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AIM AND SCOPE

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A candidate targeting molecule of insulin-like growth factor- I receptor for gastrointestinal cancers

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therapeutic target, IGF-IR. The IGF/IGF-IR axis is an important modifier of tumor cell proliferation, survival, growth, and treatment sensitivity in many malignant diseases, including human GI cancers. Preclinical studies demonstrated that downregulation of IGF-IR signals reversed the neoplastic phenotype and sensitized cells to anticancer treatments. These results were mainly obtained through our strategy of adenoviruses expressing dominant negative IGF-IR (IGF-IR/dn) against gastrointestinal cancers, including esophagus, stomach, colon, and pancreas. We also summarize a variety of strategies to interrupt the IGFs/IGF-IR axis and their preclinical experiences. Several mAbs and TKIs targeting IGF-IR have entered clinical trials, and early results have suggested that these agents have generally acceptable safety profiles as single agents. We summarize the advantages and disadvantages of each strategy and discuss the merits/demerits of dual targeting of IGF-IR and other growth factor receptors, including Her2 and the insulin receptor, as well as other alternatives and possible drug combinations. Thus, IGF-IR might be a candidate for a molecular therapeutic target in human GI carcinomas.

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Abstract

Advances in molecular research in cancer have brought new therapeutic strategies into clinical usage. One new group of targets is tyrosine kinase receptors, which can be treated by several strategies, including small molecule tyrosine kinase inhibitors (TKIs) and monoclonal antibodies (mAbs). Aberrant activation of growth factors/receptors and their signal pathways are required for malignant transformation and progression in gastrointestinal (GI) carcinomas. The concept of targeting specific carcinogenic receptors has been validated by successful clinical application of many new drugs. Type I insulin-like growth factor (IGF) receptor (IGF-IR) signaling potently stimulates tumor progression and cellular differentiation, and is a promising new molecular target in human malignancies. In this review, we focus on this promising

Key words: Dominant negative; Gastrointestinal cancer; Insulin like growth factor-I receptor; Monoclonal antibody; Tyrosine kinase inhibitor

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INTRODUCTION

Signals from a variety of growth factors and their receptors are required for tumorigenesis, cancer development, and maintenance of the malignant phenotype^[1]. Those signals alter regulation of the cell cycle, induction of apoptosis, and interactions of tumor cells with their environment, which affect the continuous growth potential of gastrointestinal (GI) cancer cells^[1].

Recently, advances in molecular cancer research have brought new therapeutic arms from the bench into clinical usage. One group of new targets is the receptor tyrosine kinases (RTKs), including epidermal growth factor receptor (EGFR, erbB1), Her2/neu (c-erbB2), c-Kit (stem cell factor receptor), and vascular endothelial growth factor receptor (VEGFR). RTKs can be blocked by small molecule tyrosine kinase inhibitors (TKIs), for example gefitinib^[2] and imatinib^[3], targeting EGFR and c-kit, respectively. Multikinase inhibitors are also available for several tumors, including sorafenib (targeting Raf, VEGFR, PDGFR, c-kit, Flt-3, and RET)^[4] and sunitinib (targeting for Flt-3, c-kit, VEGFR, and PDGFR)^[5]. RTK signals can be inhibited by human or humanized monoclonal antibodies (mAb), e.g. trastuzumab^[6] and cetuximab^[7], targeting Her2 and EGFR, respectively. Bevacizumab is a mAb against VEGF-A, which is a ligand for VEGFRs, and is also in clinical use for patients with colorectal cancer^[8]. Insulin-like growth factor (IGF) receptor-I (IGF-IR) could be the next molecular target in RTKs of human neoplasms^[9].

INSULIN-LIKE GROWTH FACTOR/IGF-I RECEPTOR AXIS

IGF-IR is synthesized as a single precursor peptide of 1367 amino acid residues, which is then cleaved at residue 706, into the α -chain (containing the extracellular domain) and the β -chain (having the transmembrane and tyrosine kinase domains) (Figure 1)^[10]. IGF-IR is transported to the membrane fully assembled in the dimeric form with two α -chains and two β -subunits. IGF-I and IGF-II are the ligands of IGF-IR and are produced by the liver and by many extrahepatic sites, including tumor cells and stromal fibroblasts. After the ligands bind to IGF-IR, which is autophosphorylated to stimulate tyrosine kinase activity, IGF-IR subsequently phosphorylates intracellular substrates, including insulin receptor substrates-1 to -4 (IRS-1~4) and Shc. These early events activate multiple signaling pathways, including the mitogen-activated protein kinase [MAPK, extracellular signal-regulated kinase (ERK)] and phosphatidylinositol 3-kinase (PI3-K)/Akt-1 (protein kinase B) pathways^[11,12]. Those pathways then switch on several cellular functions, including anti-apoptosis, transcription, metabolism, proliferation, growth, and translation.

In normal cells, the IGF/IGF-IR system is controlled by multiple steps (Figure 2)^[13]. Growth hormone-releasing hormone (GHRH) stimulates the expression of growth hormone (GH), which is produced in the pituitary gland. GH then stimulates the secretion of IGFs and IGF bind-

ing proteins (IGFBPs) from hepatocytes. Activation of IGF-IR is tightly regulated by the amount of the free forms of the ligands, which is controlled by the action of IGFBP and the non-stimulatory receptor type 2 IGF receptor (IGF-IIR, also known as mannose 6-phosphate receptor)^[14,15]. IGFBP-1 to -6 circulate and modulate IGF activity by reducing IGFs bioavailability to bind to the IGF-IR. The complex balance between IGFs and IGFBPs is modulated by specific IGFBP proteases, such as matrix metalloproteinase (MMP)^[16]. IGFBPs have IGF-independent actions, but their role in cancer is not yet clear. IGF-IIR is also a negative regulator of IGF signaling, and works by as a decoy by binding IGFs.

THE ROLES OF IGF-IR SIGNALS IN HUMAN NEOPLASMS, ESPECIALLY GASTROINTESTINAL CANCERS

Dysregulation of the IGFs/IGF-IR system has been implicated in the proliferation of numerous tumors^[17]. IGF-IR appears to be essential for malignant transformation in certain systems, for example, fetal fibroblasts with a disruption of the IGF-IR gene, while viable, cannot be transformed by the potent oncogene, SV40 T antigen^[11,18]. Elevation of serum IGF-I increases the risk of developing several cancers, e.g. colon, prostate, and breast^[14,19,20]. In addition, low serum concentration of IGFBP3 increases the risk of cancer^[14]. Increased IGF-II expression has been implicated as a biomarker of colorectal cancer risk^[21]. Overexpression of IGFs and the receptor, either by gene amplification, loss of imprinting, or overexpression of convertases or transcription factors, have been observed, as well as posttranslational modifications of the IGF-IR by glycosylation. IGF-IR is also important for the maintenance, as well as the initiation, of malignancy^[11]. Moreover, reduction of IGF-IR has been shown to induce apoptosis in tumor cells, but produces only growth arrest in untransformed cells^[1], implying that receptor blockade might have a greater therapeutic index than strategies targeting fundamental cell mechanisms, such as DNA synthesis or the cell cycle. In support of this, IGF-IR knockout mice are viable (though physically smaller than normal and ultimately die of respiratory failure), indicating that relatively normal tissue development and differentiation can occur in its absence^[22].

Exogenous IGFs stimulate the proliferation of colon, gastric, esophageal, hepatocellular, and pancreatic cancer cells, whereas blocking IGF-IR inhibits tumor progression^[23-29]. Intestinal fibroblast-derived IGF-II has been shown to stimulate proliferation of intestinal epithelial cells in a paracrine manner^[30]. Both IGF-II and IGF-IR expressions are increased in gastrointestinal cancers^[23,28,29,31-33]. Soluble IGF-IIR rescues Apc(Min/+) intestinal adenoma progression induced by loss of IGF-II imprinting^[15]. Previously, we reported that detection of IGF-II/IGF-IR might be useful for the prediction of recurrence and poor prognosis of ESCC and for selecting patients for IGF-IR targeting therapy^[33]. IGF-I has also

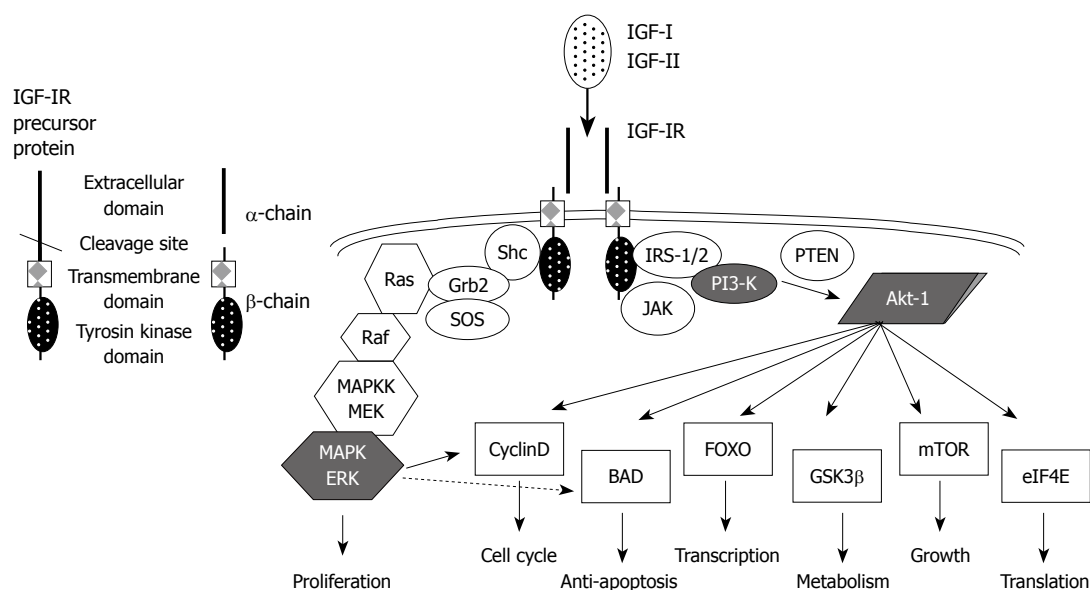


Figure 1 The structure, signal transductions, and effects of the type I insulin-like growth factor receptor system. Type I insulin-like growth factor receptor (IGF-IR) is synthesized as a single precursor peptide and then is cleaved into the α -subunit (extracellular domain) and the β -subunit (transmembrane and tyrosine kinase domains). After binding to the ligands (IGF-I and IGF-II), IGF-IR, which is constructed with two α - and two β -chains, turns on its signal transductions via two major pathways, such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3-K)/Akt, results in survival and mitogenesis. IRS: Insulin receptor substrate; Shc: Src homology and collagen-containing protein; Grb2: Growth factor receptor-bound protein 2; PTEN: Phosphatase and tensin homolog; JAK: Janus kinase; MAPKK: MAPK kinase; MEK: MAPK/ERK kinase; ERK: Extracellular signal-regulated kinase; BAD: Bcl-2-associated death promoter; FOXO: Forkhead box O; GSK3 β : Glycogen synthase kinase 3 beta; eIF4E: Eukaryotic translation initiation factor 4E.

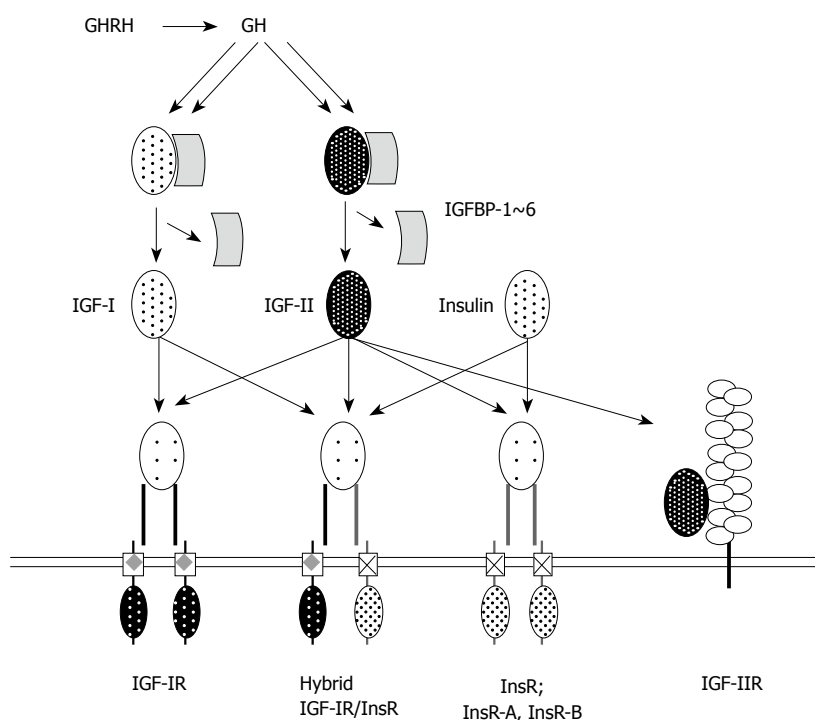


Figure 2 Insulin-like growth factor/type I insulin-like growth factor receptor and insulin/insulin receptor systems. Growth hormone-releasing hormone (GHRH) can stimulate secretion of growth hormone (GH), which upregulates insulin-like growth factors (IGFs) expression. IGF-I and IGF-II, which have about 40% sequence similarity to pro-insulin, predominantly activate type I IGF receptor (IGF-IR), which is a similar structure to insulin receptor (InsR) (59% sequence similarity). IGF-II is able to bind IR and both IGFs can bind hybrid IGF-IR/IR receptors. Ligand supply of both IGFs is regulated by two components. One is IGF binding proteins, which comprise at least six proteins [IGF binding protein (IGFBP)-1~6]. Another is IGF-IIR (lacks tyrosine kinase activity), which internalizes IGF-II for degradation in the pre-lysosomal compartment. Insulin can activate both IR and hybrid IGF-IR/InsR. Two isoforms of InsR exist, the A-isoform (InsR-A) and the B-isoform (InsR-B).

been shown to antagonize the antiproliferative effects of cyclooxygenase-2 inhibitors on pancreatic cancer cells^[34]. Thus, overexpressed IGF-IR signals are also important in tumor dissemination through the control of adhesion, migration, and metastasis.

IGF-II, in conjunction with IGF-IR, IGF-I, COX-2, and MMP-7, seems to play a key role in the early stage of colorectal carcinogenesis^[35,36]. Matrilysin (MMP-7) can

cleave all six IGFBPs and can thus cause increased IGF-mediated IGF-IR phosphorylation^[37]. Moreover, matrilysin is also able to generate bioactive IGF-II by degrading the IGF-II/IGFBP-2 complex binding to heparan sulfate proteoglycan in the ECM of HT29^[16]. We have previously reported a positive feedback loop between the IGF/IGF-IR axis and matrilysin in the progression and invasiveness of GI cancers^[38] (Figure 3).

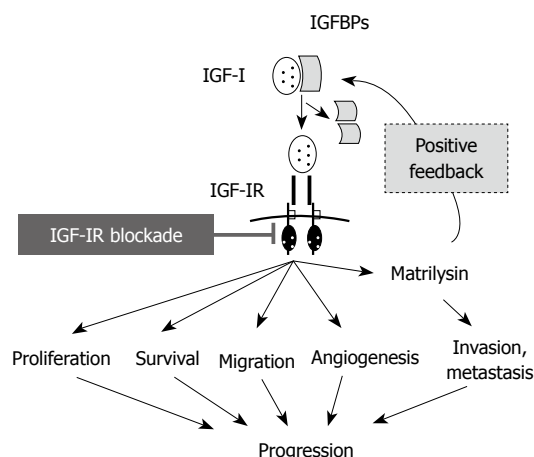


Figure 3 Blockades of type I insulin-like growth factor receptor reduce tumor progression through several interruptions of type I insulin-like growth factor receptor-mediated functions, including type I insulin-like growth factor receptor/matrilysin positive feedback system. IGF-IR: Type I insulin-like growth factor receptor; IGF-BPs: IGF binding proteins.

These findings suggest a potential basis for tumor selectivity in therapeutic applications in GI cancers.

INSULIN AND INSULIN RECEPTOR AXIS

The insulin receptor (InsR) is also a key component of the IGF signaling pathway (Figure 2). IGF-IR shares a high degree of sequence similarity to InsR. The ATP binding sites of these two receptors display 100% sequence identity, whereas the entire kinase domains share 84% sequence identity, both with each other and across species^[39]. InsR activation leads to cell proliferation in addition to glucose metabolism. In addition to insulin, InsR can also bind IGF-II and initiate mitogenic signaling^[40]. In cells that express both receptors, IGF-IR/InsR hybrids form by random association. The hybrid receptors bind both IGF-I and IGF-II at physiological concentrations.

Epidemiological studies linked long standing type 2 diabetes, obesity, and metabolic syndrome with increased risk for developing cancer, including pancreatic and colon cancer^[41]. High levels of both insulin and IGF-I are risks for breast cancer in postmenopausal women^[12,42]. Phosphorylated IGF-IR/InsR is present in all breast cancer subtypes, and is related to poor survival^[43]. InsR and IGF-IR/InsR hybrid receptors might also be involved in cancer biology, as both insulin and IGF-I contribute to the development and progression of adenomatous polyps^[44].

Two isoforms of InsR are generated by alternative splicing of exon 11^[45]. The A-isoform (InsR-A) is a fetal type and does not contain exon 11, and the B-isoform (InsR-B) is a classic form and contains exon 11^[45]. InsR-A can bind IGF-II in addition to insulin and initiates mitogenic signaling^[40]. InsR-B is able to bind IGF-I in addition to insulin. Cancers are now known to express InsRs, particularly the fetal variant InsR-A that mediates proliferation and apoptosis protection in response to IGF-II.

THE EFFECTS OF DOMINANT NEGATIVE IGF-IR IN COLORECTAL, GASTRIC, PANCREATIC, AND ESOPHAGEAL CANCER CELLS

Of the many potent strategies targeting the IGF/IGF-IR axis in GI cancer, we will first discuss data generated by our own group^[33,38,46-48]. We constructed dominant negative (dn) versions IGF-IR, which can inhibit the function rather than the expression of the naturally expressed receptor^[46,49]. We generated two different truncated IGF-IR constructs (950 and 482 amino acid residue IGF-IRs, IGF-IR/950st and IGF-IR/482st, respectively). The former lacks the tyrosine kinase domain and is thought to reside in the membrane of the transduced cells. The latter produces a defective α -chain of IGF-IR and should thus be a secreted form that may affect signal transduction in adjacent cells in addition to the transduced cells. We then constructed adenoviruses expressing two IGF-IR/dns, Ad-IGF-IR/dns (Ad-IGF-IR/482st and Ad-IGF-IR/950st).

In vitro effects and signal transduction of IGF-IR/dn

The Ad-IGF-IR/dns effectively reduced ligand dependent DNA synthesis, an index of mitogenesis, and colony formation, an index of *in vitro* tumorigenicity. IGF-IR/dns induced apoptosis and upregulated stressor (serum starvation, heat, and ethanol)-induced apoptosis.

IGF-IR/482st is a secreted protein and has a bystander effect, which suggests that IGF-IR/482st might enhance antitumor effects.

The IGF-IR/dns reduced ligands-induced phosphorylated Akt-1, but did not influence those of ERKs significantly. IGF-IR/dn can block not only IGF-I but also IGF-II stimulation, broadening the potential activity of IGF-IR/dn as an antitumor therapeutic. Although insulin induced Akt-phosphorylation, IGF-IR/482st did not block this phosphorylation, indicating that Ad-IGF-IR/dn has a high degree of receptor selectivity.

In vivo effects of IGF-IR/dn in GI tumor cells

When the GI cancer cells expressed IGF-IR/dn, the subcutaneous (SC) tumor formation was diminished significantly. Moreover, tumors derived from IGF-IR/dn expressing cells showed limited invasion into the underlying muscle. These results indicate that IGF-IR/dn effectively downregulates *in vivo* tumorigenicity and invasiveness.

Intratumoral (it) injection of Ad-IGF-IR/dn resulted in growth retardation or shrinkage of established GI tumors. The anti-tumor effect of IGF-IR/482st was stronger than that of IGF-IR/950st, undoubtedly due to the bystander effect of IGF-IR/482st. Moreover, IGF-IR/dn suppressed the invasiveness of SC tumors via downregulation of matrilysin expression and increased the number of apoptotic cells in the tumors.

In addition, GI cancer cells form peritoneal tumor

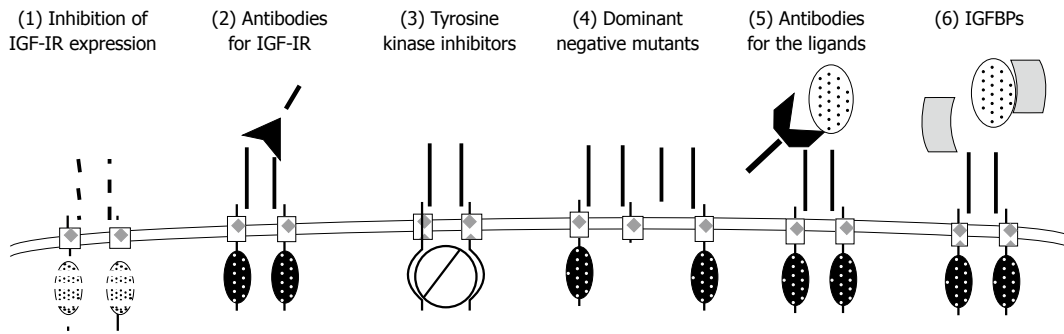


Figure 4 The summary of strategies to inactivate type I insulin-like growth factor receptor system. (1) Inhibition of type I insulin-like growth factor receptor (IGF-IR) protein expression by blocking its translation [with antisense oligodeoxynucleotides and short interfering RNA (siRNA)]; (2) IGF-IR function can be blocked with inactivating monoclonal antibodies (mAb); (3) The tyrosine kinase activity of IGF-IR can be abolished with small-molecule inhibitors (TKI); (4) IGF-IR mutants lacking β -subunits can act as dominant-negative (dn) receptors; (5) Ligand availability can be reduced by mAb for IGFs; and (6) IGF binding proteins (IGFBPs) can reduce active forms of IGFs.

nodules after intraperitoneal (ip) transplantation. Tumor bearing mice were treated by administration (ip) of Ad-IGF-IR/482st. IGF-IR/dn reduced the number of masses and resulted in a significant prolongation of survival in these mice, indicating that IGF-IR/dn can prevent and treat peritoneal cancer dissemination.

Combination effects with chemotherapy or radiotherapy

IGF-IR/dn enhanced chemotherapy (5FU and cisplatin)-induced apoptosis in GI cancer cells. The effects of the combinations were greater than addition of the effects of each monotherapy. IGF-IR/dn also upregulated radiation induced apoptosis. The combination of IGF-IR/482st (it) and 5FU (ip) for established SC tumors on mice was more effective than each single compound, and one-third of masses on the mice treated with this combo were cured; neither monotherapy cured any masses. This indicates that IGF-IR/dn has the potential to enhance the effectiveness of standard cancer therapies. Primary resistance to cytotoxic drugs is a serious problem in GI carcinomas and this approach has the potential to overcome this lack of responsiveness.

METHODS FOR THE BLOCKADE OF IGF/IGF-IR AXIS

Figure 4 shows six methods of disrupting IGF/IGF-IR signaling in cancer: (1) Blocking IGF-IR translation [with antisense (AS) oligodeoxynucleotides, AS RNA constructs, and RNA interference (RNAi)] or transcription (with triple helices) can bring about reduction or elimination of IGF-IR protein expression; (2) Binding of mAbs to IGF-IR can abolish its function; (3) Small-molecule TKIs can reduce the activity of IGF-IR; (4) Defective IGF-IRs, either mutated or lacking the tyrosine kinase domain, can act as dn receptors; (5) mAbs for the ligands can reduce their binding to the receptor; (6) Excess IGF binding proteins or inhibition of ligand expression (at the transcriptional or post-transcriptional level) can reduce the active ligands. There are several other ways to inactivate IGF-IR signals^[50]; (7) IGF mimetic peptides can compete with the natural ligands; (8) Expression of a myristoylated

IGF-IR C-terminus, which is a domain with intrinsic pro-apoptotic activity, can downregulate signals; (9) GHRH antagonists could diminish IGF-I levels^[51]; and (10) AdnectinsTM (Bristol-Myers Squibb), a novel class of targeted biologics, are proteins designed to either block or stimulate therapeutic targets of interest^[52]. Recently, an optimized Adnectins specific that specifically blocks IGF-IR has been developed.

Agents useful for blocking IGF/IGF-IR signaling in cancer are listed in Table 1. Two of the ways to inhibit IGF-IR expression are RNAi technology and the AS technique^[53,54]. We constructed a recombinant adenovirus expressing an AS to IGF-IR that decreases receptor numbers and inhibits soft agar colony forming efficiency, and treatment with this virus can significantly prolong the survival of nude mice bearing human lung tumor xenografts^[55]. ATL-1101 (Antisense therapeutics) is an AS oligodeoxynucleotide and was developed for the treatment of psoriasis (stopped after a phase I study)^[56]. We have also reported that adenoviral vectors expressing this short-hairpin RNA from IGF-IR induced effective IGF-IR silencing in lung and five GI cancers, as manifested by effective blocking of the downstream pathway of IGF-IR and by antitumor effects^[57,58]. Although an adenoviral vector has several advantages, certain side effects have been reported for gene therapy using adenovirus vectors. Thus, there are some unsolved hurdles in practical application.

Many mAbs for IGF-IR had been developed over the years. Although α IR3^[59] is a famous mAb for IGF-IR and inhibited cancer cell growth *in vitro*; however, it did not inhibit xenograft growth of breast cancer cell, MCF-7^[60]. Thus, none of the first generation mAbs had the precise characteristics for clinical use. Recently, great advances have been in the cloning and production of mAbs by several pharmaceutical companies, e.g. figitumumab (CP-751,871)^[61] by Pfizer, SCH 717454^[62] by Schering-Plough, IMC-A12^[63] by imClone systems, R1507^[64] by Roche, AMG 479^[65] by AMG, BIIB022^[13] by Biogen Idec, MK-0646^[66] by Merck, and AVE1642^[67] by Sanofi-Aventis. The first six are whole human type mAbs and the latter two are humanized mAbs. These mAbs may have the qualities necessary for clinical usage and currently under phase study. Current IGF-IR targeting mAbs seem to

Table 1 Type I insulin-like growth factor receptor targeting agents

Class	Name	Company	Other targets than IGF-IR	Clinical study	Target organs of GI	Target organs other than GI
Inhibition of IGF-IR expression	Antisense oligonucleotide Antisense RNA siRNA					
Antibodies for IGF-IR	Figitumumab (CP-751,871) ¹	Pfizer		Phase III	Colon	Lung, head and neck, breast, prostate, sarcoma, advanced solid tumor
	SCH 717454 (19D12) ¹	Schering-Plough		Phase II	Colon	sarcoma, advanced solid tumor
	IMC-A12 ¹	ImClone Systems		Phase II	Colon, HCC, pancreas, islet cell cancer	Lung, head and neck, breast, prostate, kidney, thymic, adrenocortical, sarcoma, advanced solid tumor, CMPD, leukemia, lymphoma
	R1507 (RG1507) ¹	Roche		Phase II		Lung, breast, sarcoma, advanced solid tumor
	AMG 479 ¹	Amgen		Phase II	Colon, pancreas, carcinoid, neuroendocrine cell	Lung, ovarian, prostate, sarcoma, advanced solid tumor
	BIIB022 ¹	Biogen Idec		Phase I	Liver	Lung, solid tumor
	MK-0646 (h7C10) ²	Merck		Phase II	Colon, pancreas, neuroendocrine cell	Lung, breast, myeloma, advanced solid tumor
	AVE 1642 ²	Sanofi-Aventis		Phase II	Liver	Breast
Tyrosine kinase inhibitors	NVP-AEW541 ³	Novartis				
	NVP-ADW742 ³	Novartis				
	NVP-TAE226 ³	Novartis	FAK			
	BMS-536924 ³	Bristol Myers Squibb				
	BMS-554417 ³	Bristol Myers Squibb	IR			
	BMS-754807 ³	Bristol Myers Squibb	IR	Phase I/II	Colon	Breast, head and neck, advanced solid tumor
	EGCG (tea polyphenol) ³			Phase II	Esophagus	Lung, breast, prostate, bladder, leukemia
	OSI-906 (PQIP) ³	OSI pharma		Phase III	Colon, liver	Adrenocortical, ovarian, breast, advanced solid tumor
	A-928605 ³	Abbott				
	XL-228	Exelixis	Abl, SFK, Src, Aurora kinase A	Phase I		CML, lymphoma, cancer
Dominant negative mutants	IGF-IR/482st					
	IGF-IR/486STOP					
	IGF-IR/950st					
Antibodies for the ligands	IGF-IR/952STOP					
	KM1468	Kyowa Hakko				
	KM3168	Kyowa Hakko				
IGFBPs	KM3002	Kyowa Hakko				
	Recombinant human IGFBP3 protein					

¹Fully human antibody; ²Humanized antibody; ³Adenosine triphosphate (ATP) antagonists; ⁴Non-ATP antagonists. IGF-IR: Type I insulin-like growth factor receptor; GI: Gastrointestinal; HCC: Hepatocellular carcinoma; CMPD: Chronic myeloproliferative disorder; FAK: Focal adhesion kinase; CML: Chronic myeloid leukaemia; MDS: Myelodysplastic syndromes; IGFBP: IGF binding protein.

share a common mechanism of drug action, namely to blocking ligand binding, decreasing cell surface receptor expression through receptor internalization, and blocking intracellular signaling, particularly through the PI3K/Akt pathway^[63,68]. Most mAb are IgG1 class, humanized or fully human, to reduce immunogenicity. IgG1 and IgG3

classes can mediate antibody-dependent cellular cytotoxicity^[63,68], which might strengthen anticancer activity and lymphocytic toxicity through recruitment of immune effector cells to antibody-antigen complexes. However, IGF-IR-mAb-directed cellular cytotoxicity could also enhance toxicity to normal IGF-IR-bearing tissues. As

CP-751,871 is an IgG2 subtype, which are usually poor activators of cellular immune response, and BII022 is a nonglycosylated IgG4 antibody, ongoing clinical studies should clarify whether these agents have significantly different properties from IgG1 class.

Small molecular TKIs for IGF-IR are synthesized by several companies. Novartis pharma produced three agents, NVP-ADW742^[69], NVP-AEW541^[70], and NVP-TAE226^[71], which has dual targets on IGF-IR and focal adhesion kinase (FAK). Bristol-Myers-Squibb constructed three materials, a specific inhibitor of IGF-IR, BMS-536924^[72], and dual inhibitors for InsR and IGF-IR, BMS-554417^[73] and BMS-754807^[74]. OSI-906^[75] is made by OSI pharma and A-928605^[76] by Abbott. Tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) is also identified as a TKI for IGF-IR^[77]. These nine medicines inhibit IGF-IR kinase activity by an ATP-competitive mechanism. In contrast, there are two compounds that are IGF-IR TKI non-ATP antagonists, cyclolignan picropodophyllin (PPP)^[78] and INSM-18^[13]. The latter is a dual TKI for IGF-IR and Her2. XL-228 is a multi-target TKI for IGF-IR, Bcl-Abl, SFK, Src, and Aurora kinase A^[13]. At least five TKIs are currently in clinical studies.

We used two dn inhibitors of IGF-IR, as detailed above. Several groups have used IGF-IR/486STOP^[79,80] and IGF-IR/952STOP^[81,82], the former resembles our IGF-IR/482st and the latter is similar to our IGF-IR/950st.

mAbs for IGF-I and IGF-II, KM1468^[83,84], KM3168^[85], and KM3002, are made by Kyowa Hakko. KM1468 neutralized both ligands and inhibited bone metastasis in an animal model.

The last approach is a recombinant human IGFBP-3 protein, which is available for intravenous injection^[86] and is beginning clinical testing.

TOXICITY AND COMBINATION OF IGF-IR TARGETING STRATEGIES

The two major potential toxicities of IGF-IR blockade are based on the IGF-IR expression in normal tissues and homology between IGF-IR and InsR. Long-term IGF-IR blockade might cause growth retardation during childhood and might influence the function of IGF-dependent tissues, including the myocardium and brain at any age^[87,88]. IGF-IR-inhibitory drugs are predicted to influence glucose tolerance. TKIs might directly inhibit the InsR kinase in some degree, because of binding to a well-conserved ATP binding pocket. In fact, some TKIs can inhibit both receptors, e.g. NVP-TAE226, BMS-554417, and INSM-18. Several anti-IGF-IR mAbs, such as scFv-Fc and EM164, might induce downregulation of InsR *via* endocytosis of hybrid receptors or InsR, which was observed in cancer cells expressing both receptors, but not in cells expressing InsR only^[89]. This suggests that anti-IGF-IR mAbs will not inhibit InsR function in insulin-responsible tissues, e.g. hepatocytes, which do not express IGF-IR. In addition, both IGF-IR mAbs and TKIs might result in loss of the hypoglycemic effects of IGF-I, and

blockade of pituitary IGF-IRs might result in a compensatory increase in serum concentration of GH, which could contribute to insulin resistance^[90].

Although IGF-IR mAbs are exquisitely specific inhibitors of receptor function (by inducing rapid internalization and down-regulation of the receptor), TKIs suffer from a lack of selectivity. TKIs, in general, do not lead to internalization or downregulation of IGF-IR, and will probably represent a broad spectrum of specificity against IGF-IR and InsR and a unique profile of toxicity. Possible toxicity of the central nervous system deserves particular attention during treatment with TKIs, because other molecules in this class have been shown to infiltrate the blood-brain barrier in central nervous system tumors^[91]. Nevertheless, IGF-IR TKIs offer several advantages, such as oral administration and of the ability to control the duration of drug exposure, in contrast to long-acting mAbs.

Recently, it has been revealed that the insulin/InsR axis has certain roles in carcinogenicity and tumor development. Chronic hyperinsulinaemia might be a cause of colon and pancreas cancers^[92]. IGFs have some potential for binding to and activating InsR. In addition, hybrid receptors of IGF-IR and InsR exist on malignant cells. Thus, blockade of InsR is another matter of concern to eliminate cancer cells. Thus, dual targeting TKIs for IGF-IR and InsR have merits for terminating tumor cells; however, they would, again, have adverse effects of glucose homeostasis.

According to several clinical studies, it has been reported that the adverse effects of IGF-IR mAb are hyperglycemia, mild skin toxicities (rash, flushing, pruritus, and acne), and fatigue as common toxicities of these antibodies^[64,93,94]. Other observed toxicities, such as CD4+ lymphocytopenia, thrombocytopenia, and transaminitis, do not seem to be related to the mechanism of their specific action. An IGF-IR mAb caused hyperglycemia in around 20% of patients, but was tolerable, mild to moderate (grades 1 and 2), reversible, and manageable with oral hypoglycemic drugs. Patients with previous glucose intolerance or with steroids usage were at risk of hyperglycemia.

IGF-IR is a mediator of resistance to therapy. IGF-IR activation is known to protect tumor cells against apoptosis induced by cytotoxic drugs, and might also influence the repair of DNA damage^[95,96]. There is considerable preclinical data to support the view that IGF-IR inhibition can enhance sensitivity to chemotherapy and radiotherapy. In addition, blockade of IGF-IR might have combination effects with other molecular targeting therapies, especially for RTKs.

Recently, a new role for IGF-IR has been proposed in that its signals might be an escape pathway in cancer cells for drug resistance. Many patients who achieve an initial response to trastuzumab acquire resistance within one year of treatment initiation. Two mechanisms for this trastuzumab resistance have been reported; one is overexpression of IGF-IR^[97] and the other is the formation of a IGF-IR/Her2 heterodimer^[98]. In addition, IGF-induced PI3-K/Akt activation mediates resistance to EGFR blockade in glioblastomas^[99].

Thus, there is a hypothesis for horizontal blockade of two different growth factor receptors, such as Her/EGFR and IGF-IR. Several groups have tried these dual targeting therapies. Recently, a candidate combination treatment with an IGF-IR TKI, BMS-536924, and EGFR/Her2 inhibitors was reported^[100].

On the other hand, nonselective inhibitors might have a different profile and alternative benefits. Some TKIs inhibit other kinases, such as Src (XL-228) or Her2 (INSM-18), and these multi-kinase inhibitors can expand the activity of the agent. It could also add toxicity mediated by target and off-target effects, complicating the combination with other agents.

Treatment with CP-751,871 decreased both total circulating tumor cell count and IGF-IR-positive circulating tumor cell count, suggesting that circulating tumor cells could be used as a biomarker of drug effect^[93]. High concentrations of serum free IGF-I might be a marker of high responder of patients with non small cell lung carcinoma treated with figitumumab.

Apart from mAbs and TKIs, there are individual approaches using short interfering RNA (siRNA), peptides, proteins, or antisense oligonucleotides to antagonize IGF-IR. As mentioned above, several group, including ours, have revealed that both dn and siRNA for IGF-IR show powerful anti-tumor effects. However, the delivery systems of these approaches represent a significant hurdle for clinical use. Given a suitable delivery tool for humans, we would want to start using both dn and siRNA for IGF-IR in the patients with GI cancer.

CONCLUSION

The IGF/IGF-IR axis plays pivotal roles in the carcinogenicity and progression of GI cancers. We have presented the efficacy of IGF-IR targeting strategies using our data of IGF-IR/dn against GI cancers. Blockade of IGF-IR suppresses carcinogenicity, and upregulates apoptosis-induction and the effects of chemotherapy, both *in vitro* and *in vivo*. We summarized several approaches to blocking IGF-IR signals and discussed the merits and demerits of each strategy. In addition to combination with classical chemotherapy, several attempts at dual targeting for IGF-IR and other growth factor receptors have been made. Many drugs blocking IGF-IR function are now entering clinical trials. Thus, IGF-IR might be a candidate therapeutic molecular target in gastrointestinal malignancies.

