This case report describes an unusual case of upper gastrointestinal (UGI) bleeding caused by a ruptured superior mesenteric artery (SMA) aneurysm in the duodenum in a patient with rheumatoid arthritis. The patient presented with UGI bleeding and hemorrhagic shock. Emergency UGI endoscopy could not identify the source of the bleeding because of excessive blood clots under the second portion of the duodenum. An SMA aneurysm with active contrast extravasation was diagnosed by computed tomography. The aneurysm, together with the fourth portion of the duodenum and the proximal portion of the jejunum, was surgically resected, and the SMA was skeletonized. On postoperative day 15, the patient was discharged from hospital under satisfactory conditions. Rheumatoid arthritis has been known to cause a wide spectrum of manifestations, and an SMA aneurysm is an unusual extra-articular manifestation. An SMA aneurysm rupture presenting as upper gastrointestinal bleeding is a rare complication with a high mortality rate. The clinician must be alert to this potential issue to achieve rapid diagnostic confirmation, and immediate surgical or radiological intervention.

Key words: Gastrointestinal bleeding; Aneurysm; Rheumatoid arthritis; Endoscopy; Duodenum

Core tip: Gastrointestinal bleeding is a common medical emergency. Ruptured superior mesenteric artery aneurysm is an uncommon cause of gastrointestinal bleeding, with few cases reported previously. Diagnosis is difficult before surgery. We reported the successful diagnosis and treatment of such a case.

Choo CH, Yen HH. Unusual upper gastrointestinal bleeding: Ruptured superior mesenteric artery aneurysm in rheumatoid arthritis. *World J Gastroenterol* 2013; 19(28): 4630-4632 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i28/4630.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i28.4630

INTRODUCTION

Upper gastrointestinal (UGI) bleeding is a common medical emergency. Patients usually present with hematemesis or melena, and are diagnosed after endoscopic evaluation. Peptic ulcers and esophagogastric varices account for most causes of UGI bleeding. A visceral aneurysm is an uncommon cause of gastrointestinal bleeding, and diagnosis is usually made after surgery. Here, we report an interesting case with pre-operative diagnosis of a ruptured superior mesenteric artery (SMA) aneurysm in the duodenum of a patient with rheumatoid arthritis.

CASE REPORT

A 27-year-old man with a history of rheumatoid arthritis was brought to the emergency department because of an episode of coffee ground vomitus and dizziness. He suffered from melena for 2 d. His blood pressure was 89/52 mmHg and his heart rate was 106/min. He presented with alert consciousness in the emergency department. A nasogastric tube lavage revealed coffee ground material, and further investigation revealed hemoglobin

levels of 8.1 g/dL. Emergency UGI endoscopy was performed after his hemodynamic status was stabilized with fluid hydration and blood transfusion. UGI endoscopy found excessive blood clots below the second portion of the duodenum. To identify the source of the bleeding, abdominal contrast-enhanced computed tomography (CT) was performed. The CT scan revealed an aneurysm about 3.9 cm in diameter at the jejunal mesentery; with active contrast extravasation into the fourth portion of the duodenum (Figure 1). The aneurysm, together with the fourth portion of the duodenum and the proximal portion of the jejunum, was surgically resected, and the SMA was skeletonized. Pathology revealed an aneurysm measuring 3.8 cm × 3.5 cm × 2.0 cm at the mesenteric side, with blood clots within the cystic space, as well as a focal rupture into the lumen of the small intestine.

The patient was subsequently able to eat on postoperative day 7, and his condition was satisfactory. He was discharged from the hospital on postoperative day 15.