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Pathobiology of the neutrophil-intestinal epithelial cell interaction: Role in carcinogenesis

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Abstract

The role of chronic inflammation, acting as an independent factor, on the onset of gastrointestinal carcinogenesis is now well accepted. However, even if there is an increase in the number of elements directly involving polymorphonuclear leukocytes (PMNL), as a major actor in digestive carcinogenesis, the different cellular and molecular events occurring in this process are still not completely understood. The transepithelial migration of PMNL, which is the ultimate step of the influx of PMNL into the digestive mucosa, is a complex phenomenon involving sequential interaction of molecules expressed both on PMNL and on digestive epithelial cells. Chronic inflammatory areas rich in PMNL [so-called (chronic active inflammation)] and iterative transepithelial migration of PMNL certainly evoke intracellular signals, which lead toward progressive transformation of epithelia. Among these different signals, the mutagenic effect of reactive oxygen species and nitrates, the activation of the nuclear factor- κ B pathway, and the modulation of expression of certain microRNA are key actors. Following the initiation of carcinogenesis, PMNL are involved in the progression and invasion of digestive carcinomas, with which they interact. It is noteworthy that different subpopulations of PMNL, which can have some

opposite effects on tumor growth, in association with different levels of transforming growth factor- β and with the number of CD8 positive T lymphocytes, could be present during the development of digestive carcinoma. Other factors that involve PMNL, such as massive elastase release, and the production of angiogenic factors, can participate in the progression of neoplastic cells through tissues. PMNL may play a major role in the onset of metastases, since they allow the tumor cells to cross the endothelial barrier and to migrate into the blood stream. Finally, PMNL play a role, alone or in association with other cell parameters, in the initiation, promotion, progression and dissemination of digestive carcinomas. This review focuses on the main currently accepted cellular and molecular mechanisms that involve PMNL as key actors in digestive carcinogenesis.

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Key words: Neutrophils; Intestinal epithelial cells; Carcinogenesis; Cytokines; Nuclear factor- κ B pathway; MicroRNA; Reactive oxygen species

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INTRODUCTION

The link between a chronic active inflammatory process (i.e. chronic inflammation rich in neutrophils) and the onset of carcinoma, in association or not with another factor such as a pathogen, is now convincingly demon-

strated with epidemiological, experimental, and molecular data obtained for different tissues^[1-10]. In particular, this relationship is well-established at the gastric and intestinal mucosal level^[11-18]. Different factors are involved in digestive carcinogenesis, but the association of these factors and their importance in cancer onset are certainly variable from one disease to another and among individuals. Thus, predisposing genetic factors, infectious factors and inflammatory factors can be involved in digestive carcinogenesis^[19]. Inappropriate innate immunity induces cellular infiltration of the digestive mucosa composed of polymorphonuclear leukocytes (PMNL), dendritic cells, natural killer cells, and then secondarily, an influx of adaptive immune cells such as T lymphocytes. The intensity of this polymorphous cellular infiltrate varies according to the period of the active phases of the digestive disease^[20]. In this regard, inflammatory infiltration can be present at variable time periods and at a variable frequency. Among the different populations of cells which migrate into the digestive mucosa, the PMNL play a central role in the pathophysiology of inflammatory digestive diseases^[21]. Thus, previous epidemiological and histological studies have convincingly demonstrated a direct link between the clinical symptoms (pain and diarrhea) and the presence of PMNL in the digestive mucosa. More particularly, the periods of acute diarrhea certainly correlate with transepithelial migration of PMNL into the digestive lumen. It is noteworthy that during interaction between the intestinal epithelial cells (IEC) and PMNL different intracellular events are triggered, leading to neoplastic transformation of the digestive epithelia. The molecular phases involved in PMNL transepithelial migration are complex, but it is crucial to understand these phases to better comprehend the initial steps in digestive carcinogenesis. The progression from an *in situ* carcinoma to a microinvasive and invasive digestive carcinoma is associated with several molecular events, in particular, cytoskeleton modification, modulation of adherence molecules and metalloprotease production. Among these different events, some directly implicate PMNL. Currently, the pros and cons of the role of PMNL in tumor progression are debatable^[22,23]. PMNL produce elastases^[24], which favor tumor cell extracellular matrix invasion and release of pro-angiogenic factors, which creates a favorable microenvironment for tumor progression^[25-30], but also produce defensins, which have an anti-tumor effect. Recently, a dual function of PMNL, in regard to their action on carcinoma cells, has been proposed^[31,32]. Thus, two different populations of PMNL can be present in tumors, a population that favors tumor progression, the tumor-associated neutrophils 1 (TAN1) and a population that decreases tumor progression, the TAN2^[31,32]. Accordingly to the proportion of TAN1 and TAN2 in a carcinoma the level of tumor progression can vary. This phenomenon can be present in colonic adenocarcinomas. Finally, previous studies implicate PMNL in the pathophysiology of metastases. This phenomenon can occur in colonic adenocarcinoma dissemination. In particular, PMNL allow transendothelial

migration of tumor cells and then their migration into the blood stream.

Previous studies and reviews have focused on the role of the immune system during cancer development^[33] but the impact of PMNL in the different phases of the natural history of cancer (Figure 1) has been poorly described to date. In this review, I describe the role of PMNL and the direct events induced by PMNL in the mechanisms of the different steps in digestive carcinogenesis (cancer initiation, progression and dissemination).

THE BIOLOGY OF THE NEUTROPHIL- INTESTINAL EPITHELIAL CELL INTERACTION

After transendothelial migration, following the crossing of the matrix of the lamina propria, which is mainly induced by a gradient of interleukin (IL) 8^[34], PMNL adhere to the basal side of the glandular and crypt cell epithelium, and then transmigrate to the digestive lumen. This transepithelial migration is associated with sequential steps and with dynamic and transitory interactions between some surface molecules that are present on cytoplasmic membranes of PMNL and IEC^[35,36] (Figure 2). Studies using *in vitro* models, such as the T84 model, have greatly improved our knowledge concerning these different cellular interactions. Thus, PMNL transepithelial migration can be induced by different stresses on epithelial cells, such as bacteria, bacterial products, toxins, or hypoxia^[37,38]. Using this T84 model, the different steps of PMNL transepithelial migration and the different mechanisms involved in cell-cell interactions have been described^[39-41]. Briefly, PMNL adhere to the basal side of the digestive epithelia through their CD11b/CD18 molecules (for which the ligand on epithelia is still unknown), then they migrate using a paracellular pathway through an homophilic CD47 interaction, which is expressed both on PMNL and IEC^[42,43]. A more recent study showed that CD47 regulates neutrophil transmigration through close cross-talk with one toll-like receptor, TLR-2^[44]. Other interactions occur at the desmosome and tight junction levels, which involve JAM and SIRP α ^[45-47]. After crossing the epithelial barrier PMNL interact with ICAM1 at the apical membrane through CD11b/CD18. During this transepithelial migration, the actin cytoskeleton of epithelial cells is reorganized^[48]. Activated PMNL release 5'-adenosine monophosphate, which is secondarily cleaved by an epithelial membrane ectonucleotidase into adenosine, and finally produce chloride secretion on the epithelial apical side^[49,50]. More recently, other molecular mechanisms have been described to occur during interaction between PMNL and the IEC^[44,51]. Serine protease-mediated activation of epithelial protease-activated receptors has been shown to increase permeability. It has been demonstrated that transmigrating PMNL can regulate barrier function through epithelial protease-activated receptor activation^[51]. Thus, transepithelial resistance decreased significantly after contact of PMNL with basolateral sur-

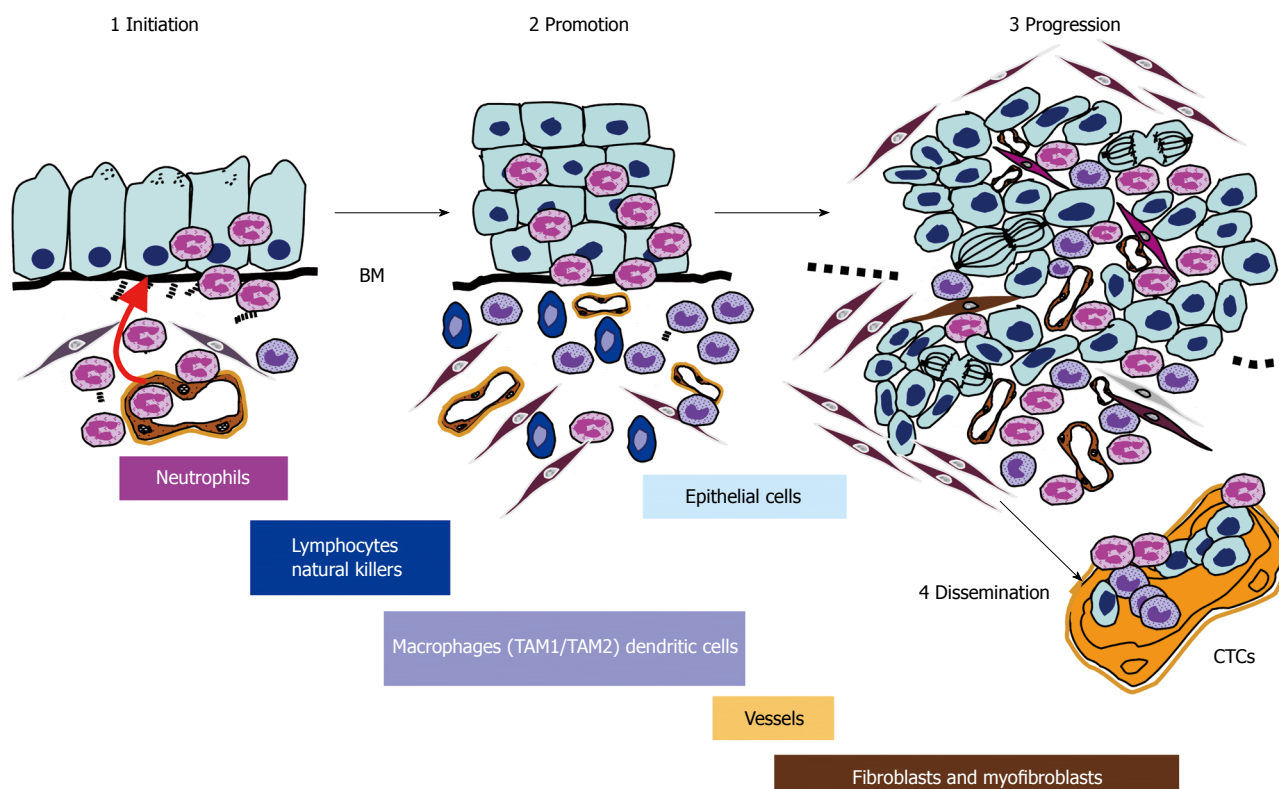


Figure 1 Involvement of a neutrophil-rich microenvironment in the different steps of digestive carcinogenesis including initiation, promotion, progression and dissemination of tumor. BM: Basement membrane; TAM: Tumor-associated macrophages; CTCs: Circulating tumor cells.

faces of T84 monolayers or after incubation with PMNL elastase and proteinase-3^[51].

ROLE OF CHRONIC ACTIVE INFLAMMATION IN INITIATION OF DIGESTIVE CARCINOGENESIS

Beside these different events, which are associated with rapid paracellular migration of PMNL, different studies using the T84 model demonstrated the modulation of different molecules expressed on epithelial cells, which may be potentially involved in the initiation of carcinogenesis in direct or indirect pathways, by inducing an amplified inflammatory response rich in PMNL^[52,53]. Moreover, paracellular migration of PMNL induced the onset of apoptosis, and, then potentially increases turnover of epithelium regeneration^[54]. Thus, there is certainly a tight association between this chronic active inflammation and the onset of digestive carcinoma. An increased level in oxidative stress is present in the mucosa of inflammatory bowel diseases^[55-57]. In this regard, an inflammatory microenvironment rich in PMNL can increase the rate of mutation, in addition to enhancing the proliferation of mutated cells^[58]. Activated PMNL serve as sources of reactive oxygen species (ROS) and reactive nitrogen intermediates that are capable of inducing DNA damage and genomic instability^[59]. Interestingly, release of ROS can occur during epithelium adhesion, but also during transepithelial migration and

during post transepithelial migration of PMNL^[60]. Alternatively, activated PMNL may use cytokines such as tumor necrosis factor (TNF)- α , which is implicated in carcinogenesis, to stimulate ROS and nitric oxide accumulation in neighboring epithelial cells^[61,62]. Moreover, nitric oxide synthase can activate cyclooxygenase-2 in epithelial cells^[63]. Different studies focus primarily on the effect of early mediators of inflammation, such as TNF- α , in stimulating tumor cell growth by activating nuclear factor (NF)- κ B^[64]. Conversely, decreased production of TNF- α in mice can reduce digestive carcinogenesis associated with chronic colitis^[65]. However, chronic inflammation involves many other cytokines in the host microenvironment, which may also affect tumor growth in an NF- κ B-dependent manner. While most inflammatory cytokines are released from activated macrophages following stimulus-induced transcription, others are secreted from intracellular pools and display later kinetics during the inflammatory response. Furthermore, the fact that NF- κ B inhibition does not completely prevent tumor formation in these studies suggests that cytokines could also promote tumorigenesis *via* alternative pathways^[66]. Mutations in p53, caused by oxidative damage, were found in both cancer cells and in a non-dysplastic epithelium in cancer associated colitis, suggesting that chronic inflammation causes genomic changes^[67]. Finally, ROS can also cause direct oxidative inactivation of mismatch repair enzymes^[5].

Other mechanisms have been described, which involve PMNL in the early steps of initiation of carcino-

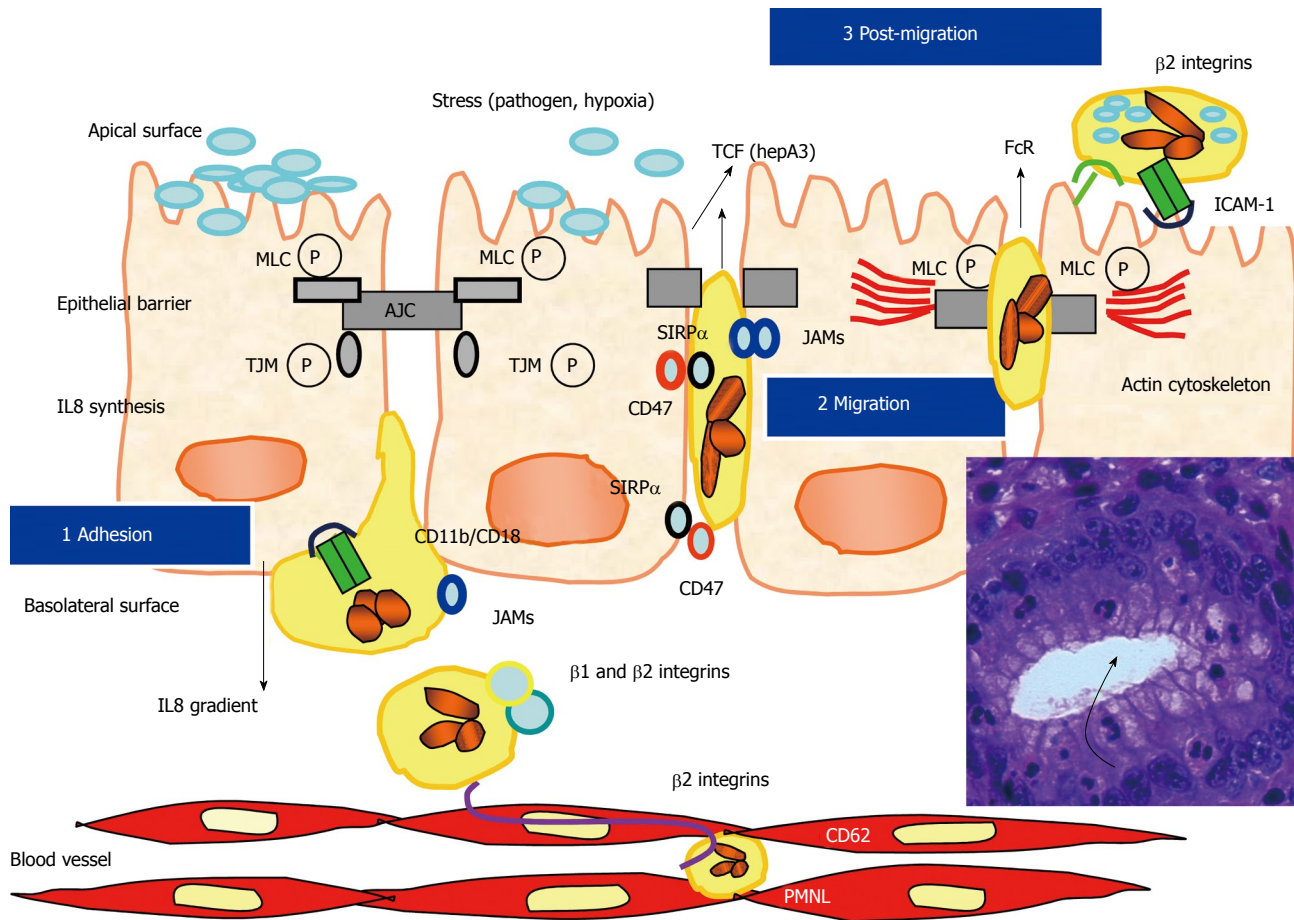


Figure 2 Cross-talk between polymorphonuclear leukocytes and intestinal epithelial cells. Different steps and molecules involvement in polymorphonuclear leukocytes transepithelial migration. The microphotograph shows polymorphonuclear leukocytes (PMNL) in an intestinal epithelium. TJM: Tight junction molecules; AJC: Apical junction complex; MLC: Myosin light chain kinase; JAM: Junctional adhesion molecules; TCF: Transcellular chemotactic factor; SIRP: Signal regulatory protein; IL: Interleukin.

genesis. Using animal models that reproduce digestive carcinogenesis linked to colitis, the molecule vanin 1 has been recently implicated in the onset of carcinoma^[68]. Interestingly, it has been described that protein expression of cyclooxygenase-2 and the hypoxia-inducible factor-1 is up-regulated and associated with inflammation in early steps of digestive carcinoma^[69]. The role of ROS and nitrates, largely suggested by previous studies, has been highlighted by different recent studies^[70-76]. Interestingly, the myeloperoxidase (MPO) released by activated PMNL can inhibit nucleotide excision repair in certain epithelial cell lines^[77]. In this regard, mutagenic products of MPO such as 5-chlorouracil and 5-bromouracil are released into inflammatory tissues. Moreover, the role of PMNL in initiation of carcinogenesis is probably more complex^[78-80].

MicroRNA have been mainly investigated in oncology. However, microRNA are also implicated in inflammatory mechanisms, and their deregulation during some inflammatory diseases, in particular at the digestive level, could be associated with the molecular events that link chronic inflammation to cancer development^[81-87]. The action of PMNL in this process is currently difficult to define, but through ROS release, and/or by the production of different enzymes, PMNL probably participate in deregulation of the RNA network in digestive epithelial cells.

IMPLICATION OF NEUTROPHILS IN PROGRESSION OF DIGESTIVE CARCINOMA

Recent studies have demonstrated that the presence of intratumoral PMNL can be associated with shorter disease specific survival in certain cancer patients^[88]. Following the initiation of digestive carcinoma, processes allow the tumor to grow from a single initiated cell into a developed primary adenocarcinoma. In this context, tumor growth depends on increased cell proliferation and reduced cell death, both of which can be stimulated by PMNL-driven mechanisms. This inflammation-induced tumor promotion may occur early or late in tumor development and leads to activation of premalignant lesions that have been dormant for many years. As for tumor-associated macrophages^[89-91], PMNL probably promote tumor growth but the putative mechanisms have not yet been determined. However, it has been shown that accelerated intestinal epithelial cell turnover caused by chronic active inflammation and epithelial damage might predispose the mucosa to DNA damage, resulting in an elevated risk of mutation, which is in line with dysplasia and carcinoma development in patients with ulcerative

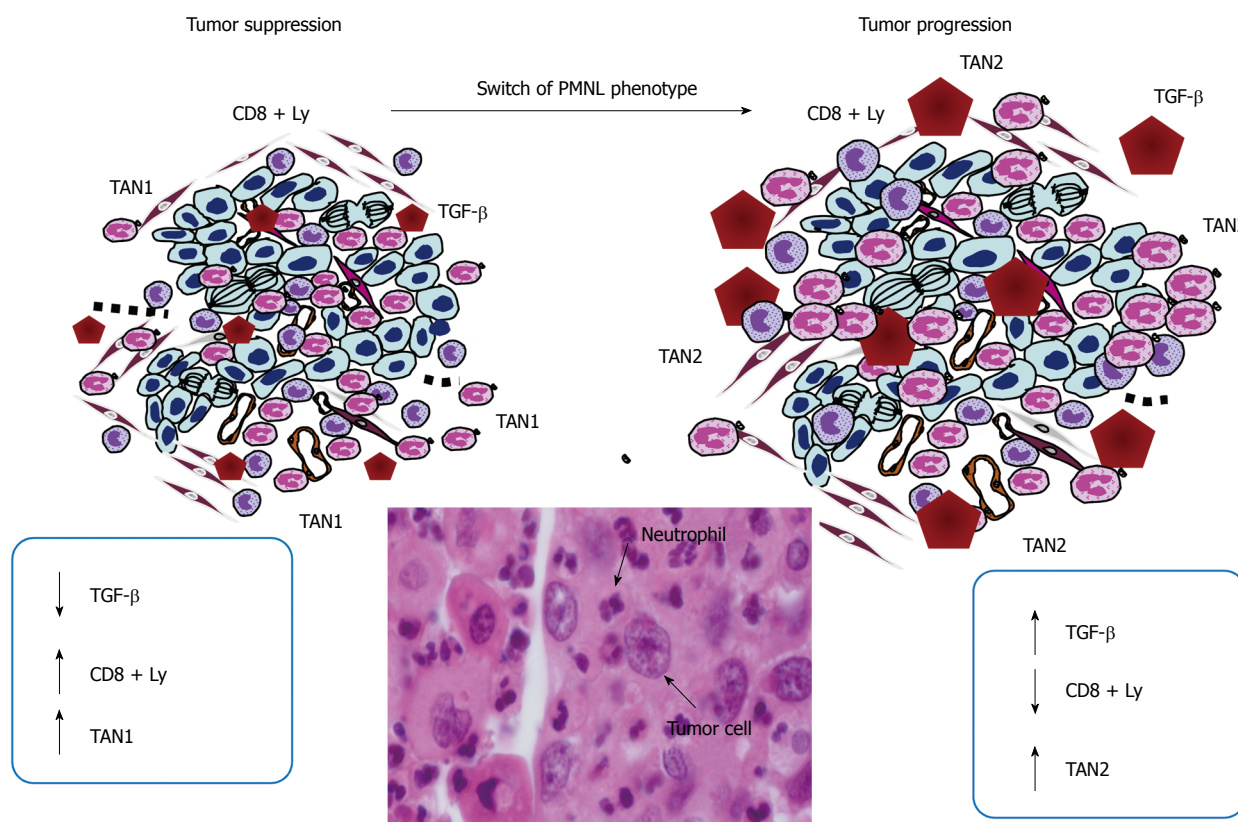


Figure 3 Speculative role of tumor-associated neutrophils in progression of digestive carcinoma. The microphotograph shows neutrophils tightly associated with digestive carcinoma cells. TAN: Tumor-associated neutrophils; TGF: Transforming growth factor.

colitis^[92]. In parallel, the repeated inflammatory process could act on COX-2 expression which is down-regulated by the adenomatous polyposis coli (APC) gene and up-regulated by nuclear beta-catenin accumulation, and additionally implicate the Wnt signaling transduction pathway in colon carcinogenesis^[93].

Secreted PMNL factors, such as human neutrophil peptides 1-3 (HNP1-3), have been found to be elevated in patients with digestive carcinoma, both in tissues and plasma, and to correlate with Dukes' stages^[94]. Other molecules such as neutrophil gelatinase-associated lipocalin or neutrophil elastase are able to suppress or to increase the invasion of carcinoma cells^[95-97]. Among the cytokines involved in carcinoma progression, Transforming growth factor (TGF)- β is certainly one of the most studied, to date. It has been reported recently in a mouse model of carcinoma that TGF- β controls maturation of a sub-type of PMNL, the so-called TAN-2. TANs could function in parallel with tumor-associated macrophages (TAMs)^[98,99]. Conversely, inhibition of the TGF- β activity leads to the differentiation of PMNL in anti-tumor TAN-1 cells (Figure 3). While TAN-2 inhibit the cytotoxic response of CD8+T lymphocytes, which infiltrate the intestinal mucosa and thereby allow tumor cells to circumvent immune surveillance, TAN-1 enhance the anti-tumor action of CD8+ T-lymphocytes. TGF- β blockade not only activates CD8+T cells, but also increases the recruitment of hyper-segmented neutrophils, their NI polarization (high expres-

sion of TNF- α , ICAM-1 and FAS) and their anti-tumor activity. Moreover, N1 neutrophils produce T cell-attracting chemokines including CCL3, CXCL9 and CXCL10. By contrast, TGF- β stimulation polarizes PMNL to the so-called N2 state with increased expression of arginase and chemokines such as CCL2 and CCL5. N1 are cytotoxic for tumors, whereas N2 display pro-tumor properties.

We may speculate that this mechanism is universally found in carcinomas arising in different organs. Finally, it is noteworthy that the prognostic value of a high number of PMNL in different carcinomas correlates with poor outcome in previous studies^[100].

In addition to TGF- β , other cytokines produced by PMNL may be involved in carcinoma progression. Thus, TNF- β secreted by PMNL can stimulate a positive loop of inflammation by inducing production of chemokines such as IL8 and Gro α by epithelial tumor cells and probably inducing renewed recruitment of PMNL^[101]. Moreover, other mechanisms may exist such as carcinoma cell stimulation of PMNL to produce oncostatin M^[102].

Although it is not yet established, we can speculate that some miRNA expressed by PMNL, in particular mir-223, may also play a crucial role in modulating progression of digestive tumors. Mir-223 was found to possess a crucial role in regulating neutrophil proliferation and activation^[103]. Moreover, the expression of mir-223 may be modulated by some cytokines released by tumor cells and may influence the phenotype of TAN-1 or

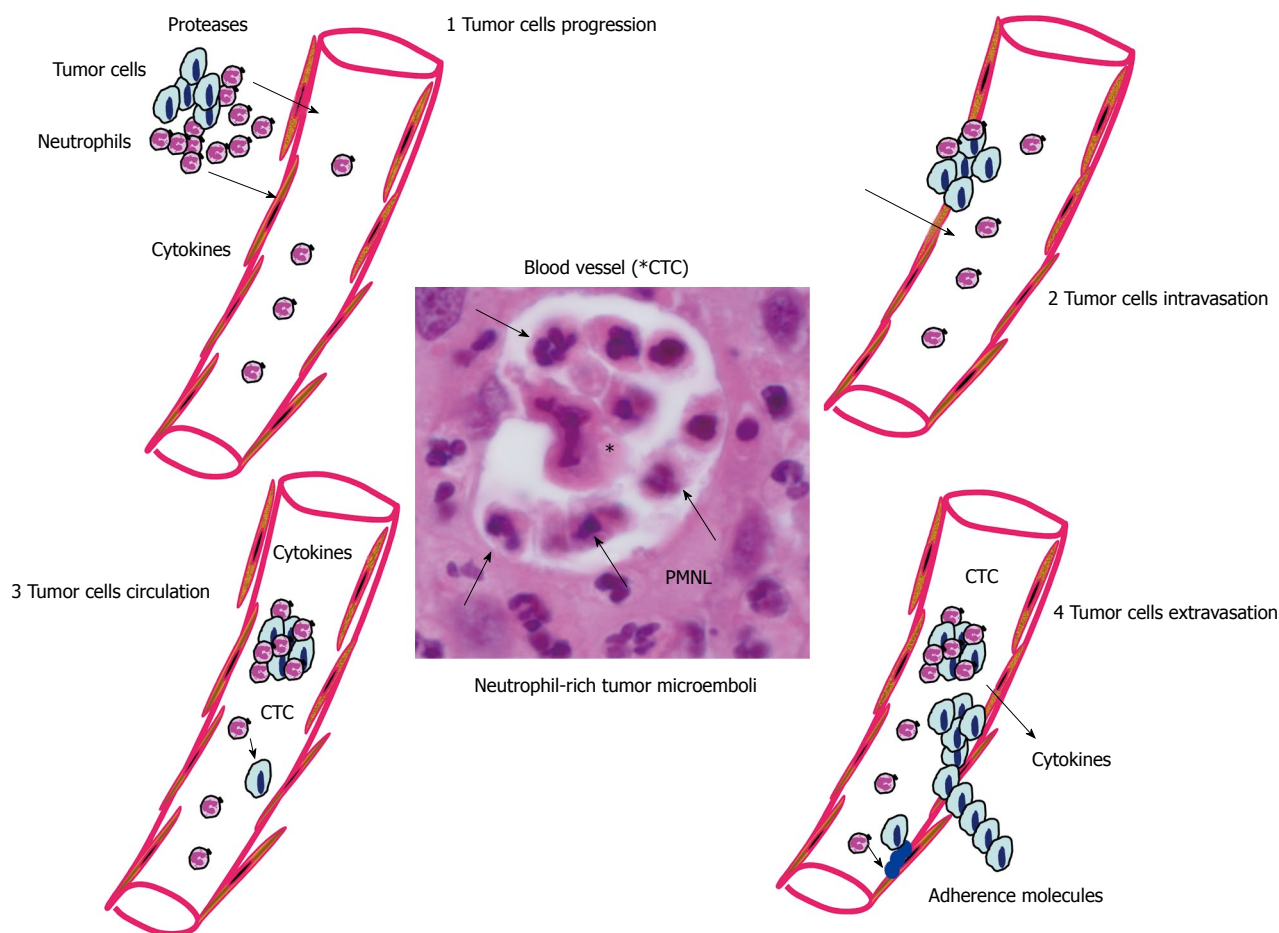


Figure 4 Speculative role of polymorphonuclear leukocytes in digestive carcinoma dissemination. The microphotograph shows circulating tumor cells associated with polymorphonuclear leukocytes (PMNL). CTC: Circulating tumor cells.

TAN-2. In this regard, different molecules have recently been reported as markers and/or promoters of inflammation-associated cancers^[104]. Thus, we can speculate that the level of expression of mir-223 in carcinoma might be a marker of tumor progression.

THE NEUTROPHIL AS AN ACTOR OF THE PATHOBIOLOGY OF DIGESTIVE CARCINOMA METASTASIS

Inflammation is a key actor of metastasis onset^[105]. In this regard, different studies have demonstrated the role of PMNL in tumor metastasis through different steps^[106,107]. PMNL can participate in the transendothelial migration of adenocarcinoma cells, as well as their dissemination into the blood (Figure 4)^[108,109]. Cytokines produced by PMNL can increase vascular permeability and upregulation of certain adhesion molecules located on endothelial cells^[110]. In addition, PMNL are important sources of proteases that degrade the extracellular matrix and may alter the vascular barrier allowing entry of tumor cells into the blood stream. Interestingly, in a model of invasive colon cancer, CCR1+ myeloid cells, the recruitment of which is driven by the chemokine CCL9 produced by cancer cells, promote inva-

siveness through secretion of the matrix metalloproteinases MMP2 and MMP9^[111]. It has been demonstrated that extracellular ATP can be released by activated PMNL^[112]. This release of ATP occurs through a conformational opening of membrane Cx43 hemichannels in response to PMNL activation^[113]. Moreover, the extracellular ATP released by activated PMNL may act both on epithelial cells, through activation of some purinergic receptors expressed by epithelial cells^[53], and on endothelial cells^[112]. More specifically, ATP released by activated PMNL is auto-hydrolyzed to AMP through CD39 on the surface of PMNL. CD39 may function as an immunomodulatory control point, requiring a close and special relationship with CD73-positive cells, such as endothelial cells. In addition to regulating the endothelial barrier function, a role for PMNL-dependent ATP release in directed movement of PMNL has been reported^[114]. ROS released by activated PMNL can generate mitochondrial DNA mutations that regulate tumor cell metastasis^[115].

Once metastatic cells enter the circulation, they need to survive in suspension and resist detachment-induced cell death or anoikis. The survival of circulating cancer cells is affected by inflammatory mediators released by immune cells in response to cancer-derived stimuli^[116]. In the same way, the presence of a variety of cytokines

released by activated PMNL present in the tumor micro-environment, including $\text{TNF-}\alpha$, can promote the survival of circulating metastatic seeds^[117]. PMNL can also favor the circulation in the blood of tumor cells, in a similar way to that of platelets or blood macrophages which can be physically linked to cancer cells, allowing them to travel together through the circulation^[118]. Thus, single circulating tumor cells (CTC), which are no longer present in an immunosuppressive environment, may be targeted again by immunosurveillance. In this regard, the interaction of circulating cancer cells with PMNL may protect them from cell death, thereby overcoming immunosurveillance^[119]. The journey of CTC ends upon integrin-dependent arrest on the endothelium, followed by extravasation. In this regard, systemic inflammation enhances attachment of CTC to endothelial cells, and this process is governed by neutrophil-dependent upregulation of adhesion molecules^[120]. Thus, the production of high levels of proinflammatory cytokines by the PMNL can upregulate expression of certain adhesion molecules on endothelial cells and thereby increase the probability of metastatic cell attachment and potentialize the passage of tumor cells from the circulation into the extracellular space and then to develop micrometastases^[90,105].

CONCLUSIONS AND PERSPECTIVES IN THERAPIES TARGETING NEUTROPHILS

Different proinflammatory molecules and inflammatory cells have been suggested to be potential candidate targets for therapeutic strategies for cancer^[99,121,122]. One study has shown that different drugs that prevent inflammation can inhibit carcinogenesis^[123].

The role of PMNL in the onset and progression of digestive carcinoma, in particular those occurring in inflammatory bowel diseases, is complex. However, recent studies highlight new aspects of the pathophysiology of the PMNL-epithelial cells interaction, in particular, the effect of ROS release by activated PMNL on digestive epithelial cells at the molecular level or the effect of different TAN on tumor progression. Interestingly, these novel findings on the role of PMNL in the initiation and progression of carcinogenesis open up therapeutic avenues for the treatment of digestive cancers^[124]. It is noteworthy that immunotherapy against cancer has been explored as a coadjuvant and has been based mostly on the properties of the adaptive immune system (i.e. B and T lymphocytes, dendritic cells) and of some components of the innate system (macrophages, NK cells, or complement proteins)^[125,126]. PMNL have been rarely considered as a weapon against cancer. However, studies highlighting the anti-tumor efficacy of PMNL have been published. For example, suppression of the secreted protein acidic and rich in cysteine, which is associated with the capacity of tumor cells to migrate and invade tissues, in malignant cells, led to the promotion of PMNL recruitment and induced tumor rejection^[127]. However, the mode of action of PMNL that leads to the killing of tumor cells is not fully understood.

It probably depends on the maturation of PMNL since in an animal model of lung tumors, only a subpopulation of PMNL i.e. TAN2 had an anti-tumor effect^[31]. PMNL produce cytotoxic agents such as proteases, ROS, and defensins, all of which can directly damage the target cells. However, the cytotoxic effect of PMNL on tumors is greatly enhanced in the presence of target-specific antibodies. Finally, another strong argument for the anti-cancer effect of PMNL comes from studies using animal models in which tumor cells were genetically engineered to release immunoregulatory molecules (cytokines and chemokines). These molecules did not affect the proliferation of the tumors directly, but activated a host immune reaction that was strong enough to overcome their oncogenic capacity. For instance, G-CSF-releasing colon adenocarcinoma cells were found to lose their tumorigenic activity through the massive attraction of PMNL to the tumor injection site^[128]. These PMNL distinguished between G-CSF-producing and nonproducing cancer cells. Moreover, tumor inhibition *in vivo* was accompanied by intimate physical contact between PMNL and G-CSF-producing tumor cells^[129]. However, future research should be done in order to better target the different subpopulations of TAN, since only one population of PMNL would have an anti-tumor effect and should be considered.

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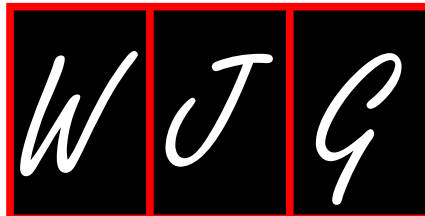
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Leptin in hepatocellular carcinoma

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Abstract

The risk factors for hepatocellular carcinoma (HCC) development have been established, and include chronic hepatitis B and C, heavy alcohol consumption, and aflatoxins. In fact, 5%-30% of patients with HCC still lack a readily identifiable risk factor. It has been reported that the majority of "cryptogenic" HCC may be attributed to nonalcoholic fatty liver disease, the hepatic presentation of the metabolic syndrome (MS). Obesity is associated with the development of the MS. Recently, adipose tissue has been considered as an endocrine organ because

of its capacity to secrete a variety of cytokines, which are collectively known as the adipokines. Leptin, the product of the obese gene, is mainly produced by adipose tissue. Since leptin was first characterized in 1994, accumulated literature has demonstrated the involvement of this adipokine in several areas of human physiology. After binding to its receptor, leptin initiates a cascade of signaling events and subsequent cellular effects. In addition to being the regulatory mediator of energy homeostasis, several *in vitro* studies have demonstrated the fibrogenic role of leptin in the liver. Furthermore, the deregulated expression of leptin and its receptor have been demonstrated to be associated with a variety of metabolic disorders as well as human cancers. Most importantly, direct evidence supporting the inhibitory and/or activating role of leptin in the process of carcinogenesis and progression of human HCC has been accumulating rapidly. This review aims to provide important insights into the potential mechanisms of leptin in the development of HCC. Hopefully, further investigations will shed light on a new therapeutic target in HCC.

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Key words: Adipokine; Hepatocellular carcinoma; Leptin; Liver cirrhosis; Metabolic syndrome; Obesity; Steatohepatitis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most com-

mon cancer worldwide and the third leading cause of cancer death^[1]. Due to its high mortality, the annual fatality ratio is close to 1.0, indicating that the patients who develop HCC will die within 1 year^[2]. Furthermore, recent data have shown that there are 662 000 deaths per year from liver cancer^[3]. In addition, using the linked Surveillance, Epidemiology and End Results and Medicare dataset to estimate the annual direct and indirect costs associated with HCC, Lang *et al*^[4] pointed out the considerable economic impact of HCC on the health care system in the United States. Thus, further understanding of the causation and potential mechanisms of HCC is urgently needed.

Recently, obesity has become a worldwide health issue, because it increases the risk for a variety of human diseases. It is said that the prevalence of obesity has increased substantially over the past decade in most industrialized countries, and a further increase is expected in the future^[5]. The International Association for the Study of Obesity reported that approximately 40%-50% of men and 25%-35% of women in the EU were overweight [defined as a body mass index (BMI) between 25.0 and 29.9 kg/m²], and an additional 15%-25% of men and 15%-25% of women were obese (BMI \geq 30.0 kg/m²)^[6]. A similar observation was also found in the US population and the prevalence continues to increase despite all efforts to oppose it^[7]. Diseases which have been associated with obesity include hypertension, type 2 diabetes, dyslipidemia and coronary heart disease^[8,9]. Further evidence suggests that obesity is also a risk factor for certain types of cancer^[10].

In spite of many well-defined risk factors for HCC [including hepatitis B virus (HBV), hepatitis C virus (HCV), and alcohol], Caldwell *et al*^[11] have shown that 5%-30% of patients with HCC lack a readily identifiable risk factor. The majority of "cryptogenic" HCC in the United States is attributed to nonalcoholic fatty liver disease (NAFLD)^[12]. In addition, a number of studies have observed an increased risk (1.5 to 4-fold) of HCC among obese individuals^[13-15]. Therefore, experts have attempted to elucidate the possible events by which obesity might be linked to these diseases. Of note, it should be remembered that adipose tissue is central to the understanding of metabolic abnormalities associated with the development of obesity. In recent years, adipose tissue has been considered as an endocrine organ because of its capacity to secrete a variety of proteins with broad biological activities^[16]. These proteins, collectively referred to as adipokines, play an important role in the physiology of adipose tissue, including food intake and nutrient metabolism, insulin sensitivity, stress responses, reproduction, bone growth, and inflammation.

Leptin, the product of the obese (*ob*) gene, has undoubtedly been the most studied adipokine since this protein was first characterized by Zhang *et al*^[17] in 1994. Leptin is best known as a regulator of food intake and energy expenditure *via* hypothalamic-mediated effects. It is currently appreciated that this adipokine has many additional effects, often as a consequence of direct peripheral actions. These include angiogenesis, hematopoiesis, lipid and carbohydrate metabolism and effects on the reproductive, cardiovascular and immune systems. More im-

portantly, a recent study considered leptin as a fibrogenic factor in all types of chronic liver disease^[18]. In addition, leptin has been demonstrated to be crucial in the progression of NAFLD, the hepatic presentation of the metabolic syndrome (MS), into liver fibrosis^[19]. Thus, the aim of this review is to discuss the updated information on leptin and its receptor, the proinflammatory effects of leptin on chronic liver disease of different etiologies, and the potential impact of leptin on HCC progression. Hopefully, leptin will shed light on a new therapeutic target in HCC treatment.

LEPTIN - A VERSATILE ADIPOKINE

Leptin, the product of the *ob* gene, is mainly produced by adipose tissues and, to a lesser extent, by tissues such as the stomach, skeletal tissue and placenta^[17]. Leptin is secreted into the blood stream in a circadian rhythm and proportional to body fat mass. Although leptin serves as a regulatory mediator between the brain and the periphery through modulating the hypothalamo-pituitary-adrenal (HPA) axis, its circulating level is also regulated by hormones secreted by the HPA system, including corticosteroids, prolactin, and insulin^[20,21]. Furthermore, leptin expression can be negatively regulated by fasting, beta-adrenergic agonists and thiazolidinediones. In addition, leptin secretion is higher in females than in males for any given age and body fat mass, i.e. it is sexually dimorphic.

Leptin is known to regulate energy homeostasis^[22]. However, leptin-deficient (*db/db*) mice are not only severely obese, but also have a number of abnormalities. Research efforts have since expanded to elucidate leptin's role in human physiology and have resulted in a fundamentally renewed understanding of its role in the regulation of neuroendocrine function, reproduction, gastroduodenal mucosa defense, and metabolism of bone^[23]. Indeed, changes in plasma leptin concentrations or in leptin action have important and wide-ranging physiological implications.

Recently, mounting evidence has advocated leptin to have a regulatory function in immunity similar to the function of a pro-inflammatory cytokine. Several studies have found that circulating leptin levels increase during infection and inflammation, suggesting that leptin is part of the immune response and host defense mechanisms. Leptin levels are acutely increased by many acute phase factors, such as tumor necrosis factor (TNF), interleukin (IL)-1 and IL-6, and during bacterial infection, or lipopolysaccharide (LPS) challenge^[24]. Leptin acts on monocytes/macrophages by inducing eicosanoid synthesis, nitric oxide and several pro-inflammatory cytokines. Moreover, leptin induces chemotaxis of neutrophils and the release of oxygen radicals. The role of leptin in the innate and adaptive immune responses has also been reviewed recently^[25].

LEPTIN RECEPTOR AND ITS SIGNALING PATHWAY

The leptin receptor (OBR), belongs to the class I cytokine

receptor family (which includes receptors for IL-6, IL-12 and prolactin), and exists in at least six alternatively spliced forms with cytoplasmic domains of different length, known as OBRa, OBRb, OBRc, OBRd, OBRe and OBRf. These receptors are membrane-spanning glycoproteins with fibronectin type III domains in the extracellular region and with a shared 200-amino-acid module containing four conserved cysteine residues and two membrane proximal cytokine-like binding motifs, Trp-Ser-Xaa-Trp-Ser^[26]. Only the long form of the leptin receptor can signal intracellularly, whereas the short forms do not^[27]. The short forms of the leptin receptor are expressed by several non-immune tissues and seem to mediate the transport and degradation of leptin. The long form of OBR, known as OBRb, is expressed by the central nervous system in areas that are responsible for the secretion of neuropeptides and neurotransmitters that regulate appetite, body weight and energy homeostasis^[28]. Interestingly, OBRb is also expressed by endothelial cells, pancreatic β -cells, the ovary, CD34+ hematopoietic bone-marrow precursors, monocytes/macrophages, and T and B cells^[25].

After binding leptin, OBRb initiates a cascade of signaling events. Foremost, the receptor-associated Janus-family tyrosine kinase 2 becomes activated by auto- or cross-phosphorylation, and subsequently tyrosine phosphorylates the cytoplasmic domain of the receptor^[29]. At least three phosphorylated tyrosine residues in the cytoplasmic domain of OBRb function as docking sites for cytoplasmic adaptors: Tyr⁹⁸⁵, Tyr¹⁰⁷⁷ and Tyr¹¹³⁸. Each of these phosphorylation sites lies in a unique amino acid motif, and each of these residues thus recruits a distinct set of downstream signaling proteins when phosphorylated.

In cultured cells, phosphorylated Tyr⁹⁸⁵ recruits the SRC homology 2 domain-containing phosphatase 2 to mediate the first step in the activation of the extracellular signal regulated kinase cascade^[30]. Phosphorylated Tyr¹¹³⁸ recruits the signal transducer and activator of transcription-3 (STAT3), a latent transcription factor that then becomes phosphorylated, translocates to the nucleus, and mediates the regulation of gene expression^[31]. Tyr¹¹³⁸→STAT3 signaling promotes the expression of SOCS3, as the afferent arm of a feedback loop that attenuates OBRb signaling^[32]. The phosphorylation of Tyr¹⁰⁷⁷ promotes the recruitment, tyrosine phosphorylation and transcriptional activation of STAT5, although Tyr¹¹³⁸ may also play a minor role in the regulation of STAT5 phosphorylation^[33].

LEPTIN AND MS

Obesity, particularly abdominal obesity, is associated with resistance to the effects of insulin on peripheral glucose and fatty acid utilization, often leading to type 2 diabetes mellitus. Insulin resistance, the associated hyperinsulinemia and hyperglycemia, and the production of adipokines may lead to vascular endothelial dysfunction, an abnormal lipid profile, hypertension, and vascular inflammation, all of which promote the development of atherosclerotic cardio-

vascular disease (CVD). Therefore, the co-occurrence of metabolic risk factors for both type 2 diabetes and CVD (abdominal obesity, hyperglycemia, dyslipidemia, and hypertension) suggests the existence of a "MS"^[34].

A study by Zimmet *et al.*^[35] reported the association of leptin with fasting insulin in several populations, raising the possibility that hyperleptinemia was an additional component of the MS, or perhaps even underlay the syndrome. Subsequent study also found that leptin was strongly, positively correlated with BMI, fasting insulin, and mean blood pressure after adjusting for age and sex irrespective of glucose tolerance status. Linear regression models indicated that leptin was associated with insulin sensitivity independent of age, BMI, waist/hip ratio, triglycerides, HDL cholesterol, and hypertension^[36]. The associations between leptin and components of the MS (insulin, blood pressure, triglycerides), independent of obesity measures, suggest that leptin is more than a mere "iostat" or indicator of obesity. Thus, hyperleptinemia or leptin resistance may also be an important etiological component of the MS, either directly or *via* its influence in regulating insulin sensitivity.

ESTABLISHED RISK FACTORS OF HCC AND THEIR POTENTIAL LINKS TO LEPTIN

The established risk factors for HCC include HBV or HCV infection, alcohol intake, tobacco smoking, and aflatoxins. Their respective hepatocarcinogenesis and potential relationship with leptin will be reviewed in this section.

Chronic hepatitis B

To date, two major HBV-specific mechanisms have been indicated to contribute to HCC development. The first is the integration of the viral genome into the host chromosome causing cis-effects, resulting in loss of tumor suppressor gene functions, and/or activation of tumor-promoting genes^[37]. The second mechanism involves the expression of trans-activating factors derived from the HBV genome, which have the potential to influence intracellular signal transduction pathways and alter host gene expression. A major player involved in this form of viral transactivation is the X protein (HBx). The HBx protein has been found to display pleiotropic functions and has been implicated in the malignant transformation of chronically-infected liver cells. By disrupting cellular gene expression, viral products such as HBx may modulate cellular growth, repair and death, consequently resulting in the transformation of hepatocytes to an oncogenic state^[37,38].

Recent clinical data, which investigated serum leptin concentrations in patients with chronic viral hepatitis, indicated that cirrhotic patients due to HBV infection had significantly higher leptin levels compared to the controls, and serum leptin levels were associated with the stage of liver fibrosis. In addition, it was suggested that increased serum leptin levels might represent a negative prognostic factor for response to lamivudine monotherapy in patients

with chronic hepatitis B^[39]. Another report, which evaluated the expression of leptin and ObR in patients with chronic viral hepatitis, found that the HBV patients expressed significantly lower ObR mRNA levels in peripheral blood mononuclear cells and had decreased serum leptin levels in comparison to the healthy controls. This implied involvement of the leptin system in the immunopathology of chronic viral hepatitis^[40].

Chronic hepatitis C

Chronic HCV infection is characterized by inflammatory lesions in the liver, often accompanied by intrahepatic lipid accumulation (steatosis) and progressive fibrosis of variable degree, and long-term progression to cirrhosis and HCC^[41]. The mechanisms underlying the progression of HCV infection to HCC still remain ill-defined. Unlike HBV, HCV does not integrate into its host genome and has a predominantly cytoplasmic life cycle^[42]. Hepatocarcinogenesis of HCV, therefore, must involve several indirect mechanisms including the interplay between chronic inflammation, steatosis, fibrosis and oxidative stress and their pathological consequences. For example, the accumulation of oxidative stress and DNA damage in a setting of restricted cell cycle checkpoint control and/or accelerated cell division is thought to compromise gene and chromosome stability and to form the genomic basis for malignant transformation. Markers of intracellular oxidative stress have also been found to be increased in patients with chronic HCV infection as well as HCV core transgenic mice^[43]. In addition, several HCV proteins have been shown to have direct oncogenic effects and to up-regulate mitogenic processes^[44]. In fact, direct interactions of the various HCV proteins with host cell factors have also been shown to lead to changes in cellular signaling cascades involved in regulation of cell metabolism and division and seem to be sufficient to induce hepatocarcinogenesis. Overall, it is thought that the synergism between chronic inflammation and direct virus-host cell interactions triggers the malignant transformation of hepatocytes. The requirement for such a synergism would also explain the slow “multi-step” transformation process that underlies human HCC development^[45,46].

A previous study has demonstrated that chronic HCV infection could induce abnormal lipid accumulation in the liver^[41]. Therefore, the association of leptin with this metabolic disorder has been reviewed. Some authors showed the link between leptin and obesity as well as hepatic steatosis development in patients with chronic HCV infection, however, this observation was not found in another report^[47,48]. Likewise, higher leptin levels were shown to be associated with cirrhosis development due to chronic HCV infection^[39], but not with their histological features^[49]. These controversial results regarding the association of leptin with chronic HCV hepatitis needs further investigation.

Alcohol consumption

Chronic alcohol consumption has long been associated

with the development of hepatic cirrhosis and subsequent HCC. Many deleterious effects of alcohol have been attributed to alcohol metabolism in hepatocytes. In general, alcohol is almost metabolized by alcohol dehydrogenase (ADH) located in the cytoplasm of hepatocytes. Acetaldehyde, which forms *via* ADH-dependent alcohol metabolism, is clearly of great significance during the initiation and progression of alcohol-related liver disease. It is said that acetaldehyde can alter the integrity of DNA in a variety of ways^[50]. Chronic alcohol consumption can alter the balance of bacterial flora within the GI tract and the permeability to LPS^[51]. The vascular architecture linking the GI tract to the liver, thus, leads to increased intrahepatic LPS levels and the stimulation/activation of the liver's resident macrophage kupffer cell (KC) population^[52]. Once activated, KCs synthesize and release a range of proinflammatory cytokines, which can act in both an autocrine and paracrine manner to further activate KCs or neighboring cell populations^[53,54]. The activation of KCs and associated cytokine release may affect hepatic responsiveness to alcohol in several different ways. For example, hepatic stellate cells (HSCs) can undergo rapid activation in the presence of hepatic insult including increases in proinflammatory cytokines, oxidative stress, and/or levels of hepatotoxins^[55]. A central mechanism underlying ethanol-induced activation of HSCs is dependent on the generation of reactive oxygen species^[56].

As mentioned above, chronic alcohol consumption activates KCs and subsequently releases a number of proinflammatory cytokines. A study from India, investigating the effect of exogenous leptin and/or ethanol on the secretion of TNF- α , IL-6 and transforming growth factor (TGF)- β 1 both *in vivo* and *in vitro*, found that leptin could downregulate ethanol-induced secretion of proinflammatory cytokines and growth factor^[57]. This implies that leptin could be useful in preventing the damage produced by ethanol, which might be of therapeutic interest.

Aflatoxins

Ecological studies in the 1970s and 1980s first reported correlations between aflatoxin levels in crops or food and HCC rates^[58]. Aflatoxin B1 (AFB1) is the most common and potent of the aflatoxins. In areas of high aflatoxin exposure, up to 50% of HCC patients have been shown to harbor a specific AGG to AGT point mutation in codon 249 of the TP53 tumor suppressor gene (codon 249^{ser} mutation)^[59,60]. Interestingly, one prospective epidemiological study has shown a more than multiplicative interaction between HBV and aflatoxins in terms of HCC risk^[61]. A number of potential mechanisms have been mentioned, for example, the fixation of AFB1-induced mutations in the presence of liver regeneration and hyperplasia induced by chronic HBV infection, and predisposition of HBV-infected hepatocytes to aflatoxin-induced DNA damage^[62]. On the other hand, one recent study also suggested that aflatoxin-albumin adducts were associated with more advanced liver disease in individuals infected with HCV^[63].

In addition to being a strong carcinogen, AFB1 is also

known to evoke a decrease in food intake and body weight gain. In one *in vitro* experiment, it was demonstrated that AFB1 had a weak effect on adipocytes, but no significant influence on leptin release^[64]. Another animal model found that AFB1 could decrease food intake and body weight, and significantly depress serum leptin levels^[65].

NAFLD: A NEW RISK FACTOR FOR HCC

NAFLD is a spectrum of disorders ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis. It is believed to account for up to 90% of cases of elevated liver function tests in patients without an identifiable cause of liver disease (e.g. viral hepatitis, alcohol, inherited liver disease, and medications)^[66]. Given the fact that patients with NASH can enter a final cirrhotic pathway similar to that in patients with alcoholic cirrhosis or in patients suffering from chronic hepatitis B or C, it is not surprising that NASH appears to be a new risk factor for HCC^[67].

A close relationship between NFLD, obesity and the MS

Obesity is found in 30%-100% of subjects with NAFLD. In obese persons, steatosis is 4.6-fold higher than in normal weight persons^[66]. Clinical, epidemiological and biochemical data strongly support the concept that NAFLD is the hepatic manifestation of the MS. According to Kotronen *et al.*^[68], 90% of individuals with NAFLD have at least one risk factor for MS, and 33% have all the features of MS. In addition, liver fat content is significantly increased in subjects with the MS as compared with those without the syndrome, independently of age, gender, and BMI. The presence of multiple metabolic disorders such as diabetes mellitus, obesity, dyslipidemia and hypertension is associated with a potentially progressive, severe liver disease^[69].

Pathogenesis of NAFLD

Insulin resistance, oxidative stress, and an inflammatory cascade are believed to play integral roles in the pathogenesis and progression of NAFLD^[70]. In insulin-resistant states, adipose and muscle cells preferentially oxidize lipids, resulting in the release of FFA. FFA can then be taken up by the liver, resulting in steatosis. Animal studies show that FFA, once released from muscle and adipose cells, can be incorporated into triglycerides in the liver or undergo oxidation in mitochondria, peroxisomes or microsomes. Oxidized by-products are harmful adducts that can cause liver injury, resulting in subsequent fibrosis^[71]. Lipid peroxidation and oxidative stress result in increased production of hydroxynonenal and malondialdehyde that upregulate liver fibrosis *via* activation of stellate cells and result in increased production of TGF- β ^[72].

Recently, scientists have focused on the role of KCs in the pathogenesis of NAFLD. KCs are the resident macrophages of the liver and function in both innate and adaptive immunity as active phagocytosing agents and antigen-presenting cells (*via* toll-like receptors, among others) to T-cells. While inactivation of KCs is associated

with NAFLD and impaired hepatic regenerative capacity, elimination of resident KCs has been associated with improvement of NASH, suggesting that overactivation of a Kupffer-cell-mediated immune response might underlie liver injury in NAFLD. It is thought that KC physiology becomes altered in the setting of increased hepatic lipid content possibly due to overcrowding of liver sinusoids resulting in prolonged exposure of KCs to antigens, reduced KC outflow, and a resulting sustained inflammatory response. Uncoupling proteins, are molecules that dissipate the proton gradient in the inner mitochondrial membrane and thereby reduce the energy needed for oxidative phosphorylation. Insufficient uncoupling protein production in KCs, possibly due to LPS-induced activity, might contribute to the pathogenesis of NAFLD^[73].

Leptin in the pathogenesis of NAFLD

Leptin is thought to participate in the development of NAFLD. In animal models of NAFLD, leptin contributes to the development of insulin resistance and subsequently steatosis. Furthermore, in the context of liver insult, leptin has a proinflammatory role and is considered to be an essential mediator of liver fibrosis. In rats treated with carbon tetrachloride (CCl₄), leptin injections have been shown to result in the increased expression of procollagen-I, TGF β 1 and smooth muscle actin, a marker of activated HSCs, and eventually to increased liver fibrosis^[74].

In human studies, leptin levels were initially found to be significantly higher in 47 NASH patients than in 47 controls and correlated with the severity of hepatic steatosis but not to necroinflammation or fibrosis^[75]. A subsequent study showed that leptin levels are significantly higher in NASH patients than in patients with chronic viral hepatitis and correlate with more severe fibrosis in univariate analysis^[76]. However, another study failed to show any significant difference in leptin levels between NASH patients and controls or any independent association with liver fibrosis^[77]. Thus, it is doubtful whether leptin is up-regulated in patients with NASH and larger studies with a homogenous population and carefully matched healthy controls are needed. For the time being, leptin cannot be used as a noninvasive marker for the diagnosis of NASH.

LEPTIN PLAYS A FIBROGENIC ROLE IN THE LIVER

The development of fibrosis, which is critical for the progression of all chronic liver diseases, comprises a series of events, including inflammation, activation of fibrogenic myofibroblasts (e.g. HSCs), deposition of fibrillar extracellular matrix, and possibly neo-angiogenesis^[78]. The data on the role of leptin in the regulation of these steps have been accumulating rapidly. Several *in vivo* studies, which evaluated the effect of leptin in animal models of chronic liver injury, including dietary steatohepatitis, bile duct ligation, and infection with eggs of *Schistosoma mansoni*, provided obvious support to the role of leptin as a critical mediator of fibrosis^[79-81]. Importantly, when assessing

the response of ob/ob mice, the decreased fibrogenic response was reverted by supplementation with recombinant leptin. These findings suggest that leptin is a critical factor for the development of fibrogenesis in rodents.

Different cell types have been mentioned to participate in the response of leptin to liver injury, including KCs, sinusoidal endothelial cells and myofibroblast-like cells, which are derived from the activation of HSCs and from other mesenchymal cells. Ikejima *et al.*^[82] demonstrated that ObRb is expressed by sinusoidal endothelial cells and KCs, where exposure to recombinant leptin up-regulates the expression of TGF- β . A number of successive studies also indicated that HSCs express functional ObRb and are directly responsive to leptin. Expression of ObRb is low in quiescent HSCs, and increases during the activation process, suggesting that in activated HSCs the effects of leptin are amplified^[83]. In addition, incubation of HSCs with recombinant leptin stimulates the expression of type I procollagen, potentiates the effects of TGF- β , and up-regulates expression of the tissue inhibitor of metalloproteinase-1, thus blocking collagen degradation^[84,85].

A recently identified effect of leptin on fibrogenic cells is the induction of vascular endothelial growth factor (VEGF) *via* oxygen-independent activation of hypoxia-inducible factor 1 α , a master switch of the angiogenic response^[86]. These observations may potentially have an impact on liver fibrosis, as formation of new blood vessels is a key component of the wound-healing response and has been suggested to play a role in the irreversibility of established cirrhosis. Together, these observations suggest the fibrogenic role of leptin in the liver.

EVIDENCE REGARDING THE ASSOCIATION OF LEPTIN WITH HCC DEVELOPMENT

Yang *et al.*^[87] first explored whether obesity might increase the risk for HCC, and found that ob/ob mice developed liver hyperplasia at the earliest stage of NAFLD and eventually HCC. This observation raised the intriguing issue that obesity-related fatty liver, itself, might be a premalignant condition. A number of studies have attempted to elucidate the possible effects of leptin in HCC development. Wang *et al.*^[88], who investigated whether leptin might be involved in the etiology of HCC in cirrhotic patients, found that increased serum leptin was significantly correlated with cirrhotic change, but not with HCC occurrence. This finding was consistent with previous studies, indicating the fibrogenic effect of leptin in the liver. Another study, which evaluated the expression of leptin and its receptor in HCC specimens and adjacent non-tumorous tissues, first pointed out the involvement of leptin in the carcinogenesis of HCC^[89]. However, the authors suggested further investigations should be carried out to define the inhibitory and/or activating role of leptin in the process of carcinogenesis and progression of human HCC.

A recent study found that, without leptin signaling,

neither fibrosis nor HCC developed in the rat NASH experimental model, suggesting that leptin might play a pivotal role in the progression of fibrogenesis and carcinogenesis in NASH^[90]. Further *in vitro* assay demonstrated the necessity of leptin-mediated neo-vascularization coordinated with VEGF in this progression. The involvement of leptin/OBR in the angiogenesis of human HCC was also shown in a recent study^[90]. Notably, one *in vitro* assay demonstrated the proliferative and anti-apoptotic effects of leptin in HCC cells *via* Janus kinase 2-linked signaling^[91]. Taken together, these findings implied that leptin-induced effects implicated in HCC development seemed to be inhibitory.

In fact, several studies have reported the contradictory effects of leptin in HCC growth. Elinav *et al.*^[92] first showed that exogenous leptin significantly decreased tumor size and improved survival rate in a murine model of HCC. The authors further demonstrated that the majority of these leptin-induced inhibitory effects might be mediated by the induction of natural killer cell proliferation and activation. Moreover, Wang *et al.*^[93,94], who evaluated the expression of leptin and its receptor in HCC specimens by immunostaining, further correlated the expression profile with Ki-67 expression, intratumor MVD, as well as overall survival, provided clinical evidence on the prognostic roles of leptin and OBR in HCC patients. First, OBR expression was inversely correlated with vascular invasion of HCC. Furthermore, high leptin expression was associated with better survival in patients with HCC, treated postoperatively with medroxyprogesterone acetate, a synthetic variant of human progesterone. As a result, it was suggested that both high leptin and OBR expression in HCC tissues could predict better overall survival.

CONCLUSIONS AND FUTURE DIRECTIONS

Leptin has an increasingly crucial role in a variety of human metabolic disorders and cancers. This parallels the increasing prevalence of NAFLD in patients with HCC. This work reviews the updated information on leptin, including its receptor and related signaling pathway, and provides important insight into the association between leptin and the MS and NAFLD as well as well-known risk factors for HCC. Moreover, research studies have demonstrated that leptin can exert a fibrogenic effect in the liver. In addition, evidence regarding the direct link between leptin and HCC development has been accumulating rapidly. This demonstrates the potential of the leptin-mediated effects in the carcinogenesis and progression of HCC, however, there is ample room for further research on its inhibitory and/or activating role. In addition, the role of leptin in the response of HCC to hormonal therapy deserves further research.

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Reproductive changes associated with celiac disease

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Abstract

Celiac disease is a mucosal disorder of the small intestine that may be triggered by dietary exposure to gluten in genetically-susceptible individuals. The disorder is often associated with diarrhea, malabsorption and weight loss along with other extra-intestinal complications. Reproductive changes have been described, including impaired fertility and adverse pregnancy outcomes possibly related to immune-mediated mechanisms or nutrient deficiency. Other possible pathogenetic factors that may alter placental function include maternal celiac disease autoantibodies binding to placental transglutaminase, and genetic mutations that may facilitate microthrombus formation. Reports noting activation during pregnancy or the puerperium may be important, and suggest that celiac disease may also be hypothetically precipitated by maternal exposure to one or more fetal antigens.

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Key words: Celiac disease; Infertility; Pregnancy; Post-partum celiac disease; Fetal outcome

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INTRODUCTION

Celiac disease is an immune-mediated mucosal disorder primarily affecting the small intestine in genetically-susceptible individuals^[1]. It may be triggered by dietary exposure to gluten, and frequently causes chronic diarrhea, malabsorption and weight loss^[1]. In some patients, extra-intestinal or autoimmune changes may occur, e.g. hepatobiliary^[2], neurological^[3], or endocrine disorders, such as hypothyroidism and insulin-dependent diabetes in children^[4]. Of particular interest has been the effect of celiac disease and its treatment on fertility and pregnancy^[5]. In recent years, there has been an increased recognition of possible changes in male and female fertility in celiac disease as well as the potential for adverse outcomes in pregnancy and the post-partum period that may lead to miscarriages and premature low birth weight fetal deliveries (Table 1).

ALTERATIONS IN FEMALE FERTILITY

Celiac disease continues to be increasingly recognized as a clinically silent disorder with limited or few intestinal symptoms, such as mild diarrhea. Often, females with reproductive disorders or pregnancy complications have no overt symptoms, or at most, fatigue associated with iron-deficiency anemia. As a result, reduced fertility in females or changes that include delayed menarche, amenorrhea and early menopause may conceivably be the initial clinical feature that ultimately results in a diagnosis of celiac disease. As serological screening has resulted in an appreciation that celiac disease may occur in up to 1%-2% of the general population, it is not surprising that this disorder is more readily detected in young women of childbearing age. Indeed, young women are still the most common

Table 1 Reproductive changes in celiac disease

Altered female fertility	Delayed onset of menarche, amenorrhea, early menopause, recurrent abortions, reduced rates of pregnancy
Altered male fertility	Gonadal dysfunction, altered sperm morphology and motility, reduced sexual activity
Alterations in pregnancy	Repeated miscarriages, premature delivery and impaired fetal growth with low birth weight, abnormal placental function
Postpartum activation of celiac disease	Hormonal or immune changes

group diagnosed with celiac disease. If changes in fertility can be documented in celiac disease, these may reflect underlying autoimmune complications of celiac disease or the negative nutritional effects of untreated disease.

Although some early case reports noted a possible association between celiac disease and infertility^[6,7], systematic investigations have been limited and the precise definition of infertility *per se* may vary between studies. An extensive serological evaluation of 150 women with infertility from Finland demonstrated an apparently increased rate of celiac disease (i.e. overall rate, 2.7%)^[8]. Similar results were later reported in 99 couples from Northern Sardinia (i.e. 3.03% of females)^[9], and later, using more modern serological screening methods [i.e. tissue transglutaminase (tTGA), endomysial (EMA) antibodies] in 192 Arab women with unexplained infertility from Israel (i.e. 2.65%)^[10]. In all three of these studies, small bowel biopsies were positive if abnormal serological screening results were present. As in most screening studies, however, biopsies in the serologically-negative screened populations were not done. Moreover, other studies have suggested that the evidence may not be quite as strong for a definite association with celiac disease. In a report from another center in Finland^[11], a higher frequency of celiac disease in women with infertility or recurrent miscarriage could not be defined. A Czech study showed increased seropositivity in women with infertility, but unfortunately, biopsies were not reported^[12]. Finally, in a selected Italian cohort of infertile women undergoing assisted reproduction techniques, a statistically significant result was not achieved^[13].

Delayed onset of menarche, amenorrhea, early menopause, recurrent abortions and reduced rates of pregnancy in celiac disease may reflect an impairment of fertility. In 74 celiac patients from the United Kingdom^[5], the reproductive period was longer for those on a gluten-free diet compared to those not on a diet but maternal health was not seriously impaired. A lower incidence of spontaneous abortions in celiacs on a gluten-free diet was also recorded. Similar results were reported in an Italian study^[14]. In consecutively diagnosed celiacs compared to age- and "sexual behavior"-matched healthy controls, there was a significant delay in the mean age of menarche in untreated celiac patients (13.5 years compared to 12.1 years). Amenorrhea and repeated abortions were more common in the celiac group, but onset of menopause did not significantly differ. Studies from Poland and Italy^[15,16] also evaluated menarcheal age of celiac girls with reference to mater-

nal menarcheal age. In one^[15], menarcheal age of celiac girls appeared to be regulated by a gluten-free diet, while in the other^[16], menarcheal age in celiac disease was not delayed, but was affected by maternal menarcheal age. A further evaluation from the United Kingdom^[17] suggested that celiacs are subfertile with an increased incidence of stillbirths and perinatal deaths. However, after diagnosis of celiac disease and treatment with a gluten-free diet, some markers of infertility (e.g. miscarriage rates) may be corrected. Finally, in a study from Brazil^[18], adherence to a gluten-free diet and resultant nutritional status was emphasized as an important and relevant factor in reproductive disorders developing in untreated celiac disease.

Nutritional studies in celiac disease during pregnancy are very limited. For example, zinc, selenium and folic acid deficiency have been noted in some studies^[19-21], but most of these have been completed in children so that these studies do not appear to offer a definitive explanation for altered fertility in women during their reproductive years with untreated disease. Others have offered contradictory data in untreated celiac disease: reduced vitamins and trace elements were not evident, or significant malnutrition was not present^[7-10]. In another report, evidence of poor vitamin status in celiacs despite a gluten free diet was reported^[22], but this was contradicted by a detailed and more recent and important evaluation that documented histological recovery^[23]. An alternative mechanism for reduced fertility may be immune-mediated, possibly by compromising placental function^[24].

Clearly, further studies are needed to precisely define the role of altered absorption and resultant nutritional changes on female fertility in untreated celiac disease as well as the effects of a gluten-free diet, especially with restoration of normal nutritional status. In addition, immune-mediated changes in placental function need to be explored in celiac disease.

ALTERATIONS IN MALE FERTILITY

Studies estimating prevalence of male infertility in celiac disease have been rare. In an infertile couples study from Northern Sardinia^[9], a single male out of 99 (or about 1%) tested positive for celiac antibodies, including EMA. Later the typical small bowel biopsy findings of untreated celiac disease were detected. Although the prevalence in a comparable control population was not provided, it is likely that an effective evaluation for infertility in a couple would best include assessment of both sexes for underlying celiac disease.

Early studies from the United Kingdom on male gonadal dysfunction described a reversible state of androgen resistance in celiac disease^[25]. Later, in a further series of studies^[26-29] on male gonadal function, consecutive males with celiac disease were evaluated and compared to males of similar age and nutritional status with Crohn's disease. Almost 20% of married celiacs had infertile marriages^[26]. Semen analysis revealed marked abnormalities in sperm morphology and motility, similar to Crohn's disease, with sperm morphology apparently improving following removal of dietary gluten^[26]. Others reported the presence

of oligospermia^[27]. Specific nutrient deficiencies and detection of anti-sperm antibodies did not appear to be a factor in male infertility^[26]. Plasma hormone levels were also determined^[28]. Plasma testosterone and free testosterone index were increased while dihydrotestosterone was reduced. These hormone levels appeared to normalize, with an improved small bowel architecture on a gluten-free diet. Serum luteinizing hormone was also raised and interpreted to reflect androgen resistance. These endocrine changes, suggestive of androgen resistance and hypothalamic-pituitary dysfunction, were interpreted to be relatively specific to celiac disease, but an association with disordered spermatogenesis was not determined^[28]. Further studies of gonadotropins were also performed^[29]. Exaggerated gonadotrophin responses were apparently unrelated to plasma concentrations of testosterone, dihydrotestosterone, estradiol or the free testosterone index. Elevated prolactin levels were also noted but these were not related to impotence or infertility. These studies that suggested deranged pituitary regulation of gonadal function in celiac disease in males were hypothesized to be part of a wider disturbance of central regulatory mechanisms of endocrine function in celiac disease. Interestingly, less significant, but similar alterations in male sex hormone status occur in dermatitis herpetiformis^[30], a skin disorder closely linked to celiac disease. These changes possibly also reflect, in part, the recognition of an autoimmune pituitary process associated with celiac disease and reported to be directly associated with an impairment in linear growth^[31].

As many of these studies appeared almost three decades ago, it is striking that very little additional new, even descriptive, information on male infertility in celiac disease has appeared. However, two more recent Italian publications have explored sexual behavior^[32,33]. In one study, sexual behaviors in treated and untreated celiac disease patients were examined using a questionnaire and compared to healthy controls^[32]. Sexual satisfaction, including frequency of intercourse, was reduced in celiac patients, but improved after a year of treatment with a gluten-free diet. In the other^[33], sexual habits appeared to be very different in celiacs who were never treated with a gluten-free diet.

ALTERATIONS IN PREGNANCY

Celiac disease, especially if untreated, appears to increase the risk of repeated miscarriages and premature deliveries, and impaired fetal growth with reduced birthweight^[34]. In addition, adverse effects on the mother may also occur, as indicated by a recent German study which demonstrated that the rate of cesarean delivery was increased if the parents had celiac disease compared to other digestive disease controls as well as controls from eye or dental outpatient clinics^[35].

In a case-control study from Italy that evaluated 94 untreated and 31 treated celiacs, the relative risks of either abortion or delivering a low birthweight baby were increased while the duration of breast feeding was significantly reduced^[36]. All of these changes were apparently

corrected with a gluten-free diet^[36]. Higher incidences of either miscarriages or spontaneous abortions were also recorded from other centers located in different countries including Argentina, Italy and the United Kingdom^[5,14,17,37].

Reduced birthweight and intrauterine growth retardation have also been recorded in several other studies from different European centers^[38-41]. In one of these studies from Italy^[39], the investigators also noted that celiac disease was a more common disorder than most of the diseases normally screened for in pregnant women in their healthcare facility. In another of these studies^[40], undiagnosed maternal celiac disease appeared to be a far greater risk factor than diagnosed celiac disease, but in a subsequent report, undiagnosed celiac disease was not associated with an unfavorable outcome of pregnancy^[42]. Other studies noted that paternal celiac disease did not appear to be a risk for an adverse pregnancy outcome^[43,44].

Mechanisms involved in the impairment of pregnancy outcome in celiac disease have been explored to a limited degree. The placentas in mothers affected with celiac disease appear to be abnormal. In particular, tTG expression and apoptosis were reported to be increased in trophoblast cells using immunohistochemical analysis and *in situ* hybridization methods, suggesting a possible mechanism of injury in both the fetal and maternal parts of the placenta^[45]. Others have noted that maternal celiac disease autoantibodies bind directly to the syncytiotrophoblast and inhibit placental tissue transglutaminase activity suggesting a possible mechanism for compromised placental function^[24]. Furthermore, recent studies in celiac women on the role of genetic prothrombin variants in early pregnancy loss suggested that the 4G variant of the plasminogen activator inhibitor-1 gene may predispose to miscarriage^[46]. Early pregnancy loss could conceivably relate to some alteration in coagulation affecting placental or fetal microvascular function. Additional studies are needed to further explore and elucidate these mechanisms.

FURTHER CONCLUDING ISSUES

Interestingly, delivery of a preterm infant has been conversely linked to later definition of underlying celiac disease in the parent, particularly underlying maternal celiac disease. In 905 preterm infants born in Lombardy, Italy, 1714 parents (868 women, 846 men) were screened for celiac disease using EMA and tTGA (followed by duodenal biopsy confirmation). In these, a higher prevalence of celiac disease in mothers of low birthweight infants was defined^[47]. Thus, selective screening for celiac disease may be useful. However, the potential value and cost-effectiveness of screening for celiac disease in all women of reproductive age has not been fully determined.