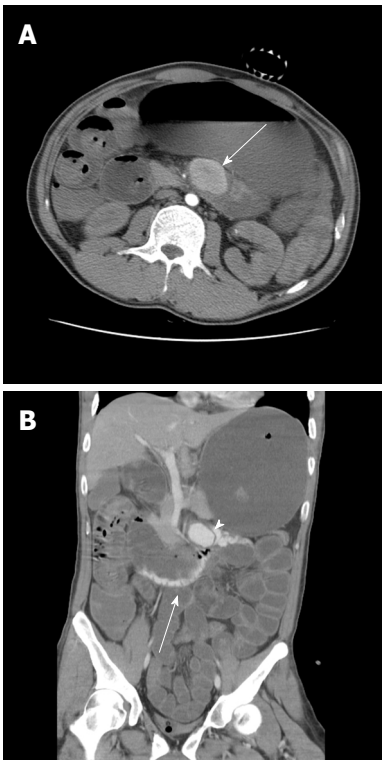

Figure 1 Computed tomography scans. A: Computed tomography (CT) scanning revealed a superior mesentery artery aneurysm about 3.9 cm in diameter at the jejunal mesentery (white arrow); B: CT scan showing a superior mesentery artery aneurysm (arrow head) with active contrast extravasation into the fourth portion of the duodenum (white arrow).

DISCUSSION

UGI bleeding is a common medical condition that results in high patient morbidity. UGI bleeding commonly presents with hematemesis or melena. It is important to take a careful history and perform a thorough physical examination to identify the possible source and etiology of the bleeding. Common causes of UGI bleeding include peptic ulcers, esophagogastric varices, erosive gastritis, Mallory-Weiss syndrome, angiodysplasia or Dieulafoy's lesion. Peptic ulcers are responsible for approximately half of UGI bleedings[1].

Our patient with an SMA aneurysm rupture in the duodenum presenting as UGI bleeding was extremely rare; there have been only a few reported cases[2-4]. Overall, only 0.2% of the general population is found to have an aneurysm of the visceral arteries, and SMA aneurysms represent only 5.5% of all visceral aneurysms[5]. Unlike other splanchnic artery aneurysms, which are mostly asymptomatic, more than 90.0% of SMA aneurysms are symptomatic, manifesting primarily as nonspecific abdominal pain[6]. Rarely, an abdominal mobile pulsatile mass or abdominal bruit maybe found on physical examination. Common causes of SMA aneurysms include septic emboli, atherosclerosis, pancreatitis and trauma. Septic emboli account for about one-third of SMA aneurysms. Other uncommon causes are collagen vascular disease, connective tissue disease and neurofibromatosis[7]. Abdominal ultrasonography and CT are useful in identifying the type of aneurysm. However, up to 50.0% of SMA aneurysms present with a rupture, where hypovolemic shock, hemoperitoneum or acute abdomen are the first manifestations. Once an SMA aneurysm ruptures, a high intra-operative mortality rate of more than 30.0% has been reported in previous studies[8]. Therefore, in patients diagnosed with an SMA aneurysm, surgical resection or radiological intervention should be recommended before the aneurysm ruptures.

An SMA aneurysm is an unusual extra-articular manifestation of rheumatoid arthritis, and our patient had no other history contributing to the formation of an aneurysm, including septic embolic, trauma or atherosclerosis. Rheumatoid arthritis can cause a wide spectrum of manifestations, from clinically insignificant to life-threatening. In postmortem examinations, rheumatoid vasculitis occurred in approximately 25.0% of all patients with rheumatoid arthritis, whereas less than 1% of rheumatoid arthritis patients developed clinical signs of vasculitis[9]. Among these patients, 10%-38% will have gastrointestinal manifestations of vasculitis and this may be the first manifestation[10,11]. Cases of abdominal aneurysm rupture with syncope and hemorrhage, bowel infarction, and intestinal perforation have been described [10-13].

Therefore, in patients with rheumatoid arthritis who have UGI bleeding, diagnosis of a ruptured SMA aneurysm in the duodenum should be considered, and the clinician should be alert to this issue to achieve rapid confirmation and to save lives.

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Conscious sedation: A dying practice?