Also intriguing is the repeated, but uncommon, definition of underlying maternal celiac disease following delivery in the puerperium^[48-51]. Activation of celiac disease during the puerperium has been hypothesized to be related to immunologic or hormonal factors, or both^[48]. Occasionally, anemia is evident during the prior pregnancy^[49] or the presentation may be acute^[50] leading to speculation

that activation of celiac disease may result from maternal exposure to fetal antigens^[50]. In addition, presentation of underlying celiac disease may not be evident during an initial pregnancy, but may only appear after a second or later pregnancy^[51]. Additional studies that explore the specific immunological changes occurring during the post-partum period may shed additional light on immune-mediated changes that occur in unrecognized celiac disease.

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Toll-like receptor 9 gene mutations and polymorphisms in Japanese ulcerative colitis patients

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Abstract

AIM: To investigate gene mutations and polymorphisms of *TLR9* in Japanese ulcerative colitis (UC) patients.

METHODS: Three single nucleotide polymorphisms (SNPs) in *TLR9* were identified in healthy controls, and were assessed in 48 UC patients and 47 healthy controls. Control subjects were matched for age, sex and date of blood sampling from among a subgroup of participants.

RESULTS: *TLR9* -1486CC, 1174GG and 2848AA increase the risk of UC [odds ratio (OR) 2.64, 95% confidence interval (95% CI): 1.73-6.53, $P = 0.042$], and *TLR9* -1486TT, 1174AA and 2848GG decrease the risk of UC (OR 0.30, 95% CI: 0.10-0.94, $P = 0.039$), although there were no correlations between SNPs and disease phenotype or *TLR9* mRNA expression.

CONCLUSION: *TLR9* polymorphisms are associated with increased susceptibility to UC.

INTRODUCTION

Inflammatory bowel diseases (IBDs), which include ulcerative colitis (UC) and Crohn's disease (CD), are chronic inflammatory disorders of the digestive tract. Although the pathogenesis of IBDs is complex and remains unclear, it has been suggested that immunologic, environmental and genetic factors contribute to their etiology^[1]. Both clinical studies of IBD and studies of animal colitis models implicate luminal bacteria as necessary for initiating and perpetuating intestinal inflammation^[2,3].

Among several candidate IBD-related chromosomal regions and genes, the caspase recruitment domain 15 (*CARD15*) gene coding for the nucleotide oligomerization domain 2 (*NOD2*) gene was identified as having the strongest linkage for CD susceptibility^[4,5]. *NOD2/CARD15* is an intracytoplasmic receptor that binds bacterial peptidoglycan-derived muramyl dipeptide. While three common variants of this gene (R702W, G908R and L1007fsinsC) have been reported in CD patients in Western countries, there were no variants found in Japanese patients^[6].

Similarly to NOD2, Toll-like receptors (TLRs) are essential components of innate immunity that recognize microbial compounds from bacteria, fungi and viruses^[7-9]. While TLR activation leads to transcription of inflammatory and immunoregulatory genes, recent studies have demonstrated that TLR signaling in intestinal sites can inhibit inflammatory responses and maintain colonic homeostasis^[10-12]. *TLR9*, which recognizes unmethylated CpG DNA in bacteria and viruses^[13,14], and its signaling pathway protect mice from experimental colitis through production of type I interferons (IFN)^[15]. Therefore, we need to know whether *TLR9* is a protective molecule not only in a mouse model but also in human IBD.

As the relationships between UC and *TLR9* gene variation have not been reported to date, we focused on *TLR9* gene mutations or polymorphisms in UC. Our results demonstrate that *TLR9* genetic polymorphisms are associated with an increased risk of UC in the Japanese population.

MATERIALS AND METHODS

Human subjects

After obtaining written informed consent based on the Declaration of Helsinki of the World Medical Association, 48 UC patients and 47 healthy controls were enrolled in this study. All subjects were Japanese, and visited our hospital between December 2005 and January 2007. UC diagnoses were confirmed by review of medical charts by clinicians. Standard clinical, endoscopic and histological criteria were used^[16-18]. The following clinical characteristics were analyzed: sex, age at diagnosis, disease location and disease severity. Control subjects were matched for age, sex and date of blood sampling from a subgroup of participants who were healthy volunteers, free from IBD and malignant tumors. Clinical characteristics of these subjects are shown in Table 1. This study was approved by the Ethics Committee of Fukushima Medical University.

Quantitative real-time reverse transcription-polymerase chain reaction

Peripheral blood mononuclear cells (PBMC) from UC patients and healthy controls were isolated from heparinized blood by Ficoll Paque density-gradient centrifugation (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway). Total cellular RNA was extracted from isolated PBMC using TRIzol[®] Reagent (Invitrogen, Carlsbad, CA, USA).

cDNA was generated by using the HotStarTaq Master Mix Kit (QIAGEN GmbH, Hilden, Germany). Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using a TaqMan[®] fast universal PCR master mix (Applied Biosystems, Foster City, CA, USA) and on-demand gene-specific primers, assessed using StepOne[™] real time PCR system (Applied Biosystems). The primers were as follows: *TLR9* (Hs00370913_s1) and *GAPDH* (Hs02786624_g1). Both primers were purchased from Applied Biosystems. Rela-

Table 1 Basic subject data (mean \pm SD) *n* (%)

	UC (<i>n</i> = 48)	Controls (<i>n</i> = 47)
Age (yr)	43.49 \pm 15.48	41.21 \pm 15.36
Sex (M/F)	21 (43.8)/27 (56.2)	21 (44.7)/26 (55.3)
Age at diagnosis (yr)	35.07 \pm 14.39	
Disease duration (yr)	8.42 \pm 8.49	
Familial disease	0 (0)	0 (0)
Disease location		
Proctitis	3 (6.25)	
Left colitis	27 (56.3)	
Right colitis	1 (2.1)	
Pancolitis	17 (35.4)	
Disease severity		
Light	17 (35.4)	
Mild	18 (37.5)	
Severe	13 (27.1)	
History of colectomy	5 (10.4)	
History of immunosuppressant	6 (12.5)	
History of colon cancer	3 (6.3)	

UC: Ulcerative colitis.

tive quantification was achieved by normalizing to the values of the *GAPDH* gene.

SNP discovery

Genomic DNA from UC patients and healthy controls was extracted from heparinized whole blood samples using the PUREGENE[®] DNA purification kit (Gentra Systems, Minneapolis, MN, USA).

All exons, introns and approximately 1500 bases of the 5'-flanking region and 1500 bases of the 3'-flanking region of the *TLR9* gene were amplified from the genomic DNA of one healthy control. The genomic sequences were based on the GenBank reference sequence NM_017442 and AC097637.2 for *TLR9*. The subject was selected at random. Thirteen *TLR9* gene segments were amplified by PCR with the primer pairs shown in Table 2, Taq DNA polymerase (QIAGEN GmbH, Hilden, Germany) and genomic DNA from a healthy control as a template. Amplified products were purified using the GFX[™] PCR DNA and Gel Band Purification Kit (Amersham Biosciences UK Limited, Buckinghamshire, UK), and were subjected to direct sequencing with the GenomeLab[™] DTCS-Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) on a CEQ2000 DNA Sequencer (Beckman Coulter). SNP offsets were calculated relative to the A base of the *TLR9* ATG start codon, such that SNPs in the promoter region upstream of the first intron have negative position numbers.

Genotyping

Three common SNPs (-1486T/C, 1174A/G, 2848G/A) were analyzed for genotyping. The -1486T/C SNP was genotyped by restriction fragment length polymorphism (RFLP). Briefly, genomic DNA fragments containing -1486T/C SNP were amplified by PCR. PCR products were digested with the restriction enzyme Afl II (TAKARA SHUZO Co., Ltd., Shiga, Japan), run on a 3% aga-

Table 2 Primers used in polymerase chain reaction for genotyping *Toll-like receptor 9* polymorphisms and three single nucleotide polymorphisms

Fragment (product)	Forward	Reverse
<i>TLR9</i> polymorphisms		
-2109 to -1293 (817 bp)	5'-CCAAGGGACTCTGGGAAAG-3'	5'-CATGTCACCCCTCTCAACAGGG-3'
-1626 to -1095 (532 bp)	5'-CAGCCTTCACTCAGAAATACCC-3'	5'-GGCCAACAAGGCCCTATG-3'
-1296 to -553 (662 bp)	5'-CATGGGAGCAGAGACATAATG-3'	5'-GCCAGGGGTAGCTTGA-3'
-795 to -134 (662 bp)	5'-GAGTCTCTCACCTAGATCAG-3'	5'-TATACCAGCCTAGTAGC-3'
79 to 962 (884 bp)	5'-CTGCAAGCAACAGTGACGG-3'	5'-AGCTTTCACTTAACCAATCCC-3'
-393 to 1698 (2092 bp)	5'-TACCCGCTACTGGTGCTATC-3'	5'-TGGCAGAGTCTAGCATCAGG-3'
996 to 1687 (692 bp)	5'-CTGGTTCTGAAGCCTAATTC-3'	5'-AGCATGAGGATGTTGGTATGG-3'
976 to 1899 (924 bp)	5'-CTGGATTCTAGGTCTCAGTCC-3'	5'-CTGGATTCTAGGTCTCAGTCC-3'
1540 to 2368 (829 bp)	5'-CCTGCCACATGACCATCGAG-3'	5'-CCTGCCACATGACCATCGAG-3'
1812 to 2520 (709 bp)	5'-AACCTCACCCACCTGTCAC-3'	5'-AACCTCACCCACCTGTCAC-3'
2407 to 3350 (944 bp)	5'-TGCAGATGAACCTCATCAACC-3'	5'-GCTGTTGCAGCTGACATC-3'
3267 to 4057 (791 bp)	5'-CAGGAAACCAGCTGAAGG-3'	5'-CAGGAAACCAGCTGAAGG-3'
3867 to 4701 (835 bp)	5'-GACTGGGTGTACAACGAGCTT-3'	5'-TCTGCATGGGAAAGGTAGG-3'
SNP		
-1486	5'-CAGCCTTCACTCAGAAATACCC-3'	5'-GGCCAACAAGGCCCTATG-3'
1174	5'-CTGGTTCTGAAGCCTAATTC-3'	5'-AGCATGAGGATGTTGGTATGG-3'
2848	5'-TGCAGATGAACCTCATCAACC-3'	5'-GCTGTTGCAGCTGACATC-3'

TLR9: Toll-like receptor 9; SNP: Single nucleotide polymorphism.

Table 3 Allele frequencies in ulcerative colitis cases and disease-free controls for *Toll-like receptor 9* single nucleotide polymorphisms at -1486, 1174, 2848

SNP ¹	Allele	UC (n = 48)	Controls (n = 47)	dbSNP ²	JSNP ³
-1486	T/C	0.344/0.656 ^a	0.532/0.468	0.647/0.353	ND
1174	A/G	0.344/0.656 ^a	0.532/0.468	0.512/0.488	0.517/0.483
2848	G/A	0.354/0.646 ^a	0.543/0.457	0.481/0.519	0.517/0.483
					^a P = 0.013

¹Positions were calculated taking the A of the Toll-like receptor 9 (TLR9) ATG start codon as position 1 based on Genbank Accession No. NM_017442; ²dbSNP public database: <http://www.ncbi.nlm.nih.gov/SNP/>; ³JSNP is a database of Japanese SNPs: http://snp.ims.u-tokyo.ac.jp/index_ja.html; ^aP value by Fisher's Exact test, cases *vs* controls. SNP: Single nucleotide polymorphisms; UC: Ulcerative colitis; ND: No data.

rose gel and subsequently stained with ethidium bromide to visualize the bands. The other two SNPs (1174A/G and 2848G/A) were confirmed by direct sequencing. The primers used for PCR or sequencing are shown in Table 2.

Statistical analysis

Statistical analyses were performed using SPSS version 11.0.1 and SNPalyze version 6.0 (Dynacom Co., Ltd. Yokohama, Japan). Cases and controls were compared using Fisher's exact test (Tables 3-6) for categorical items. Simple logistic regression models were used to analyze Genotype-Diplotype associations. The results are expressed as odds ratio (OR) with corresponding 95% confidence intervals (95% CI). The level of significance was set at 5%. We examined Lewontin's D' (|D'|) and the linkage disequilibrium coefficient r^2 between each pair of SNPs using the expectation-maximization (EM) algorithm in SNPalyze software.

RESULTS

TLR9 mRNA expression

In order to confirm whether there were differences in

TLR9 expression, we performed quantitative RT-PCR of *TLR9* using PBMC from UC patients and healthy controls. There were no significant differences in *TLR9* mRNA expression (Figure 1).

SNPs and their allele frequencies

We sequenced both exons and approximately 1500 bases of the 5'- and 3'-regions of *TLR9* in one healthy control. Offsets for each SNP were calculated relative to the transcription start site of *TLR9* mRNA (GenBank Accession No. NM_017442). We identified three SNPs in the regions sequenced. Their locations in chromosome 3 and in the *TLR9* gene are illustrated in Figure 2. These SNPs were confirmed in dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>) and JSNP (http://snp.ims.u-tokyo.ac.jp/index_ja.html). The remainder of the analysis will focus on these three relatively common SNPs.

We found a marginally significant association between the SNPs at -1486, 1174, 2848 and UC. Elevated risk for UC was associated with a C allele at -1486 (Fisher's exact test, $P = 0.013$), a G allele at 1174 (Fisher's exact test, $P = 0.013$), and an A allele at 2848 (Fisher's exact test, $P = 0.013$) (Table 3).

Table 4 Genotype frequencies in ulcerative colitis cases and disease-free controls for *Toll-like receptor 9* single nucleotide polymorphisms at -1486, 1174, 2848 *n* (%)

Genotype at <i>TLR9</i>		UC (<i>n</i> = 48)	Controls (<i>n</i> = 47)
-1486	CC	20 (41.7) ^a	10 (21.3)
	CT	23 (47.9)	24 (51.1)
	TT	5 (10.4) ^a	13 (27.7)
1174	GG	20 (41.7) ^a	10 (21.3)
	GA	23 (47.9)	24 (51.1)
	AA	5 (10.4) ^a	13 (27.7)
2848	AA	19 (39.6) ^a	9 (19.1)
	AG	24 (51.1)	25 (53.2)
	GG	5 (10.4) ^a	13 (27.7)

^a*P* < 0.05^a*P* value by Fisher's exact test, ulcerative colitis (UC) cases *vs* controls. *TLR9*: *Toll-like receptor 9*.**Table 5** Haplotype frequencies in ulcerative colitis cases and disease-free controls for *Toll-like receptor 9* single nucleotide polymorphisms at -1486, 1174, 2848 *n* (%)

Haplotype			UC (<i>n</i> = 96)	Controls (<i>n</i> = 94)
-1486	1174	2848		
C	G	A	62 (64.6) ^a	43 (45.7)
T	A	G	33 (34.4) ^a	50 (53.2)
C	G	G	1 (1.04)	1 (1.06)

^a*P* < 0.05^a*P* value by Fisher's exact test, ulcerative colitis (UC) cases *vs* controls.**Table 6** Diplotype frequencies in ulcerative colitis cases and disease-free controls for *Toll-like receptor 9* single nucleotide polymorphisms at -1486, 1174, 2848 *n* (%)

Diplotype			UC (<i>n</i> = 48)	Controls (<i>n</i> = 47)
-1486	1174	2848		
CC	GG	AA	19 (39.6) ^a	9 (19.1)
CC	GG	AG	1 (2.1)	1 (2.1)
CT	GA	AG	23 (47.9)	24 (51.1)
TT	AA	GG	5 (10.4) ^a	13 (27.7)

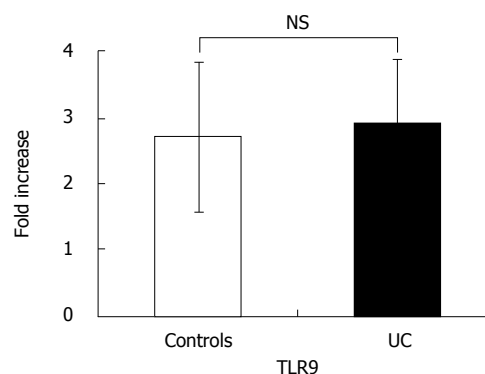
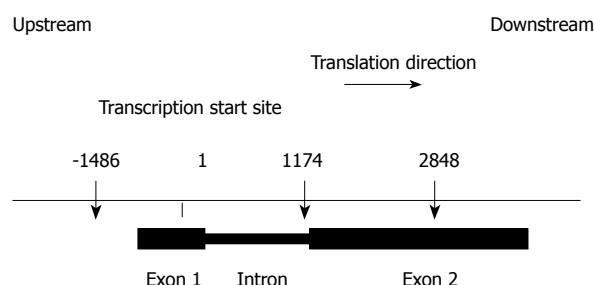
^a*P* < 0.05^a*P* value by Fisher's exact test, ulcerative colitis (UC) cases *vs* controls.

Genotype frequencies

We then analyzed genotype frequencies between UC patients and healthy controls. As shown in Table 4, a pattern of increased prevalence of CC genotypes at -1486 (Fisher's exact test, *P* = 0.047), GG genotypes at 1174 (Fisher's exact test, *P* = 0.047), AA genotypes at 2848 (Fisher's exact test, *P* = 0.042), and a decreased prevalence of TT at -1486 (Fisher's exact test, *P* = 0.039), AA genotypes at 1174 (Fisher's exact test, *P* = 0.039), GG genotypes at 2848 (Fisher's exact test, *P* = 0.039) was observed in UC patients, as compared with controls.

Pairwise linkage disequilibrium and estimation of haplotypes

We observed absolutely identical allele frequencies for the

**Figure 1** *Toll-like receptor 9* mRNA expression in peripheral blood mononuclear cells from ulcerative colitis patients and controls. Total RNA was prepared from peripheral blood mononuclear cells and *Toll-like receptor 9* (*TLR9*) mRNA expression was analyzed by quantitative reverse transcriptase-polymerase chain reaction. There were no significant differences between ulcerative colitis (UC) patients and controls. NS: No significance.**Figure 2** Gene structure and location of identified *Toll-like receptor 9* single nucleotide polymorphisms. We sequenced both exons and approximately 1500 bases of the 5'- and 3'-regions of *Toll-like receptor 9* in one healthy control. We identified three single nucleotide polymorphisms (-1486, 1174, 2848) in the regions sequenced.**Table 7** Linkage disequilibrium among single nucleotide polymorphisms of *Toll-like receptor 9* in ulcerative colitis cases and disease-free controls

SNPs		UC (<i>n</i> = 48)		Controls (<i>n</i> = 47)	
		D'	r ²	D'	r ²
-1486 T/C	1174 A/G	1	1	1	1
-1486 T/C	2848 G/A	1	0.9522	1	0.9581
1174 A/G	2848 G/A	1	0.9522	1	0.9581

Lewontin's *D'* (|D'|) and the linkage disequilibrium coefficient *r*² between each pair of single nucleotide polymorphisms (SNPs) were examined by the expectation-maximization algorithm of SNPalyze software. UC: Ulcerative colitis.

three common SNPs. Table 7 shows that three SNPs are in strong linkage disequilibrium (LD) in both UC patients and healthy controls. We also inferred three SNP haplotypes by using an expectation-maximization algorithm. The number of statistically inferred haplotypes is eight (i.e. 2³). A total of three of the eight possible haplotypes are observed in Table 5. The usage of haplotype C-G-A was more frequent in UC patients when compared with healthy controls, and the difference was statistically significant. (Fisher's exact test, *P* = 0.013, Table 5).

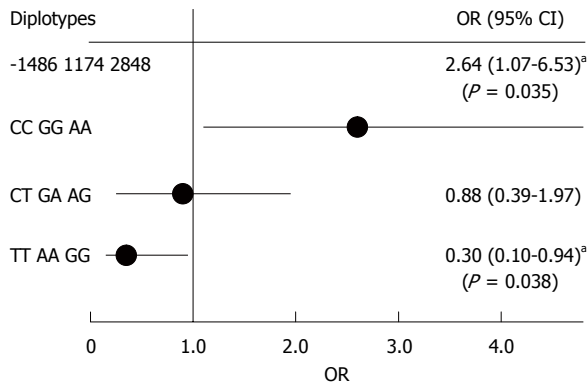


Figure 3 Toll-like receptor 9 gene diplotypes and disease sensitivity (expressed as odds ratios). There was an increased risk of ulcerative colitis with the CCGGAA diplotype, and a decreased risk with the TTAAGG diplotype, as compared with controls. OR: Odds ratio; CI: Confidence interval. ^a $P < 0.05$.

Diploype frequencies

We next analyzed diploype frequencies as pairs of haplotypes. Table 6 shows the distribution of three diploype loci by disease status; an increased prevalence of CCGGAA diploypes (Fisher's exact test, $P = 0.042$) and a decreased prevalence of TTAAGG diploypes (Fisher's exact test, $P = 0.039$) was noted among UC cases, as compared with controls. Thus, we found an increased UC risk among CCGGAA diploypes (OR 2.64, 95% CI: 1.73-6.53, $P = 0.042$) and a decreased UC risk among TTAAGG diploypes (OR 0.30, 95% CI: 0.10-0.94, $P = 0.039$) (Figure 3). These data suggest that CCGGAA diploypes are more susceptible to UC.

Genetic association with clinical phenotypes

In order to further examine the contribution of genetic variations in *TLR9* to UC phenotypes, we analyzed various clinical characteristics among UC patients: sex, age at diagnosis, disease location and disease severity. However, we did not observe any statistically significant correlations between disease status and diploype, genotype or specific *TLR9* allele.

DISCUSSION

In this study, we found that genetic variations of *TLR9* are associated with an increased risk of UC in the Japanese population. *TLR9*, which is activated by unmethylated CpG DNA, triggers innate immune responses^[19,20]. Although no variants have been found in Japanese patients, the *NOD2/CARD15* gene was found to indicate CD susceptibility in Western populations^[5,6], suggesting that abnormal innate immune responses toward luminal bacteria are involved in pathogenesis of IBD. With regard to TLRs, previous reports have indicated that *TLR4* Asp299Gly polymorphism is associated with CD and UC in Caucasian populations^[21]. Török *et al.*^[22] investigated possible associations between genetic variations in *TLR9* and IBD in the German population, but did not detect any associations between *TLR9* gene variations and UC susceptibility. Thus, as UC is a heterogeneous polygenic disease, association studies

are expected to reveal various sets of susceptibility genes depending on the ethnic background of the study populations. Further genetic studies in different ethnic groups will resolve the role of the TLRs in UC susceptibility.

Associations between *TLR9* and several autoimmune diseases or infectious diseases have been studied^[23-26]. These reports have suggested that the -1237T/C SNP in *TLR9* is associated with an increased risk of allergic asthma^[27], while 2848G/A was associated with myocardial infarction, deep vein thrombosis and chronic obstructive pulmonary disease^[27], and 1635A/G was associated with HIV phenotype^[28]. In addition, there were no associations between *TLR9* gene polymorphisms and susceptibility to systemic lupus erythematosus (SLE) and related phenotypes in Caucasian American subjects^[23], but such an association was observed in a Japanese population^[24]. These observations show that several candidate genes showing initial positive associations have generated negative findings in replication studies due to issues related to insufficient power or sample heterogeneity.

In this study, although relatively low numbers of subjects were enrolled, we observed statistically significant differences. According to the statistical methods used in this research, statistical differences in a category that has more than one subject indicate a reliable result. Moreover, we can expect to obtain the same results if more samples are added to the experiment. Therefore, we did not increase the power of this study, although more studies with large cohorts are necessary to characterize the role of *TLR9* SNPs in the etiology of UC.

We demonstrated that a C allele at -1486 (located in promoter region), a G allele at 1174 (located in intron 1) and an A allele at 2848 are associated with an increased risk of UC. SNPs in promoter regions potentially affect gene expression levels by altering the binding of gene transcription factors and SNPs in introns, thereby affecting mRNA splicing and/or enhancement of gene transcription. We were unable to identify down-regulation in *TLR9* expression in UC patients. Evaluating the relationship between the *TLR9* polymorphism and predefined clinical characteristics or biological markers also failed to demonstrate any impact on a particular UC phenotype. Taken together, these results indicate that *TLR9* polymorphism is associated with the development of disease itself, which is probably based on functional impairment of *TLR9*.

A recent study suggested that *TLR9*-triggered type-1 IFN protects mice from experimental colitis^[15]. It also underscores the important protective role of type-1 IFN in intestinal homeostasis, suggesting that type-1 IFN produced by *TLR9* signaling affects the pathogenesis of IBD. Although further experiments are needed in order to identify the functional roles of *TLR9* polymorphism, either downstream molecules of the *TLR9* signaling pathway or cytokine production participating in the development of IBD is expected to be affected by these gene polymorphisms.

In conclusion, we identified an association between *TLR9* polymorphisms and UC in Japanese patients. Our findings indicate the importance of *TLR9* in the genetic

control of reactions to intestinal microbes in UC. Studying SNPs among the molecules involved in bacterial recognition will be essential to understanding the individual responses to bacterial components and to define the genetic background associated with risk of IBD.

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COMMENTS

Background

The pathogenesis of inflammatory bowel diseases (IBDs), which include ulcerative colitis (UC) and Crohn's disease (CD), is multi-factorial, involving susceptibility genes as well as immunological and environmental factors. Recent studies on IBD have provided some evidence that commensal bacteria play a key role in the pathogenesis of the disease, and that the Toll-like receptor (TLR) signaling pathway activated by commensal bacteria plays an important role in maintaining colonic homeostasis. The signaling pathway of *TLR9*, which recognizes unmethylated CpG DNA in bacteria and viruses, protects mice from experimental colitis. As the relationships between UC and *TLR9* gene variation have not been reported to date, in this report we focused on *TLR9* gene mutations or polymorphisms in UC.

Research frontiers

Among several candidate IBD-related chromosomal regions and genes, the caspase recruitment domain 15 (*CARD15*) gene coding for the nucleotide oligomerization domain 2 (*NOD2*) gene was identified as having the strongest linkage for CD susceptibility. Similarly to *NOD2*, TLRs are essential components of innate immunity that recognize microbial compounds from bacteria, fungi and viruses. While TLR activation leads to transcription of inflammatory and immunoregulatory genes, recent studies have demonstrated that TLR signaling in intestinal sites can inhibit inflammatory responses and maintain colonic homeostasis. A gene variation in *NOD2/CARD15* has been reported in CD patients in Western countries, but this variation has not been identified in Japanese CD patients.

Innovations and breakthroughs

As the relationships between UC and *TLR9* gene variation have not been reported to date, in this report we focused on *TLR9* gene mutations or polymorphisms in UC. Three single nucleotide polymorphisms (SNPs) in *TLR9* were identified in healthy controls, and were assessed in 48 UC patients. The authors found that *TLR9* -1486CC, 1174GG and 2848AA increase the risk of UC, and *TLR9* -1486TT, 1174AA and 2848GG decrease the risk of UC.

Applications

The authors' findings indicate the importance of *TLR9* in the genetic control of reactions to intestinal microbes in UC. Studying SNPs among the molecules involved in bacterial recognition will be essential to understanding the individual responses to bacterial components and to define the genetic background associated with risk of IBD.

Terminology

TLR9 is a mammalian Toll-like receptor homologue that appears to function as an innate immune pattern recognition protein for motifs that are far more common in bacterial than in mammalian DNA.

Peer review

It is interesting that the authors identified an association between *TLR9* polymorphisms and UC in Japanese patients. My main concern is the sample size (not too large) and the fact that the *P* values obtained are not extremely significant (almost marginally significant on some occasions). This may limit the validity of the present work.

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Retrorectal tumors in adults: Magnetic resonance imaging findings

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Abstract

AIM: To retrospectively evaluate the magnetic resonance imaging (MRI) features of adult retrorectal tumors and compare with histopathologic findings.

METHODS: MRI features of 21 patients with preoperative suspicion of retrorectal tumors were analyzed based on the histopathological and clinical data.

RESULTS: Fourteen benign cystic lesions appeared hypointense on T1-weighted images, and hyperintense on T2-weighted images with regular peripheral rim. Epidermoid or dermoid cysts were unilocular, and tailgut cysts were multilocular. Presence of intracystic intermediate signal intensity was observed in one case of tailgut cyst with a component of adenocarcinoma. Six solid tumors were malignant lesions and showed heterogeneous intensity on MRI. Mucinous adenocarcinomas showed

high signal intensity on T2-weighted and mesh-like enhancing areas on fat-suppressed T2-weighted images. There was a fistula between the mass and anus with an internal opening in mucinous adenocarcinomas arising from anal fistula. Gastrointestinal stromal tumors displayed low signal intensity on T1-weighted images, and intermediate to high signal intensity on T2-weighted images. Central necrosis could be seen as a high signal on T2-weighted images.

CONCLUSION: MRI is a helpful technique to define the extent of the retrorectal tumor and its relationship to the surrounding structures, and also to demonstrate possible complications so as to choose the best surgical approach.

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Key words: Retrorectal tumor; Presacral lesions; Magnetic resonance imaging; Congenital cyst; Malignant tumor; Diagnosis

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INTRODUCTION

Tumors occurring in the retrorectal space are extremely rare in the adult population, with an estimated incidence of 0.0025-0.014 in the large referral centers^[1,2]. The ret-

rorectal space is a potential space that only becomes real when a mass grows within it. The boundaries of the retrorectal region include the posterior wall of the rectum anteriorly and the sacrum posteriorly. This space extends superiorly to peritoneal reflection and inferiorly to the rectosacral fascia and the supralelevator space. Laterally, the area is bordered by the ureters and the iliac vessels and the sacral nerve roots.

This region contains the confluence of the embryologic hindgut, neuroectoderm, and the bony pelvis. As such, multiple different tissue types can give rise to retrorectal tumors. Retrorectal tumors may be classified as congenital, neurogenic, osseous or miscellaneous^[3,4]. Two-thirds are congenital^[2,4], caused by embryological sequestration, abnormalities in midline fusion and incomplete embryological regression. Cystic congenital lesions include epidermoid and dermoid cysts, tailgut cyst (also called mucus-secreting cyst), enterogenic cyst, teratoma, and teratocarcinoma. Neurogenic (including anterior sacral meningoceles), osseous and miscellaneous tumors each account for approximately 10% of retrorectal tumors^[3]. Majority of these masses in adults are benign and asymptomatic, however, malignant tumors accounted for 21%^[5]. Malignant transformation has also been documented in tailgut cysts and epidermoid cysts^[6-8]. Symptoms include pain, change in bowel habit, difficulty with micturition, and neurological signs in the lower limb and perineum^[3,4,9].

Retrorectal tumors are often found late and may be managed suboptimally. The current consensus is that the cardinal therapy for patients with retrorectal tumors is surgical^[9-11]. Curative resection requires complete excision of the tumor, with an intact capsule for clinically well-circumscribed benign lesions and en bloc resection with clear resection margins for malignant tumors^[9,11]. Accurate diagnosis of these conditions before operation is crucial because it can significantly alter clinical management. MRI is a useful technique to evaluate pelvic disorders because of its multiplanar imaging capability and its good soft tissue contrast^[2,4,6,8,9,11]. We retrospectively evaluated the MRI features of retrorectal tumors in 21 patients and compared with pathological findings to further characterize the MR imaging findings encountered in retrorectal tumors.

MATERIALS AND METHODS

Patients

We reviewed the clinical and radiological findings in 21 patients (13 women, 8 men; age range, 16-74 years; mean age, 39.3 years) with preoperative suspicion of retrorectal tumor who were treated at our hospital between January 2006 and December 2008. Institutional review board permission was obtained for retrospective review without informed consent. MRI was performed in all patients with a standardized protocol. Data from clinic charts, hospital medical records, radiological and pathological reports of these patients were collected retrospectively. The clinical findings at presentation included

rectal fullness ($n = 7$), low back pain ($n = 2$), constipation ($n = 6$), symptoms due to recurrent perirectal abscess and/or fistula ($n = 5$) and no apparent symptom ($n = 1$). Only two patients had postanal dimples. Digital examination of the rectum revealed a mass located posteriorly or laterally to the rectum in 20 patients. Laboratory tests demonstrated elevated carcino-embryonic antigen (CEA) in two patients (2/21).

MR technique and parameters

All 21 patients underwent preoperative MRI. MR examinations were achieved on a 1.5-T unit using an eight-channel phased-array pelvic coil (Siemens, MR Magnetom Sonata, Germany). Neither bowel preparation nor IV contrast enhancement was performed. Before performing MR, the rectum was distended by a balloon filled with 60-80 mL saline. The MR examination was performed with the patient in a supine position. MR examination protocol included sagittal, axial, coronal T2-weighted images, and axial ($n = 21$) T1-weighted images. T2-weighted imaging (TR/TE, 4000/97; echo-train length 13, field of view 20 cm, 4 mm slice thickness, no interslice gap, 256×256 matrix) were performed in the sagittal plane ($n = 21$), axial plane ($n = 21$) and coronal plane ($n = 9$), and followed with fat-suppression in sagittal and axial plane. Imaging parameters for T1-weighted sequences were: TR/TE, 620/12; echo-train length 1, field of view 22 cm, 4 mm slice thickness, no interslice gap, 320×240 matrix. Overall acquisition time varied from 20 to 30 min.

Image review

Magnetic resonance images were reported by two experienced radiologists. Four features of the retrorectal tumor were assessed. (1) the tumor location and extent were defined by the most cephalad sacral vertebra involved; (2) tumor size was measured in the largest two dimensions; (3) tumor morphology was assessed by examining the internal signal characteristics and the tumor margin. A cystic tumor was diagnosed when the lesion displayed over 80% cystic elements and a solid tumor when the lesion showed more than 80% solid elements; the remainders were classified as heterogeneous tumors; and (4) the tumor margin was assessed as well-defined (a smooth or lobular contour without surface projections), irregular (with surface projections) or clearly invasive (the tumor breached an adjacent structure).

RESULTS

Eighteen patients underwent surgery for retrorectal tumor. One patient with a gastrointestinal stromal tumor (GIST) arising from the rectum received abdominal perineal resection (APR). Two patients, diagnosed as having mucinous adenocarcinoma arising from fistulae-in-ano, received radiochemotherapy because the patients refused surgery. Histopathological studies demonstrated 7 malignant tumors (2 mucinous adenocarcinomas arising from fistula, 2 gastrointestinal stromal tumors, 1 tailgut cyst

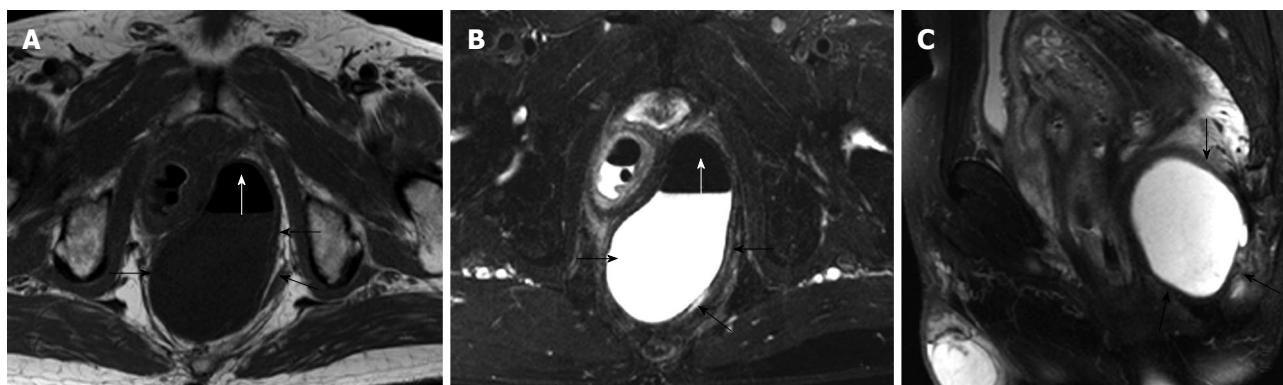


Figure 1 Dermoid cyst in a 63-year-old man, who was misdiagnosed as having abscess. Axial T1-weighted (A), T2-weighted fat-suppression (B) and Sagittal T2-weighted (C) magnetic resonance (MR) images show a thin regular peripheral rim (black arrows) circumscribing a retrorectal cyst and containing some air (white arrows). The cyst lesion extends to the left ischiorectal fossa, and is hypointense on T1-weighted (A) and hyperintense on T2-weighted MR images (B, C).

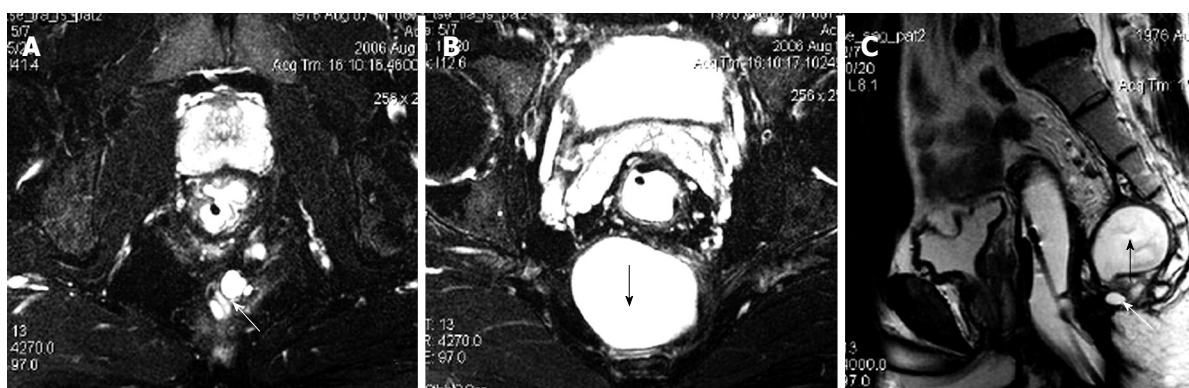


Figure 2 Tailgut cyst in a 30-year-old man. On T2-weighted fat-suppression (A, B) and Sagittal T2-weighted images (C), small cysts are composed to form a honeycomb (white arrows) adjacent to a main larger cyst (black arrows).

with component of mucinous adenocarcinoma, 1 adenocarcinoma of the anal duct, and 1 primary retrorectal adenocarcinoma) and 14 benign cysts (6 epidermoid cysts, 7 tailgut cysts and 1 dermoid cyst). Patients with malignant tumors were significantly older than those with benign tumors (52.5 years *vs* 34.6 years). There was no gender difference in malignant tumors (male/female ratio, 4/3), and women comprised a larger proportion of the patients with benign cysts (male/female ratio, 3/11).

MRI demonstrated that benign cystic lesions were primarily located in the retrorectal space in 12 cases, with downward and lateral extension to ischiorectal fossa in two cases (Figure 1). The rectum appeared anterior or lateral in five patients. The mean largest diameter of the lesions was 4.8 cm (range 0.7-10 cm). Both epidermoid cysts and dermoid cysts were unilocular (Figure 1). There were numerous cysts in all tailgut cysts, shown as a large main cyst and associated with other smaller cysts in the same location (Figure 2). All the cystic lesions appeared hypointense on T1-weighted images, and hyperintense on T2-weighted images. As far as non-complicated cysts (10/14) were concerned, the cystic mass was well-circumscribed by a regular peripheral rim. The rim was hypointense on all sequences. The borders of the cystic lesions were irregular in 4 patients, who had an operative history

with a misdiagnosis as having abscess or fistulae.

Heterogeneous tumor was found in one case of tailgut cyst with a component of adenocarcinoma (Figure 3). The rectum was compressed anteriorly but without evidence of invasion. The cystic portion appeared hypointense on T1-weighted images and hyperintense on T2-weighted images with irregular borders. The malignant portion presented as intermediate signal intensity on T2-weighted and fat-suppression with irregular margin.

The six solid tumors were malignant lesions confirmed by histopathology. Three patients were diagnosed as having mucinous adenocarcinoma, two as having mucinous adenocarcinomas arising from chronic perianal fistulae, and 1 as having adenocarcinoma of the anal duct (Figure 4). MRI showed a larger mass in the pelvis, extending to the ischiorectal fossa. The mucin, which forms the major tissue component of mucinous tumor, showed high signal intensity on T2-weighted fast SE images and mesh-like enhancing areas on fat-suppressed T2-weighted images (Figure 5). As for mucinous adenocarcinoma arising from fistula-in-ano, there was a fistula between the mass and the anus with an internal opening in anorectum (Figure 6). One was diagnosed as primary presacral adenocarcinoma after operation, and MRI identified a well-circumscribed 3.5 cm × 4 cm solid lesion with irregular margin in the

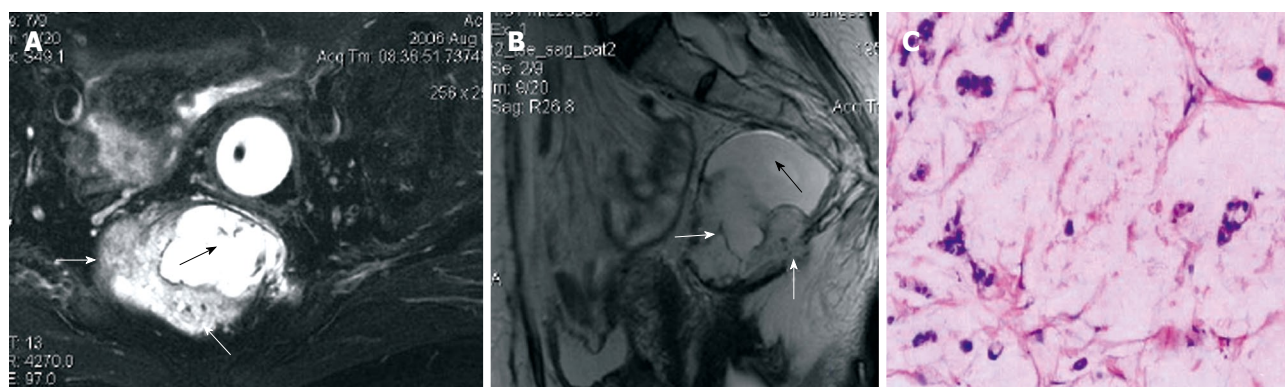


Figure 3 Tailgut cyst associated with mucinous adenocarcinoma in a 52-year-old woman. A, B: The rectum is compressed and shifted to the front but without evidence of invasion. The cystic portion (black arrows) is hyperintense, and the malignant portion (white arrows) presents as irregular margin with intermediate signal intensity on T2-weighted fat-suppression (A) and Sagittal T2-weighted images (B); C: Photomicrograph of the tumor shows a small cluster of atypical cells in stroma.

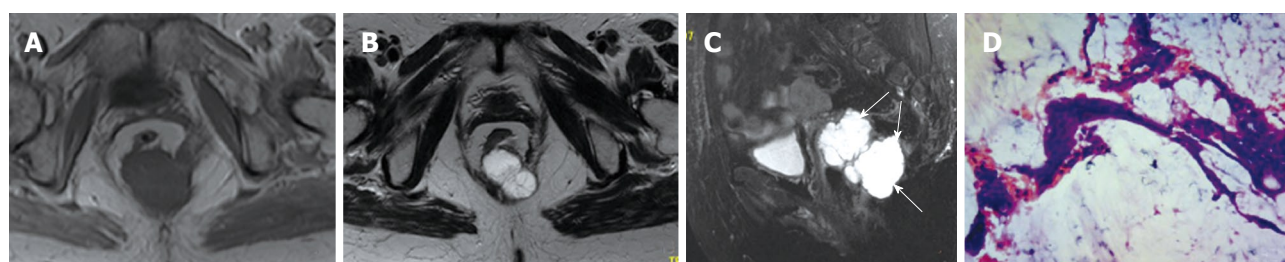


Figure 4 Adenocarcinoma of the anal duct in a 71-year-old woman. A, B: Axial T1-weighted magnetic resonance (MR) image shows a low signal mass with irregular marginal in the retrorectal space and high signal on T2-weighted MR image; C: The mucin, which forms the major tissue component of mucinous tumor, shows high signal intensity (white arrows) on fat-suppressed T2-weighted image; D: The gland shows irregular features with cellular hyperchromatin and the stroma shows marked desmoplasia.

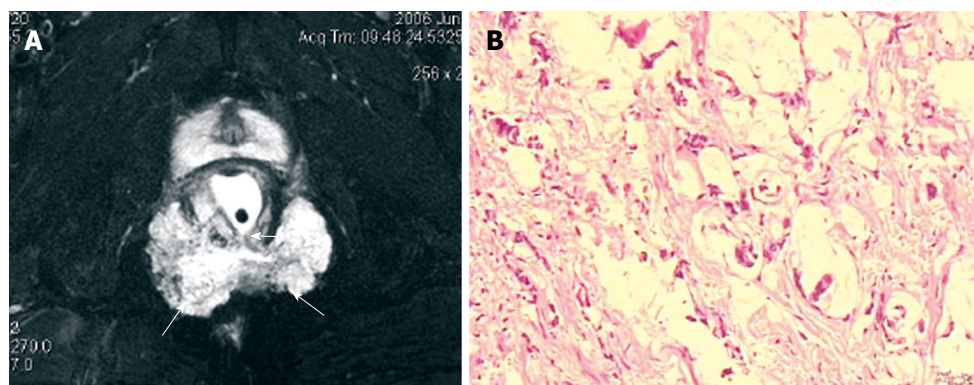


Figure 5 A 59-year-old man with mucinous adenocarcinoma caused by anal fistula. A: Axial fat-suppressed T2-weighted magnetic resonance image shows a horse-shoe mass with typical mesh-like enhancing areas (arrows). The mass has an internal fistula connected to the anorectum (arrows); B: Microscopy shows a single or small cluster of atypical cells floating in mucin pool and bundles of collagen with hyaline degeneration in stroma.

presacral space, not involving sacrum or rectum (Figure 7). The other two were diagnosed as gastrointestinal stromal tumors (GISTs), one appeared to arise from the rectum (Figure 8), and the other appeared in presacral space (Figure 9A and B). Tumors were larger than 5 cm in at least one transverse dimension. MRI showed low signal intensity on T1-weighted image, and heterogeneous signal intensity on T2-weighted image. We suspected a central necrosis based on the T2-weighted imaging with a high signal. In immunohistochemical studies, diffuse and strong immunoreactivity for CD34 and CD117 were seen throughout the tumors (Figure 9C and D). Conversely, the

tumor cells were negative for both muscle markers (smooth muscle actin, desmin) and neural markers (S-100 protein, neuron specific enolase).

DISCUSSION

The boundaries of the presacral region include the posterior wall of the rectum and the sacrum. This space extends superiorly to peritoneal reflection and inferiorly to rectosacral fascia and the supralelevator space. Laterally, the area is bordered by the ureters, the iliac vessels, and the sacral nerve roots. Because this area contains totipotential

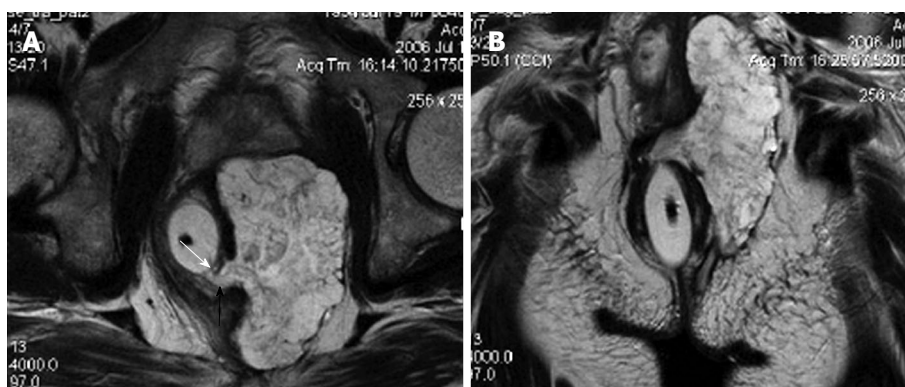


Figure 6 A 52-year-old man with mucinous adenocarcinoma caused by anal fistula. A, B: The mass displays heterogeneous intensity on T2-weighted magnetic resonance image. There is a fistula between the mass and the anus (black arrow) and an internal opening in anorectum (white arrow).

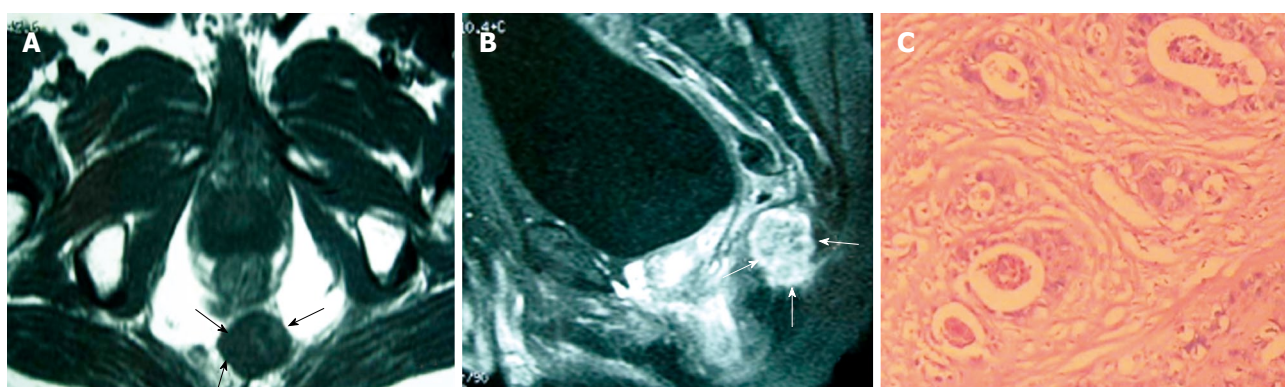


Figure 7 Primary retrorectal adenocarcinoma in a 33-year-old man. A, B: The mass displays low signal intensity, without rim (black arrows) on T1-weighted magnetic resonance (MR) image, and high signal intensity of the irregular border (white arrows) on fat-suppressed T2-weighted MR image; C: Microscopy shows irregular glands of variable sizes and atypical tumor cells. The stroma shows desmoplasia.

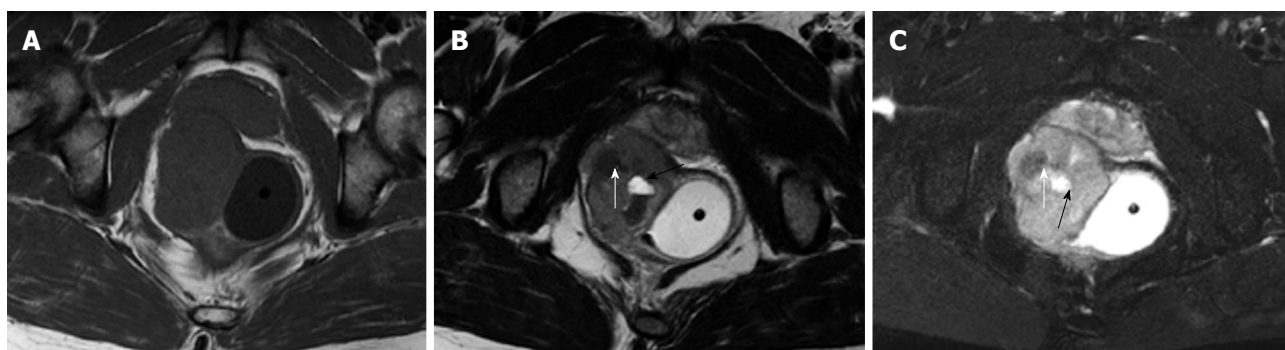


Figure 8 Gastrointestinal stromal tumors of rectum in a 44-year-old man. A well-circumscribed, smooth, intramural mass with exophytic growth expanded from the rectal wall and extended to the right ischiorectal fossa. The tumor displays low signal intensity on T1-weighted image (A), and high signal intensity on T2-weighted image (B, C). There are some areas of necrosis (black arrows) and fibrotic tissues (white arrows) in the tumor.

cells that differentiate into three germ cell layers, a multitude of tumor types may be encountered. Traditionally, these lesions are divided into congenital, inflammatory, neurogenic, osseous and miscellaneous types^[3,4]. Clinical diagnosis may be delayed because of non-specific symptoms. Symptomatic patients typically complain of vague, longstanding pain in the perineum or low back, and change in bowel habit^[1,5,8]. Because detection is often difficult and delayed, patients frequently present with tumors that have reached a considerable size and involve multiple

organ systems, thus complicating their treatment. Singer *et al*^[12] reported 7 patients who underwent an average of 4.7 invasive procedures or operations before the correct diagnosis of a retrorectal lesion was made.

The diagnosis and management of these tumors has evolved in recent years because of improved imaging modalities, especially the MRI. MRI is often used in diagnosing and managing the patients with presacral tumors, as it can provide excellent anatomic detail and soft-tissue contrast. Kim *et al*^[13] asserted that for the evaluation of a

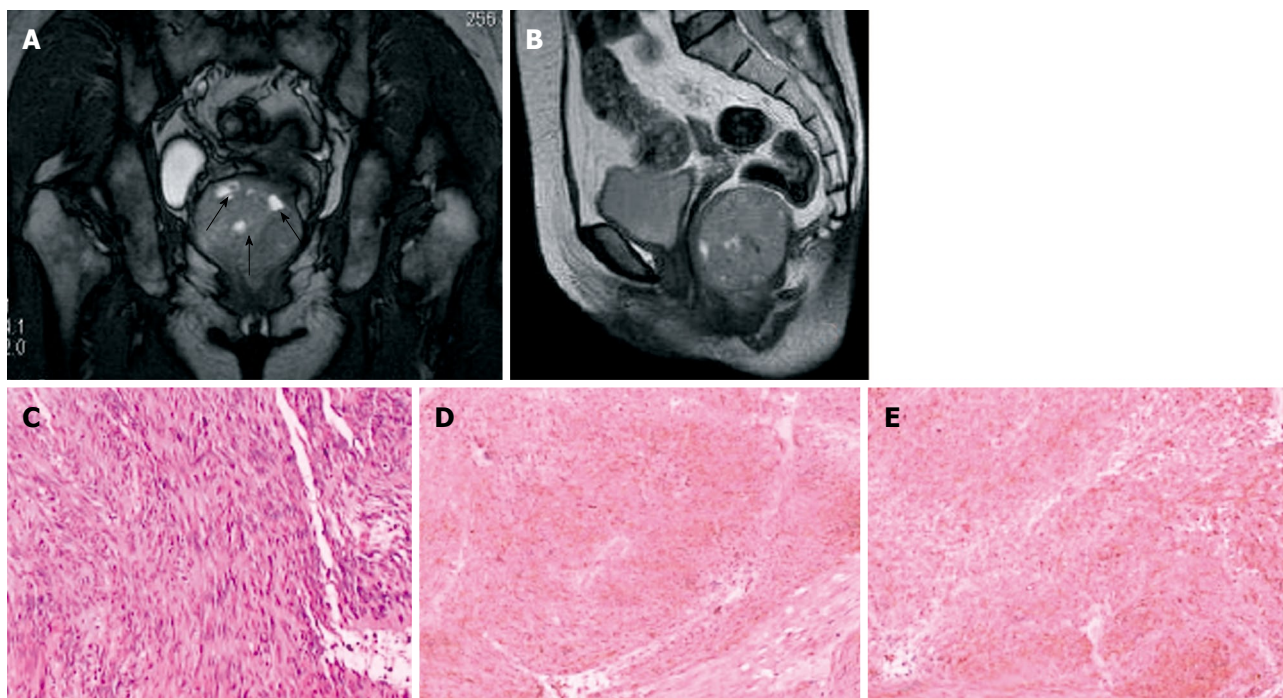


Figure 9 Retrorectal gastrointestinal stromal tumors in a 51-year-old woman. A, B: T2-weighted magnetic resonance images show a large retrorectal mass, in which some necrosis presents hyperintensity (black arrows); C: Histopathology shows spindle cells and cytoplasmic vacuoles; D, E: On immunohistochemical studies, diffuse and strong immunoreactivity for CD117 and CD34 are seen.

presacral mass, MRI has the advantage over CT of being able to offer multiplanar capabilities and good tissue contrast. MRI is a valuable tool for preoperative evaluation, imaging and characterizing lesions, estimating their extent and the risk of malignancy, distinguishing organ-confined disease from tumor spread into adjacent structures, and deciding upon the most appropriate intervention strategies and imaging follow-up requirements.

About half of the presacral tumors are congenital lesions, and most of them are developmental cysts (epidermoid, dermoid, tailgut cysts, and teratomas). On MRI, presacral cyst usually has low signal intensity on T1-weighted images and high signal intensity on T2-weighted images^[8]. However, it may have high signal intensity on T1-weighted images due to presence of mucinous materials, high protein content, or hemorrhage in the cyst^[6,8]. All of the presacral cysts in our cases were hypointense on T1-weighted images and hyperintense on T2-weighted images. Among the presacral cystic masses, epidermoid cyst, dermoid cyst, rectal duplication cyst, and meningocele are usually unilocular^[14]. The presence of fat content on fat-saturated images is suggestive of a dermoid cyst^[8]. Rectal duplication cysts often communicate with the rectal lumen and are anterior to the rectum. Anterior meningocele is a well-defined unilocular thin-walled, fluid-filled lesion of the retrorectal space with a stalk that may be seen communicating with the thecal sac. In contrast, tailgut cyst is usually multicystic^[6]. In our study, we observed that epidermoid cysts and dermoid cysts appeared unilocular on MRI, and tailgut cysts appeared as a large cyst accompanied by small peripheral cysts. We believe that the unilocular or multilocular characteristics are very important

because of the malignant potential of a tailgut cyst. A few cases of degenerated tailgut cysts have been documented in the literature^[6,15-17]. The possibility of malignant transformation must be considered in the presence of heterogeneous tumor. Our findings, that a cystic lesion displayed heterogeneous signal intensity on T2-weighted MR images with markedly irregular wall, and the malignant portion presented as irregular margin with intermediate signal intensity on T2-weighted and fat-suppression, are consistent with what has been described in the literature^[6,17].

MRI is useful in predicting whether a tumor is benign or malignant. A cystic tumor with a smooth, well-circumscribed margin and no features of invasion or enhancement with gadolinium is benign, and a heterogeneous tumor, or a solid tumor with an irregular margin, is usually malignant^[9]. The six solid tumors were malignant confirmed by histopathology in this series. Two patients with adenocarcinoma arising from fistula-in-ano displayed presacral tumor. Several characteristic MRI findings may help diagnose mucinous adenocarcinoma arising from fistula-in-ano. Histopathologically, mucinous colorectal carcinomas comprise large pools of extracellular mucin lined by columns of malignant cells, cords and vessels, which give rise to a typical mesh-like internal structure^[18]. The mucin, which forms the major tissue component of mucinous tumors, has high signal intensity on T2-weighted fast SE images. Two authors reported that a fistula between the mass and the anus is a characteristic finding of mucinous adenocarcinoma arising from fistula-in-ano on MRI^[19,20]. Two patients showed a fistula tract between the mass and the anus in agreement with the previous reports.

GIST is a non-epithelial neoplasm arising from the

wall of the gastrointestinal tract. GIST is thought to originate from the interstitial cell of Cajal, an intestinal pacemaker cell^[21]. GISTs are most often located in the stomach (39%-70%) and small intestine (20%-35%), whereas the colon and rectum (5%-12%) are less common locations^[22-25]. Some GISTs primarily arise in the omentum, mesentery, or retroperitoneum and are unrelated to the tubular gastrointestinal tract. It is even rarer that GIST originates from presacral space. On MRI, solid portions of tumor typically show low signal intensity on T1-weighted images, intermediate to high signal intensity on T2-weighted images, and enhancement after administration of gadolinium^[26]. The markedly high signal seen on T2-weighted MRI should be considered as a feature strongly indicating a diagnosis of GIST^[27]. GISTs usually involve the muscularis propria of the gastrointestinal wall, so the characteristic image is that of a well-circumscribed, smooth, intramural mass with exophytic growth. The case with rectal GIST in this series presented as well-defined, eccentric mural mass that expanded the rectal wall and extended into the right ischioanal fossa. As in GISTs at other locations, central areas of necrosis could be seen in our cases.

Adenocarcinomas of the presacral region are distinctly unusual. Most of the cases represent direct extension or metastatic spread from rectal cancer. Although malignant replacement of these cysts is possible, complete replacement of the cyst epithelium and other elements by adenocarcinoma has not been reported^[5]. However, potential sources such as gastrointestinal, pancreatic and prostatic tissues were eliminated and no evidence of a developmental cyst was found histopathologically in this case. The tumor described in the present report should be regarded as a primary presacral adenocarcinoma, as reported by Zamir *et al.*^[5] and Puccio *et al.*^[28]. Pelvic MRI demonstrated a retrorectal heterogeneous solid lesion on both T1-weighted and T2-weighted images and a markedly high signal was seen on T2-weighted images after fat-saturation.

A further benefit of MRI being confirmed is whether routine preoperative biopsy is necessary^[29]. Historically, the role of preoperative biopsy of retrorectal tumors has been a controversial topic in the general surgery. When preoperative MRI is available, the indications for biopsy can be limited to patients whose mass may represent metastatic disease or lymphoma^[9]. The risk of a routine biopsy can, therefore, be avoided.

Retrorectal tumors can be best managed by surgery. Careful surgical planning is important by selecting appropriate approaches, such as an anterior approach (abdominal), posterior approach (perineal), or a combined abdominoperineal approach. MRI will help define the margins of resection and the relationship between the tumor and the sacral level. If the tumor is positioned below the mid-body of S3, a perineal approach can be considered. All tumors that extend above S3 often require an abdominal or combined approach.

In conclusion, retrorectal tumor is a rare entity that is difficult to diagnose. Our series supports that MRI is a useful examination when a retrorectal tumor is suspected.

Cystic lesions with a smooth wall on MRI are typically benign, whereas heterogeneous or solid tumors are usually malignant. However, final diagnosis should be based on the pathological examination after surgical resection. MRI is a helpful technique to define the extent of the tumor, its relationship to the surrounding structures and also to demonstrate possible complications in order to choose the best surgical approaches.

COMMENTS

Background

Tumors occurring in the retrorectal space are extremely rare in the adult population. Retrorectal tumors may be classified as congenital, neurogenic, osseous or miscellaneous types. Majority of these masses are benign and asymptomatic, however, malignant tumors accounted for 21%. Curative resection requires a complete excision of the tumor, with an intact capsule for clinically well-circumscribed benign lesions and en bloc resection with clear resection margins for malignant tumors. Accurate diagnosis of these conditions before operation is crucial because it can significantly alter clinical management.

Research frontiers

The diagnosis and management of retrorectal tumors have evolved in recent years because of improved imaging modalities, especial magnetic resonance imaging (MRI). MRI is a valuable tool for preoperative evaluation, imaging and characterizing lesions, estimating their extent and the risk of malignancy, distinguishing organ-confined disease from tumor spread into adjacent structures, and deciding upon the most appropriate intervention strategies and imaging follow-up requirements.

Innovations and breakthroughs

This series supports that MRI is a useful examination when a retrorectal tumor is suspected. Cystic lesions with a smooth wall on MRI are typically benign, whereas heterogeneous or solid tumors are usually malignant. MRI is a helpful technique to define the extent of the tumor, its relationship to the surrounding structures and also to demonstrate possible complications in order to choose the best surgical approach.

Applications

MRI is a useful technique to evaluate retrorectal tumors. It can provide optimal information about tumor location, size, extent, the risk of malignancy and strategy for therapeutic intervention in the patients with retrorectal tumor.

Terminology

Retrorectal space, also called presacral space, is a potential space limited anteriorly by the fascia propria of the rectum and posteriorly by Waldeyer's rectosacral fascia. Laterally, it is limited by the iliac vessels and the ureters and extends along the lateral ligaments of the rectum, communicating superiorly at the level of the peritoneal reflection with the retroperitoneal space.

Peer review

This article well documented the MRI features of retrorectal lesions, and this will interest our readers.

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Pro12Ala polymorphism of the peroxisome proliferator-activated receptor γ 2 in patients with fatty liver diseases

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Abstract

AIM: To test the occurrence of the Pro12Ala mutation of the peroxisome proliferator-activated receptor- γ (PPAR γ)2-gene in patients with non-alcoholic fatty liver disease (NAFLD) or alcoholic fatty liver disease (AFLD).

METHODS: DNA from a total of 622 specimens including 259 blood samples of healthy blood donors and 363 histologically categorized liver biopsies of patients with NAFLD ($n = 263$) and AFLD ($n = 100$) were analyzed by Real-time polymerase chain reaction using allele-specific probes.

RESULTS: In the NAFLD and the AFLD collective, 3% of the patients showed homozygous occurrence of the Ala12 PPAR γ 2-allele, differing from only 1.5% cases in the healthy population. In NAFLD patients, a high incidence of the Ala12 mutant was not associated with the progression of fatty liver disease. However, we observed a significantly higher risk (odds ratio = 2.50, CI: 1.05-5.90, $P = 0.028$) in AFLD patients carrying the mutated Ala12 allele to develop inflammatory alterations. The linkage of the malfunctioning Ala12-positive PPAR γ 2 isoform to an increased risk in patients with AFLD to develop severe steatohepatitis and fibrosis indicates a more prominent anti-inflammatory impact of PPAR γ 2 in progression of AFLD than of NAFLD.

CONCLUSION: In AFLD patients, the Pro12Ala single nuclear polymorphism should be studied more extensively in order to serve as a novel candidate in biomarker screening for improved prognosis.

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Key words: Single nucleotide polymorphism; Peroxisome proliferator-activated receptor γ ; Non-alcoholic steatohepatitis; Alcoholic steatohepatitis; Inflammation; Fibrosis; Hepatitis; Steatosis; Steatohepatitis

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INTRODUCTION

Fatty liver diseases are becoming a common cause of chronic liver diseases in the Western countries encountering in about 20% of the general adult and child population^[1-5]. Excessive accumulation of triglycerides in hepatocytes occurring in etiologically diverse conditions causes hepatic steatosis characterized by more than 5%-10% fat stored either in macrovesicles or in microvesicles of hepatocytes^[6,7]. Whereas in the past, regular and excess alcohol consumption was the most common reason for hepatic steatosis^[8,9], fatty liver diseases are now most frequently associated with obesity, insulin resistance and type 2 diabetes due to an unbalanced and rich diet in industrial nations^[3,5].

The spectrum of fatty liver diseases (FLD) independent of causative agents ranges from simple steatosis to steatohepatitis, which can progress to liver fibrosis ending up in cirrhosis or hepatocellular carcinoma^[3,5,10-13]. Thus, 5%-10% of non-alcoholic fatty liver disease (NAFLD) patients with steatosis develop a steatohepatitis accompanied by a high risk of progression to fibrosis^[3,4]. Although fatty liver diseases can have various causes, features of steatohepatitis in NAFLD and alcoholic fatty liver disease (AFLD) are difficult to distinguish histologically^[6,7]. Both are characterized by foci of liver cell necrosis and lobular inflammatory infiltrates with polymorphonuclear leukocytes. Furthermore, the onset of steatohepatitis is accompanied by ballooned hepatocytes, often harboring Mallory's hyaline and megamitochondria or undergoing apoptosis^[14]. Whereas steatosis seems to be more pronounced in non-alcoholic steatohepatitis (NASH) than in alcoholic steatohepatitis (ASH), features of necroinflammatory and cholestatic activity are more prominent in ASH liver biopsies^[6]. Progression of steatohepatitis then results in pericellular fibrosis^[6,7,15] involving myofibroblastic activation of sinusoidal hepatic stellate cells responsible for elevated extracellular matrix deposition^[16].

Members of the peroxisome proliferator-activated receptors (PPAR) seem to play a key role in the pathophysiology of FLD by modulating increased glucose uptake and hepatic triglyceride accumulation, but also perform anti-inflammatory signals when steatohepatitis has occurred^[17-19]. The PPAR family consists of PPAR α , PPAR γ , and PPAR δ nuclear receptors, functioning as transcription factors, that mediate transcriptional response to insulin resulting in glucose uptake, increased fatty acid oxidation, lipogenesis and lipid storage, respectively^[17]. Whereas the PPAR α is highly present in hepatocytes, the splice variants PPAR γ 1 and 2 triggering adipogenesis are mainly expressed in adipose tissues and only to a minor extent in the liver. PPAR γ increases the expression of genes that promote fatty acid storage, whereas it represses genes that induce lipolysis in adipocytes. In patients suffering from FLD, hepatic expression of PPAR γ is shown to be involved in insulin sensitivity, triglyceride clearance and hepatic steatosis^[20].

Due to its high impact as an insulin-sensitising transcription factor involved in adipogenesis and lipogenesis, the occurrence of single nucleotide polymorphisms (SNP) in the PPAR γ gene was recently addressed by numerous

reports studying subjects with insulin resistance, type 2 diabetes, arteriosclerosis, and hypertension^[21-23]. A prevalent SNP association with impaired lipid homeostasis was observed in terms of the N-terminal proline alanine exchange (Pro12Ala) of the extra domain in the PPAR γ 2 variant. This PPAR γ splice form includes 30 additional amino acids^[24], which are responsible for a 5-6-fold increase of PPAR γ 's transcriptional activity. The Pro12Ala exchange in the activating extra region of the PPAR γ 2 is the result of a cytosine to guanine substitution in the PPAR γ gene, as a consequence encoding the Ala-allele form with a heavily reduced function^[23]. In several populations, the association of the Pro12Ala polymorphism with insulin-sensitivity, type 2 diabetes, obesity and adipositas have been shown^[25-27]. However, the role of the Pro12Ala polymorphism of PPAR γ gene in occurrence and progression of fatty liver diseases is not yet defined.

In the present study, we analyzed the frequency of the Pro12Ala polymorphism in the PPAR γ gene by a highly sensitive LNA-probe based polymerase chain reaction (PCR) approach in a total of 622 subjects of a Caucasian population, suffering from fatty liver disease ($n = 359$) or being healthy blood donors ($n = 263$). In agreement with reports showing a high Ala allele prevalence in patients with impaired lipid metabolism in obese and adipose patients^[26,28], in FLD patients the Ala allele also occurs more often than in the healthy control group. Interestingly, the interpretation and linkage of the allele frequency to histological evaluation and clinical data demonstrates a prominent risk in AFLD patients bearing the Ala allele to develop severe steatohepatitis and fibrosis. Furthermore, our data revealed for the first time a higher anti-inflammatory impact of PPAR γ in progression of human AFLD than NAFLD.

MATERIALS AND METHODS

Patients, biopsies and liver disease classification

From a total of 622 cases, 259 blood samples and 363 biopsies were studied for occurrence of the Pro12Ala exchange in the PPAR γ gene. Local research ethics guidelines were followed. We collected 363 cases from the files of the Department of Gastroenterology and Hepatology, University Hospital of Essen (GER) and the Institute for Pathology, University Hospital of Cologne (GER) according to their histological criteria of fatty liver disease (Table 1). 263 tissue specimens from patients were classified as NAFLD according to the clinical information about alcohol consumption (less than 20 g alcohol per day). One hundred specimens of patients who consume more than 20 g alcohol per day met the definition of AFLD as described by Neuschwander-Tetri *et al.*^[29]. Clinical data, such as GOT, GPT, and γ GT, were compiled along with the state of diabetes. There was no appreciable difference between the mean age of AFLD (53.93 ± 10.63 , range 20-81 years) and NAFLD (50.48 ± 15.25 , range 16-80 years). All specimens, stained with haematoxylin and eosin (HE) and by the Gomori method for visualization of reticular fibers, were independently classified by three experienced liver pathologists (Hardt A, Drebber U, Dienes HP), according to

Table 1 Scoring according to the histological features described by Kleiner *et al.*^[15]

Definition	Score
Steatosis	
Grade	
< 5%	0
5%-33%	1
33%-66%	2
> 66 %	3
Localization	
Zone 3	0
Zone 2	1
Zone 1	2
Azonal	3
Panacinar	4
Type	
Macrovesicles	In %
Microvesicles	In %
Mixed	
Inflammation	
Lobular	
No foci	0
< 2 foci	1
2-4 foci	2
> 4 foci	3
Portal	
No inflammation	0
Minimal	1
Mild	2
Moderate	3
Severe	4
Fibrosis	
None	0
Mild/moderate	1
Periportal or perisinusoidal	2
Bridging fibrosis	3
Cirrhosis	4
Liver cell damage	
Ballooning	
None	0
Moderate	1
Severe	2
Mallory bodies	
None	0
Moderate	1
Severe	2

the histological score described by Kleiner *et al.*^[15] (Table 1). Additionally, 259 DNA extracts from blood samples of healthy blood donors were taken as references for local gene distribution.

Automatic DNA extraction from formalin fixed and paraffin embedded biopsies

Extraction of DNA from 363 formalin fixed and paraffin embedded (FFPE) biopsies was performed from three 7 μ m-microtome sections after deparaffinization and proteinase K treatment, as previously described^[30]. Then, DNA was purified by means of magnetic bead technology (FormaPure™ Kit of Agentcourt, Beverly MA, USA). All DNA purification steps were carried out by the BioMek FX laboratory automatic workstation (Beckman Coulter, USA) according to the work file and recommendations of Agentcourt.

Furthermore, DNA from 259 blood samples was pre-

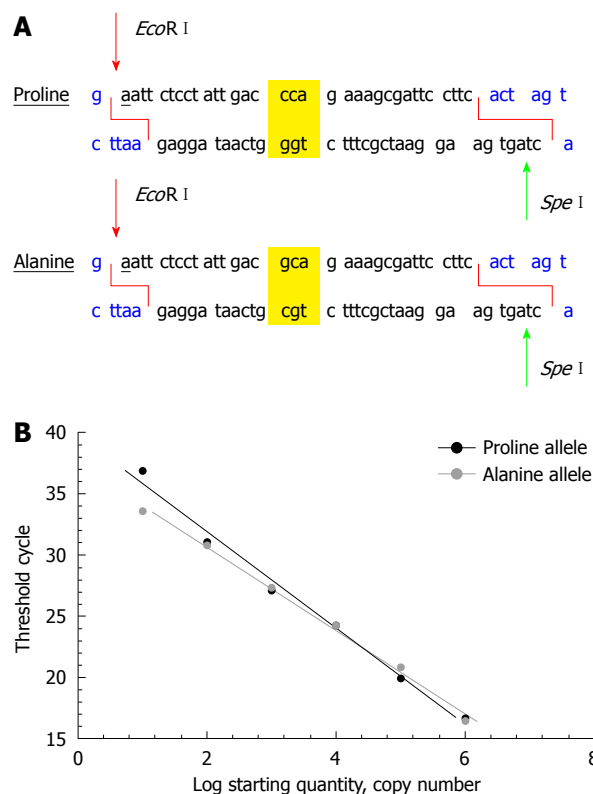


Figure 1 Cloning strategy and validation of a reference system. A: Cloning strategy for the generation of a reference system. The Pro12Ala mutation locus of the peroxisome proliferator-activated receptor- γ gene was synthesized as the proline or the alanine encoding oligonucleotides sequence. The proline or the alanine codon is indicated in yellow. The chemically synthesized oligonucleotide dimers, flanked by the overhangs of the *EcoR* I and the *Spe* I restriction sites (red/green), respectively, were used for insertion into pBluescript SKII plasmids; B: Real-time polymerase chain reaction (PCR) of the Pro12Ala locus depending on different copy numbers of reference sequences. Real-time PCR was performed using the LNA probes (Table 2) specific for the proline encoding sequence or the alanine encoding allele, respectively. 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10 copies of the plasmid reference sequences encoding either the Ala12 or the Pro12 locus were each diluted in 10 ng salmon sperm DNA and used for LNA probe based real-time PCR assays. Up to 10 copies per ng total DNA of both reference sequences were efficiently detected by the corresponding LNA probe labelled either by Hex or Fam fluorochrome.

pared by the robotic workstation using the Genfind™ Kit of Agentcourt according to the manufacturer's instructions.