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Abstract

Sedation practices vary according to countries with different health system regulations, the procedures done, and local circumstances. Interestingly, differences in the setting in which the practice of gastroenterology and endoscopy takes place (university-based *vs* academic practice) as well as other systematic practice differences influence the attitude of endoscopists concerning sedation practices. Conscious sedation using midazolam and opioids is the current standard method of sedation in diagnostic and therapeutic endoscopy. Interestingly, propofol is a commonly preferred sedation method by endoscopists due to higher satisfaction rates along with its short half-life and thus lower risk of hepatic encephalopathy. On the other hand, midazolam is the benzodiazepine of choice because of its shorter duration of action and better pharmacokinetic profile compared with diazepam. The administration of sedation under the supervision of a properly trained endoscopist could become the standard practice and the urgent development of an updated international consensus regarding the use of sedative agents like propofol is needed.

Key words: Sedation; Conscious; Endoscopy; Propofol; Fentanyl; Meperidine

Core tip: Even though the most recent "Multisociety sedation curriculum for gastrointestinal endoscopy" guidelines addressed the upper-limit range of Midazolam for proper sedation in prolonged endoscopic procedures, there are no clear-cut guidelines on the upper limit of Fentanyl dosing, especially with the risk of rigid chest syndrome and drug accumulation in skeletal muscle at high doses. Additionally, we also raised the question of whether Propofol or other agents used for deep sedation should be a routine indication for patients with chronic opioid use.

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TO THE EDITOR

While the controversy regarding the administration of sedation during gastrointestinal (GI) endoscopy continues, Triantafillidis *et al*[1] in their recent article in January 2013, provided a concise and thorough overview of the current knowledge regarding this topic. In summary, the authors concluded that the administration of sedation under the supervision of a properly trained endoscopist could become the standard practice and stressed urgent development of an updated international consensus regarding the use of sedative agents, especially propofol[1]. We would like to share some comments with the authors.

In a short survey of about sedative use in GI endoscopies conducted among thirty endoscopists at our large community-based hospital setting, Fentanyl/Midazolam and Meperidine/Midazolam are the most commonly used

sedatives for elective outpatient procedures in 63% and 36% of instances respectively. Usually, patients do not require more than 6 mg of Midazolam and 200 μg of Fentanyl to achieve moderate sedation. In a minority of patients who require more than usual dose of sedatives, 73% of the surveyed endoscopists are hesitant to go beyond the above-mentioned limits for the fear of side effects, leading to either inadequate evaluation or premature termination of the procedure. According to the recent "Multisociety sedation curriculum for gastrointestinal endoscopy" guidelines[2], the dose of Midazolam could be increased up to 6 mg and even more for prolonged endoscopic procedures[2]. However, there are no clear-cut guidelines on the upper limit of Fentanyl dosing and the risk of rigid chest syndrome[3]. During regular outpatient colonoscopies, few patients would not achieve moderate sedation despite receiving more than doses mentioned above. In such situations, 57% of the physicians responded that they would terminate the procedure and reschedule it again and 43% noted that they would proceed with increasing doses of sedatives to achieve moderate sedation. As Fentanyl can cause delayed side effects through accumulation in skeletal muscle, increasing the dose might be a concern in high-risk patients.

The second issue is with people who are chronic narcotic users. Ninety percent of the surveyed endoscopists responded that they would prefer Propofol administration in patients with chronic opioid use due to high tolerance observed in these patients. Propofol administration is usually by the anesthesia service thus adding to the patient's costs. This leads to a clinical question whether Propofol or other agents used for deep sedation should be a routine indication for patients with chronic opioid use.

Overall, this study provided a concise overview of the current knowledge and issues concerning sedation during digestive endoscopy and the authors are to be commended on their work.

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Format

Journals

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

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

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

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

In press

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

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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.00000 35706.28494.09]

Both personal authors and an organization as author

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

No author given

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

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

Issue with no volume

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

No volume or issue

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

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Personal author(s)

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

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

Author(s) and editor(s)

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

Conference proceedings

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

Conference paper

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

Electronic journal (list all authors)

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

Patent (list all authors)

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

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Write as mean ± SD or mean ± SE.

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