Cloning of reference sequences into pBluescript

For construction of a reference system with sequences of the Pro12Ala locus (rs1805192) carrying either the mutation or the wild type sequence of PPAR γ 2 gene we used oligonucleotides comprising the proline or alanine encoding sequences (Figure 1). These oligonucleotides were dimerized in 150 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L Tris pH 7.4, creating *EcoR* I and *Spe* I compatible overhangs and inserted into pBluescript SKII (+) (Stratagene, Texas, USA) by the respective restriction sites (Figure 1).

Allelic discrimination of the PPAR γ 2 by real-time PCR

The Pro12Ala exchange of the PPAR γ gene (rs1801282) was examined in DNA from normal blood donors and patients by a Taqman probe associated real-time PCR. Genomic DNA was amplified by real-time PCR in a total vol-

Table 2 Real-time polymerase chain reaction primers and probes

Name	Oligonucleotide sequence	PCR application
Plas A primer	5'-CCGCTCTAGAACTAGTGAAGGAA-3'	Reference
Plas S primer	5'-ACTCACTATAGGGCGAATTGG-3'	DNA
PPAR γ A primer	5'-TTACCTTGTGATATGTTGCAGAC A-3'	Target
PPAR γ mis primer	5'-GTTATGGGTGAAACTCTTGGAGA-3'	DNA
TM LNA probe wt	5'-6FAM-CTATTG <u>CCC</u> AGAAAGC-- BHQ1	Target and reference
TM LNA probe mut	5'-YAK-CTATTG <u>ACG</u> CAGAAAGC-- BHQ1	DNA

Bold letters indicate the single nucleic polymorphism and underlined letters indicate the insertion of locked nucleotides (LNA). PPAR γ : Peroxisome proliferator-activated receptor- γ ; PCR: Polymerase chain reaction.

ume of 10 μ L using the Eppendorf MasterMix (Hamburg, Germany) and 0.3 μ L of each primer (10 μ mol/L). The Plas A and Plas S primer set was used for amplification of reference plasmids; the PPAR γ A and PPAR γ mis primer set was used for genomic DNA samples (Table 2).

Allelic discrimination was achieved by adding 0.4 μ L of 2.5 μ mol/L LNA probes (TIB Molbiol, Berlin, Germany) recognizing the wild type and the mutant variant of the Pro12Ala locus of the PPAR γ 2 gene (Table 2). In parallel to the allelic Pro12Ala discrimination, plasmid reference sequences diluted from 10^5 to 10 copies in herring's sperm DNA (1 ng/mL) were applied to all assays as positive controls. Amplification and analyses were accomplished by the following cycling conditions using a MX3000P qPCR System of Stratagene (Texas, USA): initial denaturation at 95°C for 2 min, following 50 cycles 95°C for denaturation, 55°C for annealing, 65°C for extension, each step lasting 20 s.

Statistical analysis

Pro12Ala distribution was evaluated using the SPSS software 17 of IBM® (Chicago, USA). Significance of cross-classification was calculated by the Fisher's exact test. Odds ratios were used to describe the risk of disease progression.

RESULTS

Prominent occurrence of the PPAR γ alanine variant in patients with fatty liver disease

In order to detect the Ala12Pro polymorphism in patients with fatty liver disease we established an assay using locked nucleotide acid (LNA) probes for allelic discrimination. For this purpose, both variants of the Ala12Pro locus were cloned (Figure 1A) and the sequences, encoding either the proline or the alanine variant, were used as reference sequences for efficient allele detection and discrimination. The LNA probe hybridization assay linked to real-time PCR efficiently detected both, the proline wild type and the alanine mutated variant of the PPAR γ gene (Figure 1B). Up to 10 copies of each reference sequence were successfully proven. In addition to the high sensitivity, application of the reference sequences attested that the LNA probe

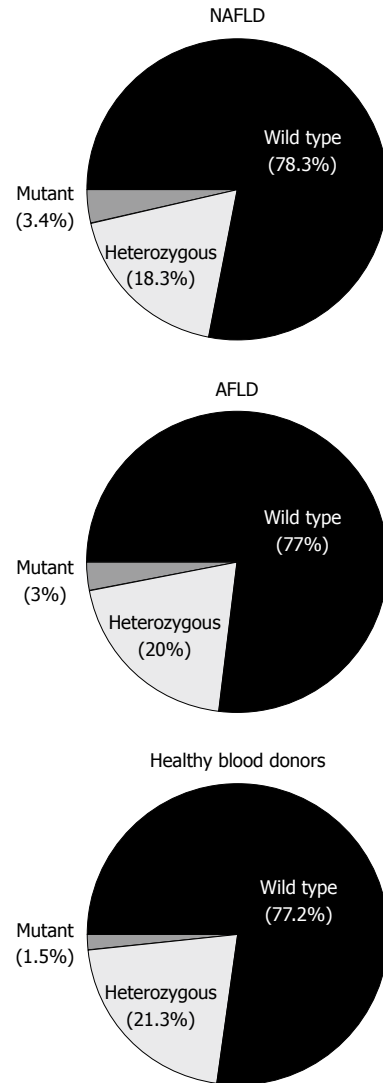


Figure 2 Frequency of the Pro12Ala polymorphism in patients with non-alcoholic fatty liver disease ($n = 263$), alcoholic fatty liver disease ($n = 100$) and in healthy blood donors ($n = 259$). The wild type allele, which is the Pro allele, is indicated in grey, the heterozygous genotype (Pro/Ala) in black and the homozygous Ala/Ala mutant in pale-grey. Genotype analyses revealed a higher prevalence of the homozygous Ala/Ala genotype in non-alcoholic fatty liver disease (NAFLD) and alcoholic fatty liver disease (AFLD) patients.

based PCR assay was highly specific, enabling the differentiation of the alanine and the proline encoding sequence.

In the 259 healthy blood donors the assay accounted for 1.5% homozygous variants carrying the alanine encoding sequence (Figure 2). Analyses of the Pro12Ala distribution in the collective of patients with fatty liver disease ($n = 363$) revealed an increased incidence of the alanine mutant (3%) compared to the healthy population (1.5%). However, the difference was not statistically significant.

In order to identify the association of enhanced alanine occurrence, clinical data of patients with fatty liver disease were considered, revealing that 263 patients suffered from NAFLD, whereas fatty liver disease was linked to alcohol consumption in 100 cases (Table 3). In both cohorts, 12%–14% of the subjects were diabetes positive.

Furthermore, we analyzed the transaminase values in the two cohorts, showing a GPT/GOT ratio of 2 in

Table 3 Occurrence of diabetes mellitus in patients with fatty liver disease *n* (%)

	No C2 (NAFLD)	C2 (AFLD)
Diabetes mellitus positive	33 (12.5)	14 (14)
Diabetes mellitus negative	144 (54.8)	51 (51)
ND	86 (32.7)	35 (35)
Total	263 (100)	100 (100)

NAFLD: Non-alcoholic fatty liver disease; AFLD: Alcoholic fatty liver disease; ND: Not determined.

Table 4 Histological features in the fatty liver diseases cohort

Cases of fatty liver disease (<i>n</i> = 363)	<i>n</i> (%)
Steatosis	
S0	9 (2.5)
S1	169 (46.6)
S2	84 (23.1)
S3	101 (27.8)
Ballooning	
No ballooning	164 (45.2)
Stage 1	124 (34.2)
Stage 2	75 (20.7)
Inflammation	
G0	4 (1.1)
G1	217 (59.8)
G2	113 (31.1)
G3	29 (8)
Fibrosis	
F0	97 (26.7)
F1	116 (32)
F2	66 (18.2)
F3	56 (15.4)
F4	28 (7.7)

NAFLD patients, but a GPT/GOT ratio of 1 in sera of AFLD patients (Figure 3). The high GPT values in NAFLD patients are in accordance with numerous reports, characterizing the progress of steatohepatitis due to non-alcoholic steatosis in comparison to alcoholic steatosis^[31]. With respect to the distribution of the Pro12 and the Ala12 alleles in these two cohorts, we found that in both the AFLD and the NAFLD collective the frequency of the alanine genotype was higher (about 3%) compared to the healthy population (Figure 2).

Association of the alanine allele with inflammation and fibrosis in fatty liver disease

We next addressed the question whether the elevated incidence of the alanine allele in the population of fatty liver diseases is associated with the grade of steatosis, ballooning, steatohepatitis, or liver fibrosis (Table 4).

The degree of steatosis is traditionally classified into mild (< 30%), moderate (30%-60%), and severe (> 60%). More than 50% of the patients with NAFLD and AFLD had developed severe steatosis, however, in patients carrying the alanine allele severe steatosis occurred slightly more often. Pronounced ballooning was observed likewise often in NAFLD patients with the mutant or the wild type isoform of the PPAR γ 2 (Table 5).

Histological scoring for inflammatory alterations and

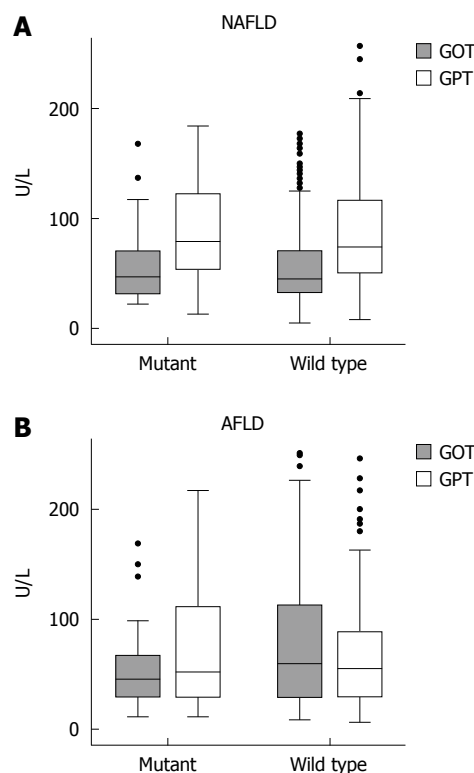


Figure 3 GOT and GPT levels in sera of non-alcoholic fatty liver disease (A) or alcoholic fatty liver disease (B) patients carrying the Ala mutated allele or the Pro wild type allele of the peroxisome-proliferator-activated receptor- γ 2 gene. NAFLD: Non-alcoholic fatty liver disease; AFLD: Alcoholic fatty liver disease. Dots (•) indicate outliers that are not included between the whiskers.

fibrosis according to the recommendations described by Kleiner *et al.*^[15] (Table 1) revealed that in most of the patients steatosis was accompanied by moderate, mild or severe steatohepatitis. In particular, in patients suffering from AFLD steatohepatitis has passed over to fibrosis in 71% of the cases.

Whereas in NAFLD patients inflammation was not significantly associated with the allelic incidence, in AFLD patients the frequency of the Ala12 variant of the PPAR γ 2 gene was significantly increased when prominent inflammation had occurred ($P = 0.028$). The higher risk of AFLD patients developing several inflammatory processes ending in liver fibrosis was also shown by elevated Odds ratios (Odd_{inflammation} = 2.50, CI: 1.05-5.90 and Odd_{fibrosis} = 2.48, CI: 0.81-7.53) (Table 6).

DISCUSSION

FLD has a high incidence of approximately 20% worldwide and is regarded as a major cause of liver-related morbidity and mortality due to its risk of progression into cirrhosis or hepatocellular carcinoma. Since the transcription factor PPAR γ has been shown to be markedly involved in adipogenesis, hepatic lipid storage and metabolism, we first analyzed the frequency of the Pro12Ala polymorphism of the PPAR γ gene in a German cohort of patients with FLD compared to German healthy blood donors. A highly sensitive and robust test was established which was certain to distinguish the Ala and the Pro alleles, even

Table 5 Occurrence of the wt and the mutated form of peroxisome proliferator-activated receptor- γ 2 depending on the grade of steatosis and ballooning *n* (%)

	Steatosis						Ballooning					
	NAFLD			AFLD			NAFLD			AFLD		
	Total	Moderate (0-1)	Severe (2-3)	Total	Moderate (0-1)	Severe (2-3)	Total	-	+	Total	-	+
Allelic discrimination												
Mutation	66 (100)	27 (41)	39 (59)	26 (100)	12 (46)	14 (54)	66 (100)	28 (42)	38 (58)	26 (100)	19 (73)	7 (27)
Wild type	460 (100)	229 (50)	231 (50)	174 (100)	88 (51)	86 (49)	460 (100)	206 (45)	254 (55)	174 (100)	121 (70)	53 (30)
<i>P</i> value		0.112			0.417			0.411			0.454	
Odds ratio (CI)		1.43 (0.85-2.42)			1.19 (0.52-2.73)			1.10 (0.65-1.85)			0.84 (0.33-2.12)	

NAFLD: Non-alcoholic fatty liver disease; AFLD: Alcoholic fatty liver disease.

Table 6 Occurrence of the wt and mutated form of the peroxisome proliferator-activated receptor- γ 2 depending on the grade of inflammation and fibrosis *n* (%)

	Inflammation						Fibrosis					
	NAFLD			AFLD			NAFLD			AFLD		
	Total	Moderate (0-1)	Severe (2-3)	Total	Moderate (0-1)	Severe (2-3)	Total	F0-F1	F2-F4	Total	F0-F1	F2-F4
Allelic discrimination												
Mutation	66 (100)	38 (58)	28 (42)	26 (100)	9 (35)	17 (65)	66 (100)	47 (71)	19 (29)	26 (100)	4 (15)	22 (85)
Wild type	460 (100)	296 (64)	164 (36)	174 (100)	99 (57)	75 (43)	460 (100)	327 (71)	133 (29)	174 (100)	54 (31)	120 (69)
<i>P</i> value		0.175			0.028			0.555			0.075	
Odds ratio (CI)		1.33 (0.79-2.25)			2.50 (1.05-5.90)			0.99 (0.56-1.76)			2.48 (0.81-7.53)	

NAFLD: Non-alcoholic fatty liver disease; AFLD: Alcoholic fatty liver disease.

though only low copy numbers from some FFPE biopsies might be available. The genotype distribution (77.5% wt, 21.2% heterozygous, and 1.5% homozygous Ala/Ala mutants) in the collective of the healthy blood donors resembles previous data collected on more than 600 Caucasians by Yen *et al*^[32] and Ghossaini *et al*^[33]. This genotype distribution in Caucasians, however, differs from the Asian or African frequency, in which less Ala alleles of the PPAR γ occur^[23,32]. In contrast to the data of healthy blood donors, PCR analyses of DNA from subjects with FLD revealed an increased frequency of the homozygous Ala-subtype up to almost 3.5% in both the AFLD and the NAFLD collectives. Recent meta-analyses summarized data of the Pro12Ala polymorphism in patients with diabetes and identified the mutated Ala variant as a protection factor of diabetes type 2^[34-36]. The malfunctioning Ala variant was also shown to be associated with coronary heart disease^[37] and with obesity indicated by significant higher BMI in homozygous Ala carriers than in subjects expressing the heterozygous or the wild type PPAR γ 2 form^[38,39]. Although in some reports higher insulin sensitivity and BMI could not be confirmed^[40,41], a comprehensive study on 1170 British patients with coronary heart disease^[42] and a meta-analysis including 19 136 subjects clearly identified the Ala carriers as individuals with significant higher BMI^[43]. Additionally, cholesterol, LDL-cholesterol and apolipoprotein B concentrations are elevated in Ala carriers^[42,44]. Therefore, this Ala-associated hyperlipidemia is assumed to be a reason for the 2-fold higher incidence of the Ala genotype in patients with FLD compared to

healthy blood donors. Since free fatty acids are shown to be involved in upregulation of Fas/CD-95 death receptor^[45], enhanced levels of circulating fatty acids due to impaired PPAR γ function in Ala/Ala patients may result in apoptosis and inflammatory processes. In contrast to NAFLD, where no or only a moderate link of inflammatory progression to the Pro12Ala polymorphism was shown, a prominent risk of developing steatohepatitis was observed in AFLD patients carrying the Ala allele. This difference in the associated frequency of the Ala variant encoding the minor active PPAR γ 2 form^[23] argues for a divergent role of the PPAR γ 2 in mechanisms of AFLD and NAFLD progression. The PPAR γ 2 isoform is up-regulated by phosphatidylinositol 3-kinase activation in response to free fatty acids or by insulin^[46]. In post-ischemic liver injury and also in alcohol-induced fibrosis, the PPAR γ 1 and γ 2 variants were shown to be downregulated and to function protectively^[47,48]. Therefore, the 2-3-fold higher risk of AFLD patients, but not of NAFLD patients, to develop inflammatory and fibrotic progression if they carry the malfunctioning Ala variant of PPAR γ 2, emphasizes a more prominent anti-inflammatory impact of the PPAR γ 2 in AFLD than in NAFLD wt-carriers.

The anti-inflammatory action of PPAR γ was also demonstrated by previous studies on hepatic stellate cells^[49,50], which take centre stage of sinusoidal liver fibrosis due to their tremendous matrix production and secretion of pro-inflammatory and pro-fibrotic mediators after myofibroblastic transition in chronic liver injury^[16]. The authors show that PPAR γ s repressed in myofibroblastic hepatic

stellate cells. Additionally, the inflammatory chemokine expression by hepatic stellate cells is markedly inhibited in response to the activation of PPAR γ by the agonistic ligand glitazone^[49,50]. Taken into account that the malfunctioning Ala variant is associated with a higher risk of progression into steatohepatitis in AFLD patients these results lead to the suggestion that in particular inflammation and fibrosis of AFLD wt-patients can be attenuated by a treatment with PPAR γ thiazolidinedione ligands such as rosiglitazone and pioglitazone. Patients with NASH, however, may benefit from glitazone therapy by other mechanisms like improved insulin sensitivity, decreased hyperlipidemia and impeded steatosis as a result of the therapeutic approach^[51,52].

In conclusion, our data of a comprehensive study of the Pro12Ala polymorphism on biopsies with FLD, well classified concerning inflammatory and fibrotic alterations, revealed for the first time an association of the Pro12Ala polymorphism with the risk of developing ASH and suggests a more prominent anti-inflammatory influence of the PPAR γ 2 on progression of human AFLD than on NAFLD. Therefore, the Pro12Ala polymorphism should be studied on an expanded cohort of AFLD patients, in order to be later integrated in a panel of genetic markers applied for future improved prognosis of disease progression.

ACKNOWLEDGMENTS

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COMMENTS

Background

Fatty liver diseases are becoming a common cause of chronic liver diseases in the Western countries encountered in about 20% of the general adult and child population. The spectrum of fatty liver diseases (FLD) independently of causative agents ranges from simple steatosis to steatohepatitis, which can progress to liver fibrosis ending up in cirrhosis or hepatocellular carcinoma. Members of the peroxisome proliferator-activated receptors (PPAR) seem to play a key role in the pathophysiology of FLD by modulating increased glucose uptake and hepatic triglyceride accumulation, but also perform anti-inflammatory signals when steatohepatitis has occurred. Since the transcription factor PPAR γ has been shown to be markedly involved in adipogenesis, hepatic lipid storage and metabolism, we analyzed the frequency of the Pro12Ala polymorphism of the PPAR γ gene in a German cohort of patients with FLD compared to German healthy blood donors.

Research frontiers

Due to its high impact as an insulin-sensitising transcription factor involved in adipogenesis and lipogenesis, the occurrence of single nucleotide polymorphisms (SNP) in the PPAR γ gene was recently addressed by numerous reports studying subjects with insulin resistance, type 2 diabetes, arteriosclerosis, and hypertension. A prevalent SNP association with impaired lipid homeostasis was observed in terms of the N-terminal proline alanine exchange (Pro12Ala) of the extra domain in the PPAR γ 2 variant. The authors analyzed the frequency of the Pro12Ala polymorphism in the PPAR γ gene by a highly sensitive LNA-probe based polymerase chain reaction approach in a total of 622 subjects of a Caucasian population, suffering from fatty liver disease ($n = 359$) or healthy blood donors ($n = 263$). In agreement with reports showing a high Ala allele prevalence in patients with impaired lipid metabolism in obese and adipose patient, the Ala allele also

occurs more often in FLD patients than in the healthy control group. Interestingly, the interpretation and linkage of the allele frequency to histological evaluation and clinical data demonstrates a prominent risk in alcoholic fatty liver disease (AFLD) patients bearing the Ala allele to develop severe steatohepatitis and fibrosis.

Innovations and breakthroughs

In order to detect the Ala12Pro polymorphism in patients with fatty liver disease the authors established an assay using locked nucleotide acid probes for allelic discrimination. The authors' data of a comprehensive study of the Pro12Ala polymorphism on biopsies with FLD, well classified concerning inflammatory and fibrotic alterations, revealed in patients with AFLD, but not with non-alcoholic fatty liver disease (NAFLD), an significant association of the Pro12Ala polymorphism with the risk to develop steatohepatitis. Therefore, PPAR γ is suggested to exert a higher anti-inflammatory impact in progression of human AFLD than NAFLD.

Applications

The association of the Pro12Ala polymorphism with the risk of developing inflammatory progression in patients with AFLD suggests a more prominent influence of PPAR γ 2 on progression of human AFLD than on NAFLD. Therefore, the Pro12Ala polymorphism should be studied on an expanded cohort of AFLD patients, in order to be later integrated in a panel of genetic markers applied for future improved prognosis of disease progression and therapy planning.

Peer review

This review article provides an overview of SLE-related gastrointestinal system involvements, and there are only few review article in the international literatures in recent years.

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Translation and validation of the Greek chronic liver disease questionnaire

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performed included: 2 independent sample *t* tests, one-way analysis of variance, reliability coefficients, explanatory factor analysis using a varimax rotation and the principal components method.

RESULTS: One hundred and twenty five (61%) patients were men, half were aged 40-59 years and > 33% were > 60 years old. Among the patients, 48 (23%) were hospitalized and 97 (47%) were cirrhotic according to the Child-Pugh score. The internal consistency of the Greek CLDQ version using Cronbach's alpha coefficient was found to be 0.93. Exploratory factor analysis identified 7 domains accounting for 65% of the variance of CLDQ items and only partially overlapping with those found in the original version. The area under the receiver operating characteristics curve was calculated at 0.813 and the logistic estimate for the threshold score of 167.50 provided a sensitivity of 74.3% and a specificity of 71.6% for the model.

CONCLUSION: Our data confirmed the validity of the Greek version of the CLDQ in identifying the QOL among patients with chronic liver disease.

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Key words: Chronic disease; Questionnaires; Validation; Quality of life; Liver cirrhosis

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Abstract

AIM: To translate into Greek and validate the chronic liver disease questionnaire (CLDQ).

METHODS: Two hundred and six consecutive adult patients with the diagnosis of a chronic liver disease from 2 general hospitals in Athens were enrolled in the study from May to September 2008. In order to assess their quality of life (QOL) the CLDQ was applied. The instrument was translated from English, back translated and reviewed in focus groups within the framework of a large multicenter study. The measurements that were

INTRODUCTION

Chronic liver disease encompasses a wide range of illnesses characterized by liver inflammation and progression to cirrhosis. Quality of life (QOL) is a concept that incorporates many aspects of an individual's experience, general well being and satisfaction, as well as social and physical functioning^[1]. Health-related QOL is important when measuring the impact or burden of a chronic disease, such as liver disease, and is highly correlated with fatigue, loss of esteem, depression and disease complications^[2]. In the last few decades, the assessment of health-related QOL has become an important outcome measure in clinical research in both gastroenterology and hepatology^[3]. Whilst a variety of generic QOL measures have been developed, there is a need to develop specific instruments endowed with sufficient sensitivity to document clinically significant changes over time^[4].

The chronic liver disease questionnaire (CLDQ) is a specific health-related QOL instrument designed for patients with liver disease, regardless of the underlying etiology and degree of disease. Its original version was developed first by Younossi *et al*^[5] and has demonstrated appropriate validity and reliability. The CLDQ has already been cross-culturally adapted and validated into different languages in recently published studies^[6-10]. Consequently, the aim of this paper was to: (1) report on the development of the Greek version of the CLDQ and on the validation procedures carried out; (2) examine the factorial structure of the Greek CLDQ; and (3) evaluate the sensitivity of the Greek CLDQ in assessing QOL over a range of cut-off scores among liver disease patients.

MATERIALS AND METHODS

CLDQ

The CLDQ is a 29-item self-reported scale consisting of statements describing QOL, and is divided into 6 domains including abdominal symptoms, fatigue, systemic symptoms, activity, emotional function and concern. All items refer to the previous 2 wk on a 7-point Likert scale, with 1 corresponding to the maximum frequency labeled as "all of the time" and 7 to the minimum labeled as "none of the time". Permission to reproduce and validate the CLDQ was provided by Younossi *et al*^[5].

Greek version of CLDQ - translation and pilot study

The 29 items of the CLDQs were translated by 2 independent bilingual translators. Another native English speaker who did not have knowledge of the original instrument then back translated the Greek version. The backward translation was sent to a group of English experts for comments. The translated questionnaire was culturally adapted through a cognitive debriefing process that was used to identify any language problems and to assess the degree of respondents understanding of the item's content that was meant to be elicited^[5].

During this stage the reconciled Greek version of the CLDQ was pilot tested among 10 patients. As part of the

cultural adaptation process, in-depth interviews were implemented with regard to the respondents understanding of the questionnaire with the purpose of revealing inappropriately interpreted items and translation alternatives. The participants gave their impression on the clarity of each item, the relevance of the content to their situation, the comprehensiveness of the instructions and their ability to complete it on their own. They were also encouraged to make suggestions whenever necessary. Finally, written comments made by the participants were included in the final Greek version of CLDQ.

Sample and data collection

Consecutive adult patients diagnosed with chronic liver disease, confirmed by laboratory tests, imaging studies and in most cases by liver histology, were asked to participate in our study. Enrolment started in May and ended in September 2008 among patients of the Gastroenterology Clinics of 2 general hospitals in Athens. Inclusion criteria were fluency in the spoken and written Greek language, age > 18 years and the existence of liver disease symptoms during the previous 3 mo. Non-Greek-speaking patients, patients who had undergone liver transplantation, patients with dementia or psychosis, and patients with refractory encephalopathy (grade II and more) were excluded. To assess the severity of liver disease, the patients' Child-Pugh scores were calculated and patients were classified as cirrhotic or not^[11]. In total, 220 patients were approached and 206 patients agreed to participate (rate of attendance 93.6%). CLDQs were also completed by healthy participants ($n = 208$, controls) in order to perform receiver operating characteristics (ROC) analysis. The control group was selected randomly from a list of the Athens county population and was matched with cases by gender, age and educational background. One control was selected for one case participant. Both patients and healthy participants completed the CLDQ in the presence of a nurse.

All participants entering the study provided written informed consent after receiving a complete description of the study and having the opportunity to ask for clarifications. Along with the questionnaires there was a cover letter explaining the purpose of the study, providing the researchers' affiliation and contact information, and clearly stating that answers would be confidential and anonymity would be guaranteed in the final data reports.

Statistical analysis

Descriptive characteristics were determined for the sociodemographic variables of the sample and Student *t* tests were performed on the descriptive characteristics of the study population and the CLDQ score. All *P*-values were based on 2-sided tests and significance was defined as $P < 0.05$. The assumptions of normality, homogeneity and independence of the sample were checked. Reliability coefficients as measured by Cronbach's alpha were calculated for the CLDQ in order to assess reproducibility and consistency of the instrument. The underlying dimensions

Table 1 Patients’ demographic and medical characteristics

	<i>n</i> (%)	Mean score	<i>P</i> -value
Sex			
Men	125 (60.7)	4.77 (1.26)	0.61
Women	81 (39.3)	4.85 (1.13)	
Age (yr)			
≤ 39	40 (19.4)	5.19 (1.15)	0.02
40-59	99 (48.1)	4.92 (1.22)	
≥ 60	67 (32.5)	4.40 (1.13)	
Educational level			
Primary	74 (35.9)	4.55 (1.19)	0.03
Secondary	85 (41.3)	4.84 (1.24)	
Higher	47 (22.8)	5.13 (1.09)	
Family status			
Married	144 (69.9)	4.74 (1.22)	0.27
Non-married	62 (30.1)	4.94 (1.17)	
Child-Pugh classification			
Class A	49 (23.8)	4.71 (1.06)	< 0.001
Class B	32 (15.5)	3.82 (1.12)	
Class C	16 (7.8)	3.36 (0.88)	
No Cirrhosis	109 (52.9)	5.34 (0.95)	
Etiology of liver disease			
Hepatitis B	78 (37.9)	5.07 (1.01)	< 0.001
Hepatitis C	65 (31.6)	4.97 (1.22)	
Autoimmune hepatitis	12 (5.8)	4.72 (1.31)	
Cirrhosis	36 (17.5)	3.92 (1.22)	
Other	15 (7.3)	4.87 (1.10)	

of the scale were checked with an explanatory factor analysis using a varimax rotation and principal components method as a descriptive method for analyzing grouped data^[12]. Factor analysis using principal component analysis with varimax rotation was carried out to determine the dimensional structure of CLDQ using the following criteria: (1) eigenvalue > 1^[13]; (2) variables should have a load > 0.50 on only one factor and < 0.40 on more factors; (3) the interpretation of the factor structure should be meaningful; and (4) the screen plot is accurate in the case where the means of communalities are above 0.60^[14]. Computations were based on a covariance matrix, as all variables were receiving values from the same measurement scale^[15]; Bartlett’s test of sphericity with *P* < 0.05 and a Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy of 0.6 were used when performing this factor analysis. A factor was considered as important if its eigenvalue exceeded 1.0^[13]. As the factor analysis found 2 independent domains, subsequent Cronbach’s alpha measurements were separately performed for each domain, highlighting how the items were grouped together.

Sensitivity and specificity

The sensitivity and specificity were calculated at several cut-off scores of the CLDQ. A ROC analysis was carried out; this method allows the display of all the pairs of sensitivity and specificity values achievable as the threshold is changed from low to high scores, plotting the true-positive rate (sensitivity) on the vertical axis and the false-positive rate (1-specificity) on the horizontal axis. The area under the ROC curve is a quantitative indicator of the information content of a test and it may be interpreted as an estimate of the probability that a liver disease patient

Table 2 The 29 Greek chronic liver disease questionnaire items

	CLDQ item	mean ± SD
Q1	Abdominal bloating	5.08 ± 2.01
Q2	Tiredness or fatigue	3.98 ± 2.14
Q3	Bodily pain	5.22 ± 2.03
Q4	Sleepiness during the day	4.96 ± 1.89
Q5	Abdominal pain	5.67 ± 1.84
Q6	Shortness of breath	5.42 ± 2.02
Q7	Not eating enough	4.78 ± 2.35
Q8	Decreased strength	4.50 ± 2.21
Q9	Trouble in carrying or lifting heavy objects	4.59 ± 2.49
Q10	Anxiety	3.40 ± 2.29
Q11	Decreased energy	4.50 ± 2.25
Q12	Unhappiness	4.67 ± 2.02
Q13	Drowsiness	5.04 ± 1.87
Q14	Bothered by a limitation of the diet	3.17 ± 2.50
Q15	Irritability	4.33 ± 2.12
Q16	Difficulty in sleeping at night	4.91 ± 2.15
Q17	Abdominal discomfort	5.16 ± 2.05
Q18	Worries about the impact of the liver disease	3.95 ± 2.22
Q19	Mood swings	4.55 ± 2.01
Q20	Difficulty falling asleep at night	5.07 ± 2.15
Q21	Muscle cramps	5.50 ± 1.87
Q22	Worries that symptoms will develop into major problems	4.21 ± 2.05
Q23	Dry mouth	5.17 ± 2.06
Q24	Depression	4.74 ± 2.04
Q25	Worries that the condition is getting worse	4.28 ± 2.12
Q26	Problems	5.75 ± 1.74
Q27	Itching	5.78 ± 1.91
Q28	Worries about never feeling any better	4.46 ± 2.22
Q29	Concerned about the availability of a liver in the case of a transplant	6.41 ± 1.42

CLDQ: Chronic liver disease questionnaire.

at random will, at each threshold, have a lower test score than a healthy participant.

RESULTS

Patients’ demographic and medical characteristics are shown in Table 1. Almost 61% of the sample were men and nearly half of the sample (*n* = 99, 48%) were aged 40-59 years with 33% (*n* = 67) aged ≥ 60 years old. Seventy four (36%) had a primary level of education, 41% (*n* = 85) a secondary level and 23% (*n* = 47) had higher education. The majority of the patients (70%) were married. Regarding the patients’ disease status, 23% were hospitalized while 47% were classified as having severe cirrhotic liver disease based on the Child-Pugh classification. The most common etiology for their liver disease was viral hepatitis (69.5%) and cirrhosis (18%). Table 1 also depicts the differences in the mean total score of CLDQ according to the patients’ characteristics. More specifically, older age, lower educational level and cirrhotic disease were found to be associated with lower CLDQ scores and therefore with lower QOL among patients.

The mean score of the CLDQ was 4.81 [standard deviation (SD) 2.01] and ranged from 3.17 to 6.41 (Table 2). The communalities for the Greek CLDQs are presented in Table 3. The internal consistency characteristics of the

Table 3 Inter-item correlation matrix for Greek chronic liver disease questionnaire

	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	Q11	Q12	Q13	Q14	Q15	Q16	Q17	Q18	Q19	Q20	Q21	Q22	Q23	Q24	Q25	Q26	Q27	Q28	Q29
Q1	1.00	0.45	0.42	0.22	0.45	0.37	0.29	0.35	0.34	0.30	0.41	0.20	0.18	0.21	0.21	0.31	0.59	0.26	0.23	0.36	0.16	0.22	0.28	0.20	0.29	0.25	0.36	0.31	0.16
Q2	0.45	1.00	0.56	0.34	0.42	0.57	0.52	0.76	0.67	0.30	0.75	0.34	0.42	0.22	0.30	0.43	0.43	0.40	0.44	0.41	0.21	0.35	0.33	0.31	0.38	0.30	0.32	0.39	0.13
Q3	0.42	0.56	1.00	0.39	0.37	0.33	0.28	0.50	0.41	0.29	0.52	0.37	0.42	0.12	0.18	0.32	0.39	0.24	0.38	0.34	0.21	0.12	0.37	0.30	0.18	0.20	0.24	0.18	0.10
Q4	0.22	0.34	0.39	1.00	0.28	0.34	0.29	0.33	0.30	0.26	0.40	0.24	0.72	0.17	0.16	0.32	0.22	0.20	0.33	0.28	0.28	0.23	0.29	0.19	0.26	0.30	0.14	0.26	0.31
Q5	0.45	0.42	0.37	0.28	1.00	0.41	0.32	0.40	0.36	0.18	0.43	0.18	0.26	0.24	0.17	0.35	0.50	0.34	0.24	0.28	0.14	0.20	0.27	0.17	0.26	0.17	0.23	0.22	0.22
Q6	0.36	0.57	0.33	0.34	0.41	1.00	0.49	0.58	0.57	0.24	0.61	0.20	0.32	0.35	0.28	0.48	0.47	0.39	0.33	0.47	0.29	0.28	0.32	0.29	0.29	0.21	0.32	0.35	0.20
Q7	0.29	0.52	0.28	0.29	0.32	0.49	1.00	0.54	0.50	0.36	0.60	0.38	0.28	0.28	0.34	0.40	0.39	0.41	0.40	0.39	0.31	0.36	0.19	0.36	0.39	0.26	0.27	0.39	0.12
Q8	0.35	0.76	0.50	0.33	0.40	0.58	0.54	1.00	0.78	0.31	0.87	0.44	0.39	0.28	0.26	0.41	0.43	0.42	0.44	0.42	0.20	0.35	0.28	0.38	0.42	0.32	0.32	0.40	0.13
Q9	0.34	0.67	0.41	0.30	0.36	0.57	0.50	0.78	1.00	0.28	0.77	0.32	0.35	0.32	0.18	0.39	0.48	0.30	0.37	0.38	0.19	0.24	0.32	0.21	0.31	0.23	0.36	0.32	0.09
Q10	0.30	0.30	0.29	0.26	0.18	0.24	0.36	0.31	0.28	1.00	0.37	0.41	0.29	0.16	0.45	0.30	0.23	0.36	0.48	0.32	0.18	0.27	0.24	0.36	0.35	0.29	0.08	0.29	0.09
Q11	0.41	0.75	0.52	0.40	0.43	0.61	0.60	0.87	0.77	0.37	1.00	0.39	0.40	0.31	0.31	0.42	0.43	0.42	0.47	0.43	0.26	0.38	0.29	0.36	0.42	0.33	0.32	0.40	0.13
Q12	0.20	0.34	0.37	0.24	0.18	0.20	0.38	0.44	0.32	0.41	0.39	1.00	0.30	0.23	0.35	0.36	0.29	0.34	0.63	0.36	0.08	0.35	0.24	0.68	0.44	0.22	0.16	0.41	0.10
Q13	0.18	0.42	0.42	0.72	0.26	0.32	0.28	0.39	0.35	0.30	0.40	0.30	1.00	0.10	0.15	0.30	0.28	0.23	0.36	0.34	0.28	0.15	0.32	0.26	0.25	0.40	0.20	0.25	0.18
Q14	0.21	0.22	0.12	0.17	0.24	0.35	0.28	0.28	0.32	0.16	0.31	0.23	0.10	1.00	0.14	0.22	0.29	0.36	0.12	0.28	0.06	0.20	0.22	0.14	0.23	0.07	0.17	0.27	0.17
Q15	0.21	0.30	0.18	0.16	0.17	0.28	0.34	0.29	0.18	0.45	0.31	0.35	0.15	0.14	1.00	0.34	0.20	0.46	0.43	0.35	0.13	0.33	0.04	0.32	0.37	0.21	0.12	0.33	0.13
Q16	0.31	0.43	0.32	0.32	0.35	0.48	0.40	0.41	0.39	0.30	0.42	0.36	0.30	0.22	0.34	1.00	0.36	0.30	0.40	0.84	0.28	0.17	0.32	0.38	0.20	0.25	0.22	0.24	0.24
Q17	0.59	0.43	0.39	0.22	0.50	0.47	0.39	0.43	0.48	0.23	0.43	0.29	0.28	0.29	0.20	0.36	1.00	0.36	0.31	0.38	0.22	0.28	0.39	0.29	0.36	0.19	0.37	0.35	0.12
Q18	0.26	0.40	0.24	0.20	0.34	0.39	0.41	0.42	0.30	0.34	0.42	0.34	0.23	0.36	0.46	0.30	0.36	1.00	0.40	0.33	0.15	0.51	0.11	0.38	0.53	0.13	0.19	0.53	0.20
Q19	0.23	0.44	0.38	0.33	0.24	0.33	0.40	0.44	0.37	0.48	0.47	0.63	0.36	0.12	0.43	0.40	0.31	0.40	1.00	0.42	0.14	0.36	0.27	0.60	0.45	0.20	0.24	0.45	0.15
Q20	0.36	0.41	0.34	0.28	0.28	0.47	0.39	0.42	0.38	0.32	0.43	0.36	0.34	0.28	0.35	0.84	0.38	0.33	0.42	1.00	0.30	0.22	0.34	0.39	0.22	0.29	0.26	0.27	0.21
Q21	0.16	0.21	0.21	0.28	0.14	0.29	0.31	0.20	0.19	0.18	0.26	0.08	0.28	0.06	0.13	0.28	0.22	0.15	0.14	0.30	1.00	0.14	0.25	0.21	0.15	0.15	0.23	0.13	0.18
Q22	0.22	0.35	0.12	0.23	0.20	0.28	0.36	0.35	0.24	0.27	0.38	0.35	0.15	0.20	0.33	0.17	0.28	0.51	0.36	0.22	0.14	1.00	0.16	0.43	0.81	0.15	0.10	0.74	0.15
Q23	0.27	0.33	0.37	0.29	0.27	0.32	0.19	0.28	0.32	0.24	0.29	0.24	0.32	0.22	0.04	0.32	0.39	0.11	0.27	0.34	0.25	0.16	1.00	0.26	0.15	0.28	0.32	0.14	0.17
Q24	0.20	0.31	0.30	0.19	0.17	0.29	0.36	0.38	0.21	0.36	0.36	0.68	0.26	0.14	0.32	0.38	0.29	0.38	0.60	0.39	0.21	0.43	0.26	1.00	0.50	0.19	0.18	0.49	0.09
Q25	0.29	0.38	0.18	0.26	0.25	0.29	0.39	0.42	0.31	0.35	0.42	0.44	0.25	0.23	0.37	0.20	0.36	0.53	0.45	0.22	0.15	0.81	0.15	0.50	1.00	0.15	0.11	0.86	0.08
Q26	0.25	0.30	0.20	0.30	0.17	0.21	0.26	0.32	0.23	0.29	0.33	0.22	0.40	0.07	0.21	0.25	0.19	0.13	0.20	0.29	0.15	0.15	0.28	0.19	0.15	1.00	0.04	0.19	0.13
Q27	0.36	0.32	0.24	0.14	0.23	0.32	0.27	0.32	0.36	0.08	0.32	0.16	0.20	0.17	0.12	0.22	0.37	0.19	0.24	0.26	0.23	0.10	0.32	0.18	0.11	0.04	1.00	0.12	0.17
Q28	0.31	0.39	0.18	0.26	0.22	0.35	0.39	0.40	0.32	0.29	0.40	0.41	0.25	0.27	0.33	0.24	0.35	0.53	0.45	0.27	0.13	0.74	0.14	0.49	0.86	0.19	0.12	10.0	0.13
Q29	0.16	0.13	0.10	0.31	0.22	0.20	0.12	0.13	0.09	0.09	0.13	0.10	0.18	0.17	0.13	0.24	0.12	0.20	0.15	0.21	0.18	0.15	0.17	0.09	0.08	0.13	0.19	0.13	1.00

CLDQ: Chronic liver disease questionnaire.

Table 4 Exploratory factors and explained variance after rotation for the Greek chronic liver disease questionnaire

Factors		Rotation sums of squared loadings				
		Rescaled loadings	Eigen values	% of variance	Cumulative variance	Cronbach's alpha
Factor I	Question 2	0.72	4.08	14.07	14.07	0.91
	Question 8	0.84				
	Question 9	0.84				
	Question 11	0.82				
	Question 18	0.55				
Factor II	Question 22	0.85	3.24	11.07	25.24	0.93
	Question 25	0.87				
	Question 28	0.85				
Factor III	Question 1	0.79	2.78	9.57	34.81	0.80
	Question 5	0.57				
	Question 17	0.74				
Factor IV	Question 4	0.83	2.49	8.6	43.41	0.91
	Question 13	0.8				
Factor V	Question 12	0.79	2.42	8.35	51.76	0.79
	Question 24	0.73				
Factor VI	Question 16	0.79	2.11	7.27	59.03	0.84
	Question 20	0.77				
Factor VII	Question 10	0.72	1.68	5.78	64.81	0.67
	Question 15	0.73				

Greek CLDQ showed good reliability as the Cronbach's alpha was 0.93 for the total scale (Items 1-29). The exploratory factor analysis on the 29 items of the CLDQ revealed 2 orthogonal d (KMO measure of sampling adequacy = 0.886 and Bartlett's test of sphericity = 3422.25, $df = 406$, $P < 0.001$). Factor analysis indicated there are 7 principal domains in the model which explained 64.81% as presented in Table 4. The first domain (F1) included the following items: 2 (fatigue), 8 (decreased strength), 9 (trouble lifting heavy objects) and 11 (decreased level of energy) and this domain was named as "Fatigue". The second domain (F2) was composed of items 18 (concern about the impact of liver disease on the family), 22 (concern that symptoms will develop into a major symptom), 25 (concern about the condition getting worse) and 28 (concern about never feeling any better). Therefore F2 represented "Concern". The third factor (F3) included the following items: 1 (abdominal bloating), 5 (abdominal pain) and 17 (abdominal discomfort) and was named as "Abdominal symptoms". The fourth domain (F4) included the following items: 4 (feeling sleepy during the day) and 13 (drowsiness) and we named this as "Activity". The fifth factor (F5) was composed of items 12 (unhappiness) and 24 (feeling depressed). Therefore F5 represents "Emotional function". The sixth domain (F6) was composed of items 20 (unable to fall asleep at night) and 16 (difficulty sleeping at night). Therefore F6 represents "Sleeping disorders". The seventh domain (F7) was composed of items 10 (anxiety) and 15 (irritability). Therefore F7 represents "Anxiety". Cronbach's alpha for the 7 domains ranged from 0.67 (F7) to 0.93 (F2).

The Greek version of the CLDQ was well accepted by the patients. It was easily and very quickly (approximately 10 min) completed. The questions appeared to be relevant, reasonable, unambiguous and clear. Therefore, face validity was considered to be very good. The overall accuracy of the Greek CLDQ, as an instrument

Table 5 Sensitivity and specificity values of different cut-off scores of the Greek chronic liver disease questionnaire for identifying level of quality of life

Threshold scores	Sensitivity (%)	Specificity (%)
147.50	57.8	99.0
157.50	64.6	82.2
167.50	74.3	71.6
175.50	85.4	55.8
185.50	92.2	37.0
194.50	98.5	16.8

for assessing QOL among liver disease patients can be described as the area under its ROC curve calculated as 0.813 (SD = 0.021, Asymp. Sig < 0.0001). Table 5 presents the sensitivity and specificity values for the different cut-off values of the ROC analysis. A 167.50 cut-off score of the CLDQ provided the best sensitivity (74.3%) and specificity (71.6%). Figure 1 depicts the accuracy of the Greek CLDQ for assessing the level of QOL among patients with liver disease.

DISCUSSION

CLDQ is a non-generic, disease-specific instrument for assessing QOL among liver disease patients. Our validation study provided a Cronbach's alpha equal to 0.93 and the factor analysis identified 7 domains with Cronbach's alpha ranging from 0.67 to 0.93 and included "Fatigue", "Concern", "Abdominal symptoms", "Emotional function", "Sleeping disorders", "Anxiety" and "Activity". Those domains explained 65% of the total variance. The ROC analysis presented the highest sensitivity and specificity at the overall score of 167.50, which can be considered as the cut-off score under which QOL can be assessed accurately.

CLDQ has already been validated in many countries

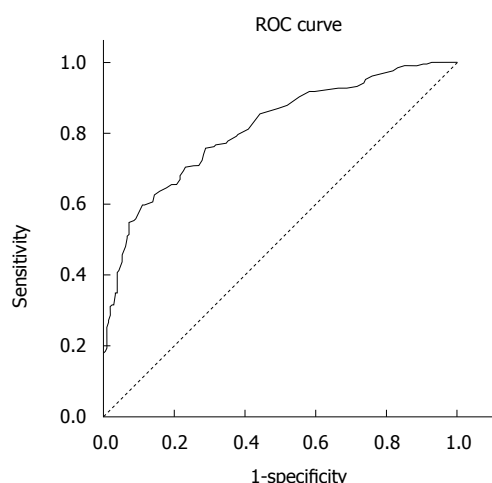


Figure 1 Receiver operating characteristics curve for the Greek chronic liver disease questionnaire. Diagonal segments are produced by ties. ROC: Receiver operating characteristics.

including Spain, Italy, Germany, Lithuania and Thailand and has shown remarkable reproducibility and comparability^[6-10]. The overall Cronbach's alpha for the Greek CLDQ was found to be the same as those reported by the Spanish, Lithuanian and Thailand validation teams whereas the German validation study reported a Cronbach's alpha ranging from 0.69 to 0.95 among different domains. The cumulative variance of the Greek validated questionnaire is similar to the Spanish (68%) and Italian (65.4%) versions.

In comparing the structure of the Greek CLDQ with those of other countries we identified both discrepancies and similarities. The factor analysis of the Greek questionnaire revealed significance in 18 out of the 29 items included in the original one. The Greek validated version of CLDQ revealed a 7 domain structure similar to the Spanish validated questionnaire as opposed to the 6 domains of the original U.S. version^[5] and the Italian version^[10]. Additionally, the Greek version did not include questions regarding systemic symptoms which are included in the original version and in the existing validated versions of the questionnaire (Italian and Spanish)^[9,10]. Sleepiness and drowsiness constitute a new domain in the Greek validation which was named "Activity" whereas in both the Spanish^[9] and in the original validation^[5] they are included under the domain "Fatigue" and in the Italian version^[10] under the domain "Somatic symptoms". "Anxiety" (including anxiety and irritability items) also consist of a separate domain in our validation whereas in the other validation studies these items are included in the domain of "Emotional symptoms". The "Fatigue" domain included the definitions of "felt drowsy" and "felt sleepy during the day" in the original and Spanish version^[9] whereas in the Greek version these items are included in a new domain which was named as "Sleeping disorders". Only the factor comprising items that explored patient concern is common among the different versions.

The ROC analysis confirmed the effectiveness of CLDQ in assessing health-related QOL in the range of

cut-off scores proposed. In our study, the high sensitivity (74.3%) of the 167.50 score allows the use of this cut-off score in the clinical assessment of QOL. If a health professional would like to use the Greek CLDQ for QOL assessment then these different cut-offs should be used. It is very important for the CLDQ to be used as a diagnostic tool in clinical practice which may allow health care professionals to understand the impact of health care interventions on the patient's everyday life, rather than the effects of treatment on their bodies^[16]. Additionally, the Greek CLDQ's use provides a basis for the holistic view of the patient and therefore may help facilitate a dialogue with patients with low QOL.

In general, this is the first study to validate the Greek CLDQ which is recommended to be incorporated into research and clinical practice to allow international comparison of the results of separate national studies^[4]. An important strength of our study is that this is the first study to perform a ROC analysis which provided us with a cut-off score for assessing QOL accurately among patients with liver disease. Our findings also revealed a different structure of the questionnaire after the factor analysis which underlines the necessity of cultural validation and adaptation of the questionnaire before its use in specific countries. A limitation of this validation study was that there was no test-retest, because it may have resulted in a low correlation due to an actual change in the QOL symptoms. Additionally, the high percentage of patients with a cirrhotic liver disease might have affected the CLDQ score as the complications associated with cirrhosis, such as hepatic encephalopathy, have been shown to negatively influence physical and mental domains of QOL^[17]. On the other hand, the exclusion of the patients with hepatic encephalopathy of grade II or more might have reduced this error. In addition, the ethnic and cultural background of the patients may have had an effect on the score of CLDQ whereas previous studies have reported no differences in the CLDQ score among different ethnic groups^[8,17].

The Greek version of the CLDQ has shown a satisfactory reliability and the factor analysis indicated 7 factors that were of interest. We can therefore assert that it is a reliable and valid tool for identifying QOL among liver disease patients and it can be used by health professionals in their clinical practice to improve assessment of patients with low scores. Our findings, however, need to be confirmed by future cross-sectional and cohort studies.

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COMMENTS

Background

Chronic liver disease questionnaire (CLDQ) is a disease specific instrument for assessing quality of life (QOL) among liver disease patients. In the last few decades, the assessment of QOL related to chronic liver disease has become an important outcome measure in clinical research.

Research frontiers

The CLDQ has already been cross-culturally adapted and validated into different languages. In the current study, the authors aimed to translate and validate the Greek version of the CLDQ.

Innovations and breakthroughs

The authors' findings revealed a different structure of the questionnaire after the factor analysis, which underlines the necessity of cultural validation and adaptation of the questionnaire before its use in specific countries. This is the first study to validate the Greek CLDQ which therefore should be incorporated into research and clinical practice so as to allow international comparison of the results of separate national studies. An important strength of the study is that this is the first study to perform a receiver operating characteristics (ROC) analysis which provided us with a cut-off score for accurately assessing QOL among patients with liver disease.

Applications

The Greek version of the CLDQ has shown a satisfactory reliability. The authors can therefore assert that it is a reliable and valid tool for identifying QOL among liver disease patients and it can be used by health professionals in their clinical practice to improve assessment of patients with low scores.

Terminology

ROC curve: a graphical plot of the sensitivity, or true positives, vs (1-specificity), or false positives, for a binary classification system as its discrimination threshold is varied.

Peer review

Dr. Zoi Kollia and colleagues validated the Greek version of the CLDQ to assess QOL factors for patients with chronic liver diseases. The establishment of QOL measures or indices is very important to assess what physical or mental status a patient is in and to compare multinational patients. This study provides an important clue to assess QOL of patients with chronic liver diseases.

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Serum immune-activation potency and response to anti-TNF- α therapy in Crohn's disease

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Abstract

AIM: To study whether immune-activation stage in serum of adult Crohn's disease (CD) patients correlates with disease activity and with treatment response to anti-tumor necrosis factor- α (TNF- α) therapy.

METHODS: Serum samples were obtained from 15 adult CD patients introduced to anti-TNF- α therapy. The individual stage of immune activation was studied applying our new *in vitro* assay, in which target cells (donor derived peripheral blood mononuclear cells) were cultured with patient serum and the T-cell activation in-

duced by the patient serum was studied using a panel of markers for effector [interferon γ (IFN γ), interleukin (IL)-5] and regulatory T-cells [forkhead transcription factor 3 (FOXP3) and glucocorticoid-induced tumour necrosis factor receptor (GITR)]. The endoscopic disease activity was assessed with the Crohn's disease endoscopic index of severity (CDEIS) before and 3 mo after therapy with an anti-TNF- α agent.

RESULTS: Low induction of FOXP3 and GITR in target cells cultured in the presence of patient serum was associated with high disease activity i.e. CDEIS assessed before therapy ($r = -0.621$, $P = 0.013$ and $r = -0.625$, $P = 0.013$, respectively). FOXP3 expression correlated inversely with pre-treatment erythrocyte sedimentation rate ($r = -0.548$, $P = 0.034$). Low serum induced FOXP3 ($r = -0.600$, $P = 0.018$) and GITR ($r = -0.589$, $P = 0.021$) expression and low IFN γ secretion from target cells ($r = -0.538$, $P = 0.039$) associated with treatment response detected as a decrease in CDEIS.

CONCLUSION: The immune-activation potency in the patient serum prior to anti-TNF- α therapy reflected intestinal inflammation and the therapeutic response.

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Key words: Crohn's disease endoscopic index of severity; Forkhead transcription factor 3; Glucocorticoid-induced tumour necrosis factor receptor; Infliximab; Inflammatory bowel disease

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INTRODUCTION

Tumor necrosis factor- α (TNF- α), a pro-inflammatory protein secreted mainly by monocytes, macrophages and T-cells, has a central role in the disease pathogenesis of Crohn's disease (CD)^[1-3]. TNF- α is elevated in stools and mucosa of CD patients^[4-6].

The chimeric TNF- α -antibody infliximab and human IgG₁ TNF- α -antibody adalimumab are indicated for the treatment of moderate to severe CD^[7,8]. During treatment with an anti-TNF- α agent, clinical improvement is accompanied by significant healing of endoscopic lesions and the disappearance of mucosal inflammatory infiltrate^[9]. The therapeutic response, however, is not uniform and a significant proportion of patients are non-responders. In the ACCENT I study, 58% of patients with moderate CD responded to the first infusion of infliximab and with adalimumab 24%-36% of TNF- α -antibody naïve patients responded to the induction of therapy^[7,8].

There are a few studies exploring immunological markers that reflect treatment response to anti-TNF- α therapy in CD. Elevated concentration of mucosal nuclear NF- κ Bp65 and high secretion of TNF- α by cells of peripheral blood cultivation precede clinical relapse^[10]. In the study by Mäkitalo *et al.*^[11], the expression profile of the macrophage tissue inhibitor of metalloproteinase (TIMP)-1 and stromal TIMP-3 in the intestine correlated positively with the Crohn's disease endoscopic index of severity (CDEIS) and the down regulation of matrix metalloproteinase-9 (MMP9) production of macrophages correlated with histological improvement during anti-TNF- α therapy. Further, in another study serum MMP9 levels seemed to decrease particularly in those CD patients who responded to infliximab therapy^[12].

Recently, we described a novel approach to study individual treatment responses at an early phase of glucocorticoid therapy. In that study we investigated pediatric patients with inflammatory bowel disease (IBD) introduced to systemic glucocorticoids and showed that patient serum modified the expression of T-cell signalling markers on target cells (peripheral blood mononuclear cells from a healthy donor)^[13].

Encouraged by this finding we applied the same method to study the individual immune-activation potency in adult CD patients starting anti-TNF therapy. We measured the expression of the regulatory T-cell markers forkhead transcription factor 3 (FOXP3) and glucocorticoid-induced tumour necrosis factor receptor (GITR), and cytokines interferon γ (IFN γ), interleukin-5 (IL-5) and IL-17 induced in the target cells by patient serum in 15 adult CD patients at the beginning of anti-TNF- α therapy. We found that prior to anti-TNF- α therapy the ability of patient serum to modulate the FOXP3 and GITR activation of the target cells mirrored the disease activity and the individual therapeutic response in the gut assessed with the CDEIS.

MATERIALS AND METHODS

Subjects

Fifteen adult patients (6 females) with established CD

Table 1 Patient characteristics

Age (yr): median (range)	25 (19-44)	
Disease duration, years median (range)	5.1 (0.4-27)	
	No. of patients	%
Disease location		
Ileum	2	13
Colon	4	27
Ileocolon	9	60
Disease type		
Inflammatory	7	47
Stricturing	5	33
Inflammatory + perianal	3	20
Prior anti-TNF-therapy	4	27
Prior bowel operation	4	27
Smokers	7	47
Baseline concomitant medication		
Azathioprine/6-mercaptopurine	10	67
Methotrexate	2	13
Corticosteroids	10	67
Mesalamine or sulphasalazine	13	87
Week 12 concomitant medication		
Azathioprine/6-mercaptopurine	13	87
Methotrexate	2	13
Corticosteroids	1	6.7
Mesalamine or sulphasalazine	11	73
CDAI at baseline, median (range)	158 (49-605)	
CDAI at week 12, median (range)	66 (24-202)	

CDAI: Crohn's disease activity index; TNF: Tumor necrosis factor.

were introduced to an anti-TNF- α agent due to an acute flare ($n = 6$), chronic active disease (6), or rapid postoperative recurrence of the disease (3; Table 1). Fourteen patients received infliximab infusion 5 mg/kg at week 0 and 8. One patient received an adalimumab induction dose 80 mg subcutaneously (*s.c.*) at week 0, followed by 40 mg *s.c.* every other week until week 8. After the beginning of the anti-TNF- α treatment, corticosteroids were tapered off.

All patients underwent an ileocolonoscopy before the introduction of anti-TNF- α therapy (median 7 d, range 1-38 d) and the endoscopic assessment of treatment response was performed at week 12 (week 10 for the adalimumab-treated patient). The endoscopic activity was graded according to the CDEIS^[14,15]. This score is based on the presence of superficial or deep ulcerations, proportion of affected and ulcerated surface, and presence of either ulcerated or non-ulcerated stenosis in the terminal ileum and four segments of the colon (right, transverse, left colon and sigmoid, and rectum)^[14]. Clinical disease activity was assessed with the Crohn's disease activity index (CDAI)^[16].

Exclusion criteria were contraindication to anti-TNF- α treatment, pregnancy, history of extensive bowel resection, ostomy, long-term use of nonsteroidal anti-inflammatory drugs, or perianal fistulating disease without luminal inflammation.

Blood samples and fecal calprotectin

A serum sample for the target cell assay was provided at the time of the first ileocolonoscopy. The routine blood samples for serum C-reactive protein (CRP, normal value < 10 mg/L), erythrocyte sedimentation rate (ESR) and

fecal samples for measurement of calprotectin (PhiCal Test, Calpro AS, Oslo, Norway^[17,18]) were obtained by the time of the endoscopies and 3 mo after the first anti-TNF- α dose^[15].

Laboratory assay for systemic immunological effects

The assay for the assessment of individual stages of immunoactivation by applying patient serum in an in vitro culture of donor derived peripheral blood mononuclear cells (PBMC, target cells) is described recently in detail^[13]. In brief, healthy donor (male 34 years) derived PBMC were separated by Ficoll-Paque (Amersham Biosciences) centrifugation ($800 \times G$, 25 min) and cultured in the presence of the patients inactivated (35 min in $56^\circ C$) serum at an end concentration of 8%, either at resting state or activated with mitogen phytohemagglutinin (PHA, 5 $\mu g/mL$). Serum of a healthy donor (male 27 years) was used as the methodological control between cell culture plates. After 72 h incubation at $37^\circ C$ in humidified atmosphere with 5% CO₂/air the supernatants were collected and stored at $-70^\circ C$. This assay was performed with serum samples drawn prior to first anti-TNF- α infusion.

ELISA for IFN γ , IL-5 and IL-17

IFN γ , IL-5 and IL-17 were measured with ELISA in duplicate from the supernatants collected from the target cell cultures (see above) incubated with patient serum. IFN γ and IL-5 was detected as described before^[19,20]. IL-17 was measured according to the manufacturer's protocol (Catalogue no: DY317; R&D Systems, United Kingdom). We subtracted the non-stimulated value from the stimulated value to obtain the Δ -value for statistical analyses.

Quantitative reverse transcriptase-polymerase chain reaction

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) process was recently described in detail^[13]. Briefly, total RNA was isolated from cell samples with the GenElute Mammalian total RNA miniprep kit (Sigma-Aldrich), and the RNA concentration was measured by a spectrophotometer (ND-1000, NanoDrop Technologies Inc, Wilmington, DE, USA). Reverse transcription was performed by using TaqMan Reverse Transcription reagents (Applied Biosystems, Foster City, CA, USA) with additional treatment of total RNA at 10 ng/ μL with DNase I (0.01 U/ μL) (Roche Diagnostics, Mannheim, Germany) to eliminate genomic DNA. Quantitative RT-PCR was performed using predesigned FAM-labelled TaqMan Gene Expression Assay reagents (Applied Biosystems) and the ABI Prism 7700 Sequence Detection System (Applied Biosystems) in triplicate wells. Assay reagents for FOXP3 (Hs00203958_m1), GITR (Hs00188346_m1), IFN γ (Hs00174143_m1) and 18s RNA (Hs99999901_s1) were used. The difference value (ΔCt) is the normalised quantitative value of the expression level of the target gene achieved by subtracting the Ct value of the reference gene (18s) from the Ct value of the target gene. An exogenous cDNA pool calibrator was collected from

PHA stimulated PBMC and considered as an interassay standard to which normalized samples were compared. $\Delta\Delta Ct$ is the difference between the ΔCt of the analyzed sample and ΔCt of the calibrator. Calculation of $2^{-\Delta\Delta Ct}$ gives a relative amount of the target gene in analyzed sample compared with the calibrator, both normalized to an endogenous control (18S). For presentations the relative amount of target genes was multiplied by 1000 and expressed as relative units.

Statistical analysis

Results are reported as median. Comparison between two dependent samples was calculated with Wilcoxon rank test and two independent samples was calculated with Mann-Whitney t -test. The two-tailed Spearman's rho was used for calculation of the correlations and Kruskal-Wallis test served in exploring associations between groups (SPSS 16.0 program). $P < 0.05$ was set for statistical significance.

Ethics

All patients gave their informed written consent for participation in this study approved by the ethics committee of the Helsinki University Central Hospital.

RESULTS

Patient serum induced IFN γ , FOXP3 and GITR specific mRNA expression and secretion of IFN γ , IL-5 and IL-17 from target cells

The expression levels of IFN γ , FOXP3 and GITR specific mRNA in both resting and activated target cells cultured in the presence of CD patient serum obtained before anti-TNF- α therapy is shown in Table 2. Also, the secretion of IFN γ , IL-5 and IL-17 from activated target cells is shown in Table 2. The secretion of IFN γ , IL-5 and IL-17 from resting target cells was below detection limits.

The type of CD or localization was not associated with the level of IFN γ , FOXP3 and GITR specific mRNA expression or IFN γ , IL-5 and IL-17 secretion from target cells (all $P = NS$).

CDEIS

During anti-TNF- α therapy the CDEIS decreased from a median of 13 points (range 1.8-25) to 4.8 points (range 0-11, $P = 0.002$). 12/15 patients responded to therapy, while 3 patients had no decrease in the CDEIS.

Correlations between the target cell responses and pre-treatment the CDEIS

The expression of regulatory T-cell markers FOXP3 and GITR specific mRNA in activated target cells cultured with patient serum correlated inversely with the pre-treatment CDEIS (FOXP3 $r = -0.621$, $P = 0.013$ and GITR $r = -0.625$, $P = 0.013$; Figure 1). A trend towards an inverse correlation between IFN γ mRNA expression and the pre-treatment CDEIS was observed ($r = -0.446$, $P = 0.095$). There was no correlation between IFN γ , IL-5 or IL-17 secretion from target cells and the pre-treatment CDEIS (P

Table 2 The effect of Crohn's disease patient serum withdrawn before anti-tumor necrosis factor- α therapy on forkhead transcription factor 3, glucocorticoid-induced tumour necrosis factor receptor and interferon γ specific mRNA expression (relative units) and interferon γ , interleukin-5 and interleukin-17 secretion (pg/mL) from peripheral blood mononuclear cells obtained from healthy volunteers (target cells)

Patient No.	RT-qPCR						ELISA		
	FOXP3	FOXP3 PHA	GITR	GITR PHA	IFN γ	IFN γ PHA	IFN γ PHA	IL-5 PHA	IL-17 PHA
1	4.9	53.8	10.8	65.0	1.8	11.7	1580.0	8.4	0.0
2	8.2	65.4	18.7	175.9	3.8	30.5	37400.0	80.5	19.4
3	12.9	127.7	30.8	452.9	11.6	128.9	172000.0	187.0	239.0
4	9.2	153.7	32.5	399.2	5.0	52.0	97600.0	154.0	265.0
5	10.0	68.4	22.8	183.2	8.2	22.6	38100.0	81.5	118.0
6	4.0	4.6	8.9	55.2	1.3	10.4	0.0	0.5	42.7
7	11.5	36.7	22.4	107.0	6.1	19.3	1460.0	8.5	20.9
8	7.9	97.0	17.93	171.0	5.8	26.2	0.0	6.5	82.0
9	4.6	40.7	9.3	66.6	3.0	4.5	0.0	1.0	0.0
10	6.4	68.3	15.8	145.8	4.2	15.8	28.8	4.1	0.0
11	7.4	96.9	17.0	251.4	3.4	36.1	173.0	7.4	0.0
12	6.9	86.4	15.1	217.4	4.3	36.2	223000.0	144.0	638.0
13	5.2	32.7	14.1	83.0	3.8	5.3	11600.0	3.3	341.0
14	3.8	81.6	12.7	180.7	2.9	13.5	1660.0	0.5	181.0
15	5.4	31.2	9.9	54.4	3.6	3.3	16100.0	5.5	310.0

Secretion of interferon (IFN) γ , interleukin (IL)-5 and IL-17 from naive target cells was below detection limit. Phytohemagglutinin (PHA) = Target cells activated with phytohemagglutinin. RT-qPCR: Quantitative reverse transcriptase-polymerase chain reaction; GITR: Glucocorticoid-induced tumour necrosis factor receptor; FOXP3: Forkhead transcription factor 3.

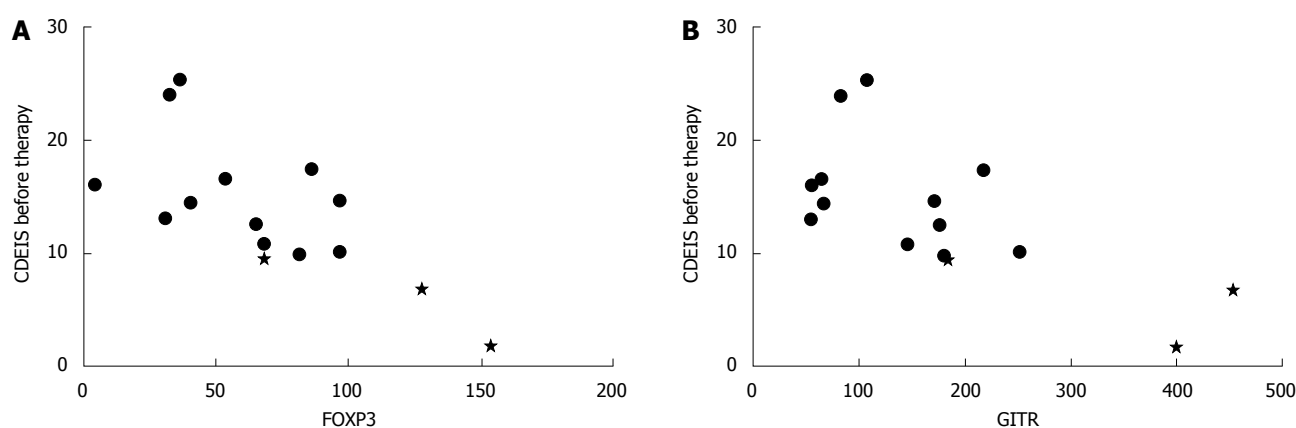


Figure 1 Patient serum withdrawn before anti-tumor necrosis factor- α therapy induced forkhead transcription factor 3 (A) and glucocorticoid-induced tumour necrosis factor receptor (B) specific mRNA expression (relative units) in activated target cells that correlated negatively with pre-treatment Crohn's disease endoscopic index of severity. [points; forkhead transcription factor 3 (FOXP3) $r = -0.621$, $P = 0.013$; glucocorticoid-induced tumour necrosis factor receptor (GITR) $r = -0.625$, $P = 0.013$]. Patients who had no decrease in Crohn's disease endoscopic index of severity (CDEIS) during therapy are marked with star.

$= 0.241$ for IFN γ , $P = 0.286$ for IL-5 and $P = 0.980$ for IL-17).

Correlations between target cell responses and the change of CDEIS during anti-TNF- α therapy

Low patient serum induced FOXP3, GITR and IFN γ specific mRNA expression in target cells was associated with a remarkable change of CDEIS observed during 3 mo therapy (FOXP3 $r = -0.600$, $P = 0.018$; GITR $r = -0.589$, $P = 0.021$; IFN γ $r = -0.486$, $P = 0.066$; Figure 2). Accordingly, in resting target cells GITR specific mRNA expression correlated with the change of CDEIS ($r = -0.550$, $P = 0.034$).

Also low serum induced IFN γ and IL-5 secretion from activated target cells was associated with a high

change of CDEIS ($r = -0.538$, $P = 0.039$; $r = -0.504$, $P = 0.055$). IL-17 secretion from activated target cells did not correlate with the change of CDEIS ($P = 0.467$).

Findings related to fecal calprotectin

Fecal calprotectin decreased from a median of 1170 $\mu\text{g/g}$ (range 88-15300) to a median of 130 $\mu\text{g/g}$ (range 13-1400) within the 3 mo anti-TNF- α treatment ($P = 0.001$). No correlation was observed between target cell responses and calprotectin levels before or after treatment.

Findings related to ESR and CRP

ESR decreased from a median of 18 mm/h (range 6-58) to a median of 10.6 mm/h (range 1-40; $P = 0.001$) and CRP decreased from a median of 10 mg/L (range 0-54)

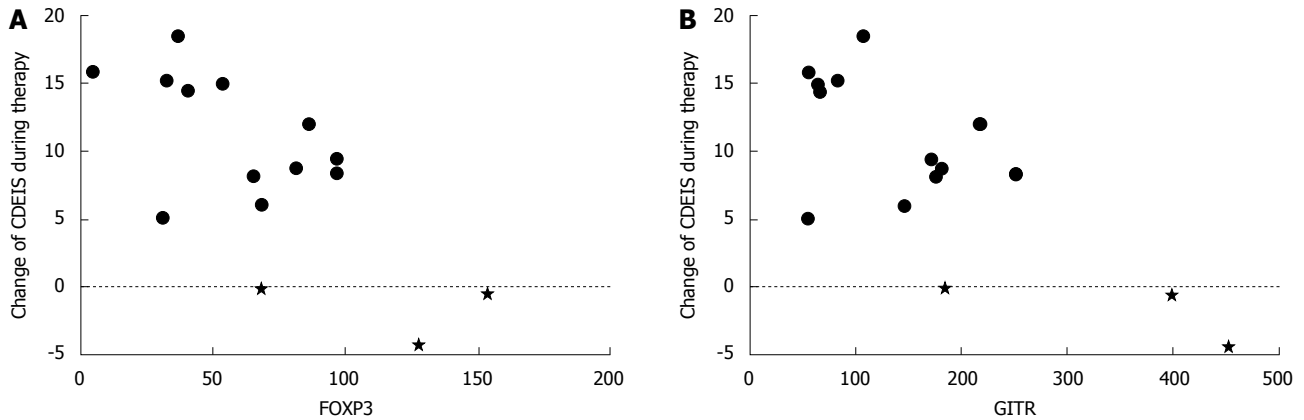


Figure 2 Patient serum withdrawn before anti-tumor necrosis factor- α therapy induced (A) forkhead transcription factor 3 ($r = -0.600$, $P = 0.018$) and (B) glucocorticoid-induced tumour necrosis factor receptor ($r = -0.589$, $P = 0.021$) specific mRNA expression (relative units) in activated target cells that had a negative correlation with the change of Crohn's disease endoscopic index of severity during three months therapy. The change of Crohn's disease endoscopic index of severity (CDEIS) corresponds with the decrease in points along improvement and is given as a positive value to illustrate the magnitude of therapeutic response. Patients who showed no decrease in the CDEIS during therapy are marked with star. GITR: Glucocorticoid-induced tumour necrosis factor receptor; FOXP3: Forkhead transcription factor 3.

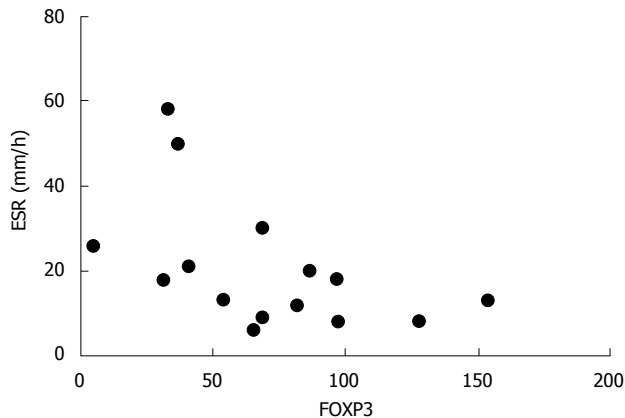


Figure 3 Patient serum withdrawn before anti-tumor necrosis factor- α therapy induced forkhead transcription factor 3 specific mRNA expression (relative units) in activated target cells that correlated negatively with erythrocyte sedimentation rate. ESR: Erythrocyte sedimentation rate. $r = -0.548$, $P = 0.034$.

to a median of < 5 mg/L (range < 5 -11, $P = 0.005$) within 3 mo after introduction of anti-TNF- α therapy.

FOXP3 specific mRNA expression in activated target cells correlated negatively with pre-treatment ESR ($r = -0.548$, $P = 0.034$, Figure 3). There was no correlation between pre-treatment ESR and GITR ($P \geq 0.210$) or IFN γ ($P \geq 0.109$) specific mRNA expression or cytokine secretion from target cells (IFN γ $P = 0.755$, IL-5 $P = 0.434$, IL-17 $P = 0.511$).

FOXP3, GITR or IFN γ specific mRNA expression in target cells or IFN γ , IL-5 or IL-17 secretion from target cells did not correlate with the change of ESR or CRP during 3 mo anti-TNF- α therapy (all $P = \text{NS}$, data not shown).

DISCUSSION

We found that serum samples obtained from patients with

CD before the introduction of anti-TNF- α therapy modulated the expression of regulatory T-cell (T-reg) markers FOXP3 and GITR and secretion of inflammatory cytokines IFN γ , IL-5 and IL-17 from target cells (peripheral blood mononuclear cells from healthy donors). The serum induced FOXP3, GITR and IFN γ responses of target cells correlated with the pre-treatment endoscopic status and also with therapeutic responses, i.e. mucosal improvement assessed with CDEIS within 3 mo.

In our previous study of children with IBD we found that the attenuation of systemic inflammation after the start of oral glucocorticoids was mirrored in the target cell responses induced by the patient serum^[13]. We underline that we measured cytokine secretion and transcription markers of the target cells (donor derived PBMCs) modulated by the patient serum and not the activation stage of the PBMC from the patient. To our knowledge the ability of patient serum to activate signalling of normal T-cells has not previously been studied in adult CD patients at induction of anti-TNF- α therapy. It is remarked that post-treatment samples are not comparable here since the serum taken after the treatment contains an anti-TNF- α agent.

We found an inverse correlation between the expression of T-reg markers FOXP3 and GITR in target cells and the endoscopic disease activity before therapy. A similar inverse correlation existed also between FOXP3 and pre-treatment ESR. Since FOXP3 inhibits T-cell activation by its suppressive effect on transcription of cytokine genes^[21] it is reasonable to assume that those patients whose serum environment seemed to mediate enhanced FOXP3 up-regulation as a response to T-cell stimulation had endoscopically milder disease. However, the serum induced expression of FOXP3 in target cells was not directly reflected in the cytokine activation of the target cells and cytokine response did not correlate with the pre-treatment CDEIS. Impaired up-regulation of GITR in the patient serum environment may be related to poor suppression of T-cell activation^[22,23].

Interestingly, we also found that low expression of FOXP3 and GITR specific mRNA induced by patient serum obtained prior to therapy was associated with a good therapeutic response within 3 mo. These parameters were associated with high clinical activity and thus enhanced inflammation *in vivo*. High inflammatory activity at the early phase of anti-TNF- α therapy has been connected to the lack of therapeutic response in rheumatoid arthritis (RA). Previously non-responders to anti-TNF- α agents had a higher number of blood T-cells expressing chemokine receptors (CCR 3 and CCR 5) before the introduction of therapy^[24]. In another study, high levels of serum IL-2 were associated with poor therapeutic response^[25]. In our study, the group of patients that had high potency for FOXP3 induction in target cells showed poor clinical response to anti-TNF treatment. It was also evident that their disease activity before treatment was milder. TNF blocking has been shown to induce FOXP3 expression in patients with RA. Recovery of regulatory mechanisms has been proposed to be one of the mechanisms of action for TNF blocking^[26]. It is possible that non-responders whose serum induced high FOXP3 up-regulation in target cells do not benefit from further activation of FOXP3 but their disease activity should be down-regulated by other mechanisms.

The majority of CD patients driven to anti-TNF- α therapy are on immunosuppressive medication such as azathioprine or methotrexate as here also. However, there was no correlation between patient serum induced expression or secretion of inflammatory cytokines from target cells and pre-treatment disease activity. We suggest that individual differences in target cell responses mediated by patient serum represents the net effect of maintenance medication, disease activity and patients immunological heterogeneity that together reflect the patients further capability to respond to biological therapy.

Fecal calprotectin correlates with the CDEIS and CRP^[15,27]. In this study we failed to find statistically significant correlations between calprotectin or CRP and target cell responses. This finding was similar to the finding in our previous study of IBD children. Fecal calprotectin excretion reflects increased neutrophils and mononuclear cell migration into the gut lumen through the inflamed mucosa^[28] and CRP is an acute phase protein produced predominantly in the liver in response to stimulation by IL-6, TNF- α and IL-1 β ^[29]. It seems that the ability of patient serum to activate target cells reflects the immunological net effects in circulation in CD patients rather than inflammatory cell accumulation in the intestine reflected in fecal calprotectin.

To conclude, there are few studies of the mechanisms of treatment failure during anti-TNF- α therapy. An impaired response to anti-TNF- α therapy in CD has been suggested to be a result of early reactivation of the inflammatory cascade caused by individual intrinsic immunological mechanisms^[10]. Also the inflammatory activity of the disease itself may play a role in the therapeutic response. We found that the immune activation potency of the patient serum that is monitored by gene expression profile

of human PBMC is individual and correlates to later mucosal healing during anti-TNF- α therapy. Characterization of the key factors in serum that mediated the effects observed with this method, such as up-regulation of FOXP3 and GITR, could be one step toward better understanding of *in vivo* actions of anti-TNF- α therapies.

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COMMENTS

Background

Since the FDA approval of infliximab in 1999 for the treatment of severe Crohn's disease (CD), the use of tumor necrosis factor- α (TNF- α)-antagonist agents has emerged in treatment of severe inflammatory bowel disease. A significant proportion of patients, however, do not respond to the treatment. To date there are no means to foresee the therapeutic response or to monitor the response at an early phase of therapy with an TNF- α -antagonist agent.

Research frontiers

Recently, the authors applied a novel immunological assay for assessment of therapeutic response to glucocorticoids in pediatric patients with inflammatory bowel disease. In that study they showed that the therapeutic effect of corticosteroid therapy can be measured from patient serum at an early phase of the therapy. In the *in vitro* assay, a sample of patient serum is used to stimulate human white blood cells and the effect on specific white blood cell (T cell) markers is assessed. This kind of testing seems a promising means to predict individual responses to immunological therapies.

Innovations and breakthroughs

There are few studies exploring the immunological markers that reflect treatment response to anti-TNF- α therapy in CD. Here the authors used a recently described assay to measure therapeutic response to TNF- α -antagonist therapy from a patient serum sample. Disease activity and response to therapy is reflected in the patient serum and can be measured before the introduction of therapy. Serum induced changes in the specific white blood cell markers [forkhead transcription factor 3 (FOXP3), glucocorticoid-induced tumour necrosis factor receptor (GITR)] seemed to reflect individual response to anti-TNF- α therapy.

Applications

The results suggest that the effect of therapy with an anti-TNF- α agent can be measured from patient serum at an early phase. The study group was small and the results are preliminary, thus more studies are warranted to establish whether this kind of serum testing is suitable for predicting the individual response to anti-TNF- α therapy in clinical practice.

Terminology

Forkhead box P3, FOXP3, is a gene regulating the development and function of specific white cells, regulatory T cells. GITR is glucocorticoid-induced tumour necrosis factor receptor. FOXP3 and GITR are commonly used markers for regulatory T-cell activity.

Peer review

This study raises some interesting points never raised before about the T regulatory cell responses with biological agents.

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Nitric oxide activation of a potassium channel (BK_{Ca}) in feline lower esophageal sphincter

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Abstract

AIM: To assess the effect of nitric oxide (NO) on the large conductance potassium channel (BK_{Ca}) in isolated circular (CM) and sling (SM) muscle cells and muscle strips from the cat lower esophageal sphincter (LES) to determine its regulation of resting tone and relaxation.

METHODS: Freshly enzymatically-digested and isolated circular smooth muscle cells were prepared from each LES region. To study outward K⁺ currents, the perforated patch clamp technique was employed. To assess LES resting tone and relaxation, muscle strips were mounted in perfused organ baths.

RESULTS: (1) Electrophysiological recordings from isolated cells: (a) CM was more depolarized than SM (-39.7 ± 0.8 mV vs -48.1 ± 1.6 mV, $P < 0.001$), and maximal outward current was similar (27.1 ± 1.5 pA/pF vs 25.7 ± 2.0 pA/pF, $P > 0.05$); (b) The NO donor sodium nitroprusside (SNP) increased outward currents only in CM (25.9 ± 1.9 to 46.7 ± 4.2 pA/pF, $P < 0.001$) but not SM (23.2 ± 3.1 to 27.0 ± 3.4 pA/pF, $P > 0.05$); (c) SNP added in the presence of the BK_{Ca} antagonist iberiotoxin (IbTX) produced no increase in the outward current in CM (17.0 ± 2.8 vs 13.7 ± 2.2 , $P > 0.05$); and (d) L-NNA caused a small insignificant inhibition of outward K⁺ currents in both muscles; and (2) Muscle strip studies: (a) Blockade of the nerves with tetrodotoxin (TTX), or BK_{Ca} with IbTX had no significant effect on resting tone of either muscle; and (b) SNP reduced tone in both muscles, and was unaffected by the presence of TTX or IbTX.

CONCLUSION: Exogenous NO activates BK_{Ca} only in CM of the cat. However, as opposed to other species, exogenous NO-induced relaxation is predominantly by a non-BK_{Ca} mechanism, and endogenous NO has minimal effect on resting tone.

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Key words: Circular smooth muscle; Feline; K⁺ channel; Lower esophageal sphincter; Nitric oxide; Sling; Tone

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INTRODUCTION

Nitric oxide (NO) is the main neurotransmitter for active inhibition and relaxation of the lower esophageal sphincter (LES)^[1-4]. In the esophageal body and in the LES circular muscle, NO activates the large conductance potassium channel (BK_{Ca}) and causes membrane hyperpolarization^[5-9] as one potential mechanism leading to LES relaxation. NO has also been shown to be one putative neurotransmitter responsible for the membrane hyperpolarization of the inhibitory junction potential (IJP) in the LES circular muscle of the opossum, guinea pig, dog and mouse^[6,10-15]. Activation of the BK_{Ca} by NO is a factor in production of the IJP membrane hyperpolarization^[15].

In the cat, the BK_{Ca} channel plays a role in setting the resting membrane potential (RMP) in LES muscles^[16]. This modulation of the RMP by the BK_{Ca} is also present in the opossum LES circular muscle and blockade of the BK_{Ca} results in an increase in tone attributed to a balance between the calcium-activated BK_{Ca} and chloride (Cl⁻) channels that raise the RMP to the point where ongoing spiking activity is produced^[17]. In the dog LES circular muscle, the activation of the BK_{Ca} in setting the RMP involves at least an endogenous source of NO^[18,19]. It is not known to what extent endogenous NO is similarly involved in BK_{Ca} regulation of LES tone in other species.

The LES is composed of at least two separate muscle components, the circular and the oblique sling muscles^[19]. In humans, the circular muscle forms only a partial ring (or semicircular clasp), whereas in other species including the cat, the circular muscle fully encircles the distal esophagus^[19,20]. The circular and the sling muscles are functionally different with unique motor and electrophysiological properties. The circular muscle has significant spontaneous myogenic resting tone but is poorly responsive to cholinergic stimulation, whereas the sling muscle has little intrinsic myogenic tone but contracts vigorously to cholinergic stimulation, whereby it maintains its resting tone *in vivo*^[21-26]. There are regional differences within the feline LES in terms of distribution, nature and function of ion channels^[16,17,27-30]. In particular, the BK_{Ca} density is greater in circular muscle than in the sling muscle^[16].

The objective of the present study was to determine in the cat, whether the BK_{Ca} in LES circular and sling muscles is influenced by exogenous and/or endogenous NO to regulate muscle resting tone and/or relaxation. These studies utilized isolated smooth muscle cells and strips taken from the feline LES circular and sling regions. The effect of endogenous *vs* exogenous sources of NO on the outward K⁺ currents was also assessed and compared. A portion of this work has appeared in abstract form^[31].

MATERIALS AND METHODS

Animal model

The cat was chosen as our animal model because of several important similarities between the cat and human esophagus. These similarities include (1) a significant portion of the distal esophageal body is composed of

smooth muscle^[20]; (2) the cholinergic sensitivity of the smooth muscle esophagus and LES are similar^[22,23]; and (3) the placement of the gastroesophageal junction is similarly placed relative to the diaphragm^[20].

Animal preparation

Experiments were approved by the University Health Network Animal Care Committee. Fasted, adult cats of either sex, weighing 2.5 to 5.0 kg were anesthetized with ketamine hydrochloride (0.15 mL/kg *im*; Bimeda-MTC, Cambridge, ON, Canada) and euthanized with pentobarbital sodium (0.5 mL/kg *iv*; Bimeda-MTC). At laparotomy, an esophago-gastric segment from 5 cm above the LES and including a 4 cm cuff of the stomach was carefully excised and placed into Krebs solution equilibrated with 95% O₂/5% CO₂ and maintained at pH 7.40 ± 0.05. The tissue was freed from surrounding fascia, stretched to its *in situ* length, and then cut along the greater curvature of the stomach. The mucosa was then gently removed to expose the LES circular and sling regions^[22]. To consider regional differences within the LES circular *vs* sling muscles, muscle strips and isolated smooth muscle cells were prepared from each region.

Muscle strip studies

Muscle strips, 2 mm wide and 8 mm long, were obtained from the long axis of the circular and oblique sling muscles. Muscle strips were individually mounted in a 25 mL water-jacketed tissue bath. For isometric tension measurement (transducer model FT-03; Grass Instruments, Quincy, MA, USA) Force transducer data were acquired (Digi-Data 1200B analog-to-digital converter, Axon Instruments, Union City, CA, USA) and analyzed using pCLAMP software (version 8; Axon Instruments, Union City, CA, USA). Transmural electrical field stimulation (EFS) was delivered (Grass stimulator SP-9) through platinum wire electrodes with 0.5 ms square-wave pulses in a 5 s train at a frequency of 10 Hz and a strength of 70 V.

Initially muscle strips were hung with 0.5 g tension for a 1 h equilibration period, and the length was then measured and defined as L₀ (initial length). Strips were then slowly stretched twice at increments of 25% of L₀ with 15 min between each stretch^[24]. At study length of 150% L₀, EFS of the sling muscle resulted in an initial contraction in all strips and EFS of circular muscle resulted in relaxation in all strips studied^[25].

The relative contribution of the BK_{Ca} channel to tension as affected by an exogenous or endogenous myogenic source of NO was assessed with the nerves intact or blocked with tetrodotoxin (TTX) and using the following protocols: (1) TTX alone (10⁻⁶ mol/L) or with blockade of the BK_{Ca} channel with iberiotoxin alone (IbTX, 10⁻⁷ mol/L), or the two in combination TTX (10⁻⁶ mol/L) + IbTX (10⁻⁷ mol/L); and (2) TTX (10⁻⁶ mol/L) + SNP [sodium nitroprusside (10⁻⁴ mol/L)] ± IbTX (10⁻⁷ mol/L) or TTX (10⁻⁶ mol/L) + IbTX (10⁻⁷ mol/L) ± SNP (10⁻⁴ mol/L). The chemicals were successively added and allowed 15-30 min to act, the strips were not washed in between experimental steps.

The data were normalized and expressed as tension: tension (mmol/L per mm²) = [tone (g) × 9.81 m/s²] / [cross-sectional area (mm²)]; where the cross-sectional area (mm²) = [tissue weight (mg)] / [1.05 mg/mm³ × study length (mm)], and where 1.05 mg/mm³ is the density of smooth muscle^[32]. The unstimulated tension was referred to as baseline resting tension. After an experiment, each muscle strip was blotted onto a filter paper and weighed.

Isolated smooth muscle cell studies

Freshly enzymatically-digested and isolated circular smooth muscle cells were prepared from each LES region^[30]. Two to three pieces (about 2 mm²) of LES tissues were cut and placed into a test tube with 1 mL of dissociation solution and stored at 4°C for up to 36 h. For cell dissociation of the cells, each of the following chemicals was added to the test tube: papain (2 mg/mL), collagenase Sigma blend type F (1.3 mg/mL), 1,4-dithio-L-threitol (154 µg/mL) and bovine serum albumin (1 mg/mL) were added and the tube was incubated at 35°C for 30–60 min. Following rinsing in enzyme-free dissociation solution and gentle mechanical agitation, isolated spindle-shaped single smooth muscle cells were allowed to settle and adhere to a 1 mL glass bottom recording chamber, mounted on the stage of an inverted microscope. After 30 min, cells were washed with external solution and used within 4 h of isolation. Recordings were performed at room temperature (22–25°C).

To study outward K⁺ currents, the perforated patch clamp technique was employed, with the pipette tip resistance between 2–4 MΩ. The pipettes were front-filled with the pipette solution and back-filled with the nystatin solution. With a giga ohm seal the cell was held at -50 mV and a whole-cell configuration was achieved within 5 min. Once a stable access resistance (i.e. below 20 MΩ) had been achieved and maintained, the RMP was measured. To study the effect of NO, two approaches were employed: (1) puffing sodium nitroprusside (SNP, NO donor, 10⁻⁴ mol/L) onto the muscle cell with a picospritzer^[29] positioned 100 µm from the cell (to mimic NO release from nerves); and (2) adding N^G-nitro-L-arginine (L-NNA; NO synthase inhibitor; 10⁻⁴ mol/L) to the recording dish (to assess the potential contribution of endogenous NO). BK_{Ca} channel currents were blocked by the addition of iberiotoxin (IbTX, 200 nmol/L).

To record outward K⁺ currents, voltage-ramp protocols (starting and ending with the holding potential of -50 mV), from -140 to +60 mV or from -70 to +70 mV over 500 ms were generated by a pClamp 9.0 software (Axon Instruments, Union City, CA, USA) and recorded by an Axopatch 200B amplifier (Axon Instruments). Before digitization, all signals were sampled at 10 kHz, filtered at 1 kHz by an on board eight-pole Bessel filter, and digitized (Digidata 1320 converter, Axon Instruments). Cell capacitance was determined by integration of the capacitance transient, and the maximal current density was normalized for cell size. Peak outward currents were compared after each pharmacological manipulation. Recordings were analyzed using Clampfit 9.0 software (Axon Instruments).

Chemicals and solutions

Atropine (atropine sulfate), TTX (Alomone Labs, Jerusalem, Israel), IbTX (Alomone Labs) and the dissociation enzymes were reconstituted into deionized distilled water. SNP was diluted into the external solution. Seventy-five mg of L-NNA was added into 5 mL of 0.1 mol/L HCl, heated gently until dissolved, and then brought up to 10 mL with water. For nystatin, 0.006 g of the powder was dissolved into 200 µL DMSO and 13 µL of that stock was further diluted into 2 mL of pipette solution. If not otherwise stated, all chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada).

For the muscle strip studies, the Krebs solution (115 mmol/L NaCl, 4.6 mmol/L KCl, 1.2 mmol/L MgSO₄·7H₂O, 1.2 mmol/L NaH₂PO₄·H₂O, 22 mmol/L NaHCO₃, 2.0 mmol/L CaCl₂·2H₂O and 11 mmol/L dextrose) was maintained at 37°C with 95% O₂/5% CO₂ in the organ bath. For the isolated smooth muscle studies, the dissociation solution (135 mmol/L KCl, 10 mmol/L HEPES, 10 mmol/L glucose, 10 mmol/L taurine, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂, 0.25 mmol/L EDTA; pH 7.0 adjusted with 1 mol/L KOH), the external solution (130 mmol/L NaCl, 5 mmol/L KCl, 20 mmol/L HEPES, 10 mmol/L glucose, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂; pH 7.4 adjusted with 1 mol/L NaOH) and the pipette solution (140 mmol/L KCl, 20 mmol/L HEPES, 10 mmol/L glucose, 0.1 mmol/L EGTA, 1 mmol/L MgCl₂; pH 7.2 adjusted with 1 N KOH) all had an osmolality between 285 and 310 mOsm.

Statistical analysis

All data are expressed as mean ± SE, where “*n*” represents the number of muscle strips (*n* = 6–11) or cells (*n* = 6–13) studied per group of 2–5 cats per experiment. Each muscle strip or cell served as its own control. SAS software (SAS Institute, Cary, NC, USA) was used to determine statistical differences between groups by a repeated measure ANOVA, followed by a *post hoc* Bonferroni adjusted paired-wise comparison test. A Student's *t*-test was also used to determine statistical differences between means. An alpha value of 0.05 was considered significant.

RESULTS

Effects of NO on the BK_{Ca} channel in LES isolated smooth muscle cells

The RMP of the LES circular smooth muscle cells (no added drugs) was significantly more depolarized than the sling smooth muscle cells (-39.7 ± 0.8 mV *vs* -48.1 ± 1.6 mV, *P* < 0.001, data not shown). Ramp protocols elicited outward potassium currents at potentials greater than -35 mV. The maximal total outward current was similar in isolated smooth muscle cells from the LES circular and sling regions (27.1 ± 1.5 pA/pF *vs* 25.7 ± 2.0 pA/pF, *P* > 0.05).

To mimic the effect of NO release from nerves, the NO donor, SNP, was puffed onto the smooth muscle cells, and a ramp protocol from -140 to +60 mV was used. SNP increased outward potassium currents of LES circular

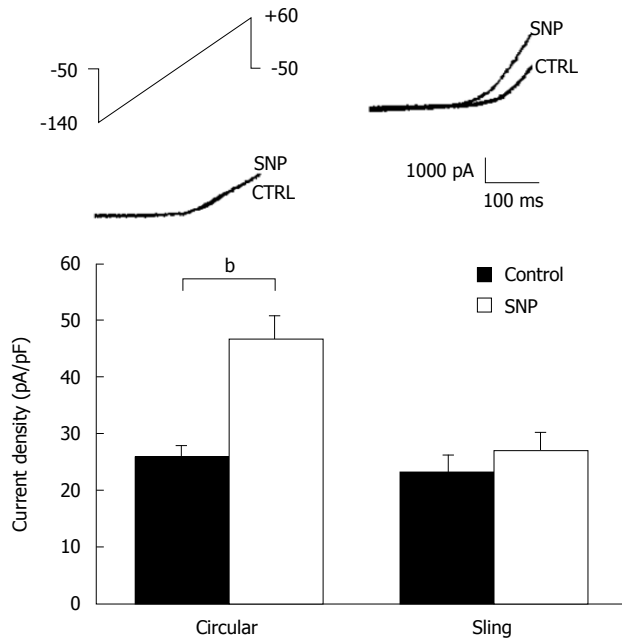


Figure 1 Representative traces (last 300 ms of trace shown) and quantification of the effect of sodium nitroprusside (10^{-4} mol/L) on elicited outward K⁺ currents using a ramp protocol from -140 to +60 mV of lower esophageal sphincter circular ($n = 13$) and sling ($n = 8$) smooth muscle cells. The maximal total outward current was similar in isolated smooth muscle cells from the lower esophageal sphincter (LES) circular and sling regions. The nitric oxide donor sodium nitroprusside (SNP) increased outward potassium currents of LES circular smooth muscle cells. In contrast, SNP did not significantly cause an increase in the outward K⁺ currents in LES sling muscle. ^b $P < 0.001$.

smooth muscle cells from 25.9 ± 1.9 to 46.7 ± 4.2 pA/pF ($P < 0.001$, Figure 1); an 80% increase, and hyperpolarized the cells (from -42.2 ± 1.3 to -63.4 ± 5.0 mV, $P < 0.05$, Table 1). In contrast, SNP did not cause a significant increase in the outward K⁺ currents (from 23.2 ± 3.1 to 27.0 ± 3.4 pA/pF, $P > 0.05$, Figure 1) of sling cells, and no significant change in the RMP (Table 1) was observed. SNP also activated outward potassium currents in circular smooth muscle from both proximal (21.9 ± 3.0 to 38.5 ± 9.6 pA/pF, $n = 5$) and distal (23.5 ± 2.3 to 39.1 ± 6.0 pA/pF, $n = 8$) esophageal body sites.

In the LES, since the effect of the exogenous NO donor (SNP) was seen only in the circular muscle cells, we then pharmacologically isolated the portion of the recorded outward potassium currents attributed to the BK_{Ca} channel currents only in the circular muscle cells^[16]. The BK_{Ca} channel was blocked with 200 nmol/L IbTX, and a voltage-ramp protocol, from -140 to +60 mV, was employed where the different steps of the protocol were performed on the same LES circular smooth muscle cells. SNP induced a significant increase in elicited outward potassium currents *vs* control current density (37.9 ± 4.9 pA/pF *vs* 26.7 ± 3.5 pA/pF, $P < 0.05$, $n = 8$, Figure 2). IbTX alone blocked a significant portion of the control outward potassium currents (13.7 ± 2.2 pA/pF *vs* 26.7 ± 3.5 pA/pF, $P < 0.05$, $n = 8$, Figure 2). SNP added in the presence of IbTX, produced no significant increase in the outward currents (17.0 ± 2.8 *vs* 13.7 ± 2.2 , $P > 0.05$ for IbTX+SNP *vs* IbTX alone, $n = 8$, Figure 2). In separate experiments, blockade of K_{DR} with

Table 1 Resting membrane potential (mV) of isolated cells from the lower esophageal sphincter circular and sling regions in the absence or presence of sodium nitroprusside or L-NNA

	Circular	Sling
Control	-42.2 ± 1.3	-53.0 ± 1.2
Sodium nitroprusside	-63.4 ± 5.0^a	-50.3 ± 3.1
Control	-40.5 ± 1.4	-44.0 ± 1.1
L-NNA	-33.8 ± 1.5^a	-38.8 ± 2.3

^a $P < 0.05$ *vs* control.

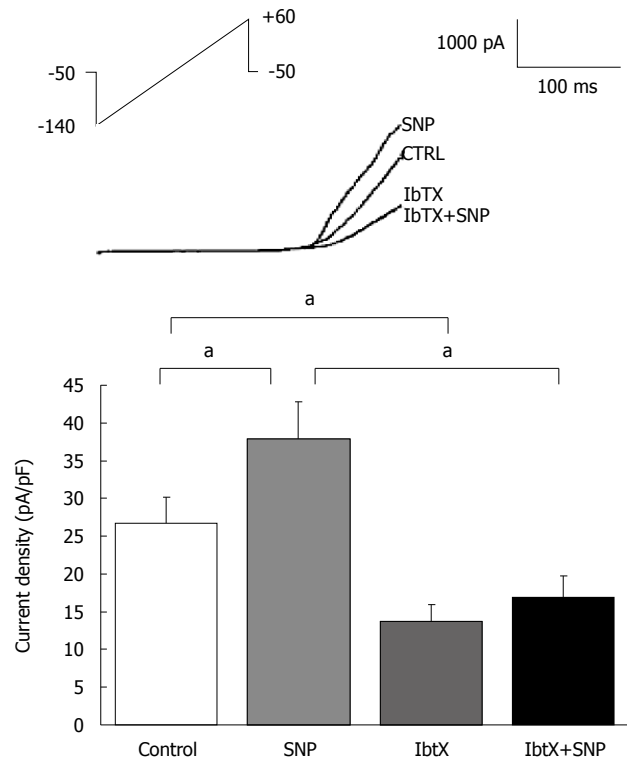


Figure 2 Pharmacological identification, with iberiotoxin (200 nmol/L), of the portion of the elicited outward K⁺ currents, using a ramp protocol from -140 to +60 mV, corresponding to BK_{Ca} channel currents in lower esophageal sphincter circular ($n = 8$) smooth muscle cells. Last 300 ms of representative traces shown. Sodium nitroprusside (SNP) induced a significant increase in elicited outward potassium currents *vs* control current density. Iberiotoxin (IbTX) alone blocked a significant portion of the control outward potassium currents. SNP added in the presence of IbTX, produced no significant increase in the outward currents. ^a $P < 0.05$.

DTX (200 nmol/L) did not inhibit SNP induced outward potassium currents. Thus, for the circular smooth muscle cells, SNP, an exogenous source of NO, produced a significant activation of the BK_{Ca} channel.

To assess the potential contribution of an endogenous source of NO in the two LES regions, the NO synthase inhibitor, L-NNA, was added and a voltage-ramp protocol from -70 to +70 mV was used. Addition of L-NNA led to an insignificant 14% decrease in outward potassium currents in the circular smooth muscle cells (from 29.1 ± 2.6 to 25.0 ± 2.4 pA/pF, $P > 0.05$, Figure 3), however, L-NNA significantly depolarized the cell (from -40.5 ± 1.4 to -33.8 ± 1.5 mV, $P < 0.05$, Table 1), a 17% change in the

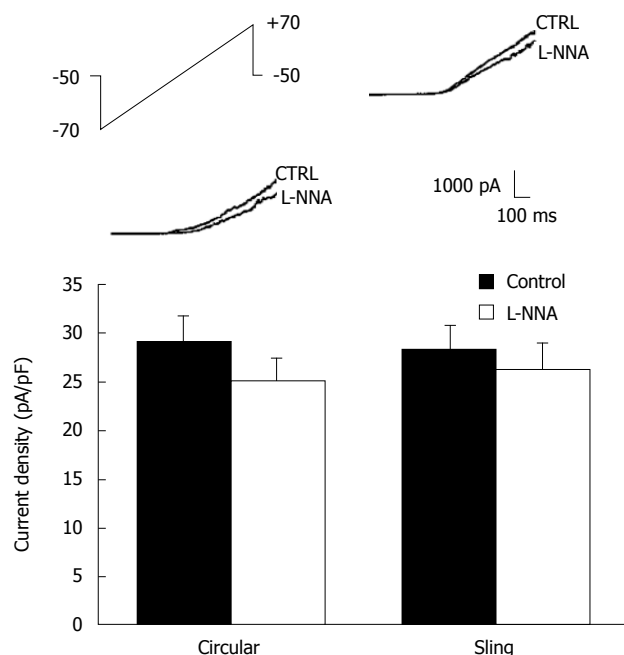


Figure 3 Representative traces and quantification of the effect of L-NNA (10^{-4} mol/L) on elicited outward K⁺ currents using a ramp protocol from -70 to +70 mV of lower esophageal sphincter circular ($n = 8$) and sling ($n = 8$) smooth muscle cells. Addition of L-NNA led to an insignificant 14% decrease in outward potassium currents in the circular smooth muscle cells. In the sling smooth muscle cells, the addition of L-NNA led to an insignificant 7% reduction in current density.

RMP. In the sling smooth muscle cells, there was a smaller reduction in current density (from 28.2 ± 2.5 to 26.3 ± 2.7 pA/pF, $P > 0.05$, Figure 3), a 7% decrease, and only a 3% increase in the RMP (from -44.0 ± 1.1 to -38.8 ± 2.3 mV, $P > 0.05$, Table 1). Thus, an endogenous source of NO may modulate outward potassium currents and RMP, but to a small extent, and the effect is more pronounced in the circular muscle.

Effects of NO on the BK_{Ca} channel in LES smooth muscle strips

In view of the small effect of endogenous NO on the RMP of both circular and sling muscle cells, we investigated the myogenic contribution of the BK_{Ca} channel to basal resting smooth muscle tone. Nerves were blocked with TTX and the BK_{Ca} channel was inhibited with IbTX. For the circular muscle, TTX (tension 14.5 ± 3.4 mmol/L per mm² vs 18.2 ± 4.7 mmol/L per mm²) or IbTX (tension 14.5 ± 2.3 mmol/L per mm² vs 15.0 ± 2.3 mmol/L per mm²) did not significantly change the resting tension ($P > 0.05$, Figure 4A). The combined blockade of the nerves and the BK_{Ca} channel also resulted in no significant change in the overall muscle tone (7.3 ± 1.3 mmol/L per mm² vs 8.3 ± 1.1 mmol/L per mm², $P > 0.05$, Figure 4A). Similarly, for the sling muscle, TTX (tension 10.3 ± 2.0 mmol/L per mm² vs 10.5 ± 2.4 mmol/L per mm²) or IbTX (tension 7.3 ± 1.2 mmol/L per mm² vs 7.3 ± 1.3 mmol/L per mm²) or TTX and IbTX together (tension 6.3 ± 1.5 mmol/L per mm² vs 8.4 ± 2.4 mmol/L per mm²) did not significantly modulate the basal resting tone ($P > 0.05$, Figure 4B).

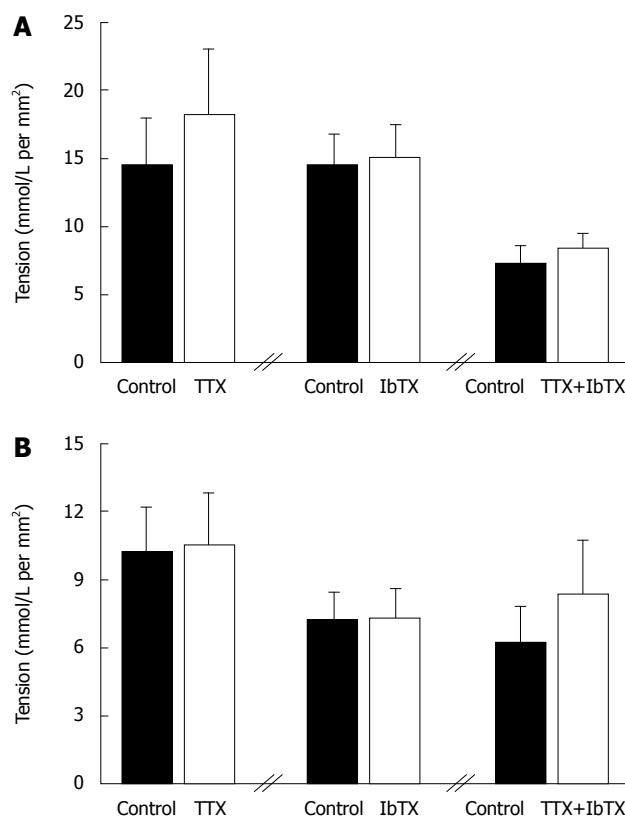


Figure 4 Effect of the blockade of the BK_{Ca} channel with iberiotoxin (10^{-7} mol/L) of lower esophageal sphincter (A) circular ($n = 6-8$) and (B) sling ($n = 7-11$) smooth muscle tone with intact nerves or blocked with tetrodotoxin (10^{-6} mol/L). A: For the circular muscle, tetrodotoxin (TTX) or iberiotoxin (IbTX) did not significantly change the resting tension ($P > 0.05$). The combined blockade of the nerves and the BK_{Ca} channel also resulted in no significant change of the overall muscle tone ($P > 0.05$); B: Similarly, for the sling muscle, TTX, IbTX or TTX and IbTX together did not significantly modulate the basal resting tone ($P > 0.05$). //: Separates different experiments.

To study the effect of exogenous NO on the BK_{Ca} channel in muscle strips, SNP was added in the presence or absence of IbTX, and *vice versa*, with the nerves blocked with TTX. In LES circular muscle, SNP decreased the resting tension by 17.4 ± 5.7 mmol/L per mm², the tension was unchanged with further addition of IbTX ($P > 0.05$, Figure 5). When IbTX was added first, there was no significant change in tension, while the subsequent addition of SNP (IbTX+SNP in the presence of TTX) significantly decreased the change in tension (1.1 ± 0.5 mmol/L per mm² vs -6.3 ± 1.2 mmol/L per mm², $P < 0.001$, Figure 5). For the sling muscle, similar changes were observed. SNP or SNP+IbTX decreased the tension to the same extent, IbTX alone produced no significant change in resting tension, and IbTX+SNP decreased the tension by 4.1 ± 1.0 mmol/L per mm² ($P < 0.001$, Figure 5). Therefore, in the cat, whether the BK_{Ca} channel is blocked or not, exogenous NO causes relaxation in both sling and circular smooth muscles.

The effects of IbTX on neural responses induced by Electrical Field Stimulation (EFS) were assessed. The addition of TTX was omitted in these studies. In circular muscle, EFS caused transient relaxation of resting tone

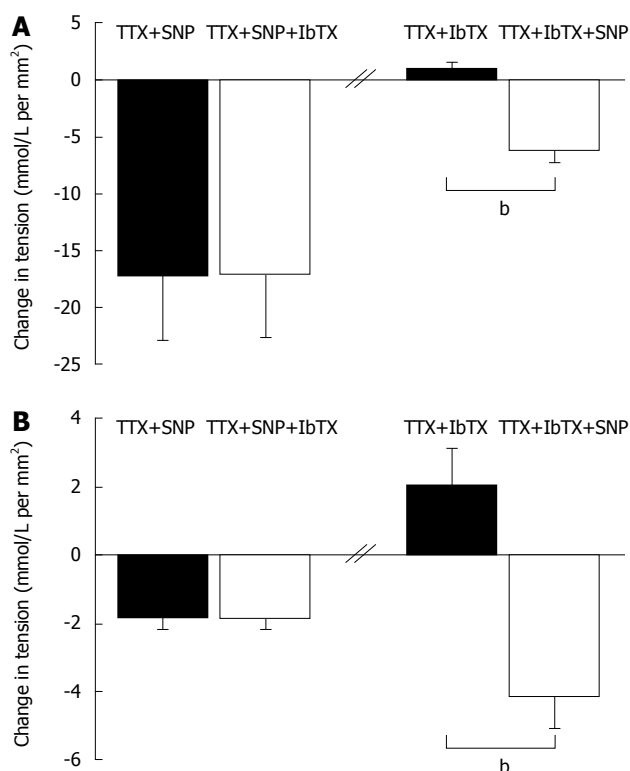


Figure 5 Effect of sodium nitroprusside (10^{-4} mol/L) \pm iberiotoxin (10^{-7} mol/L), and iberiotoxin (10^{-7} mol/L) \pm sodium nitroprusside (10^{-4} mol/L) on smooth muscle tone relative to basal tone in the presence of tetrodotoxin (10^{-6} mol/L) in muscle strips of lower esophageal sphincter (A) circular ($n = 6$) and (B) sling ($n = 6-9$) muscles. A: In lower esophageal sphincter (LES) circular muscle, sodium nitroprusside (SNP) decreased the resting tension and this tension was then unchanged with further addition of iberiotoxin (IbTX) ($P > 0.05$). When IbTX was added first, there was no significant change in tension, while the subsequent addition of SNP [IbTX+SNP in the presence of tetrodotoxin (TTX)] significantly decreased the tension ($P < 0.001$); B: In LES sling muscle, SNP or SNP+IbTX decreased the tension to the same extent, and IbTX alone produced no significant change in resting tension. IbTX+SNP decreased the tension ($P < 0.001$). //: Separates different experiments. ^b $P < 0.001$.

from 14.5 ± 2.3 to 3.8 ± 1.9 mmol/L per mm², and IbTX had no effect on LES tone (15.0 ± 2.3 vs 14.5 ± 2.3 , $P > 0.05$) or EFS induced LES relaxation (3.9 ± 1.9 mmol/L per mm² vs 3.8 ± 1.9 mmol/L per mm², $P > 0.05$). In the LES sling muscle, EFS caused a transient contractile response from resting tone of 7.3 ± 1.2 to 19.3 ± 4.1 mmol/L per mm². IbTX had no effect on resting tone (7.3 ± 1.3 mmol/L per mm² vs 7.3 ± 1.2 mmol/L per mm², $P > 0.05$) or EFS induced responses (19.3 ± 4.1 mmol/L per mm² vs 23.4 ± 5.8 mmol/L per mm², $P > 0.05$).

DISCUSSION

In view of the part played by the activation of the BK_{Ca} by NO in the modulation of resting tone and relaxation of the LES in some species, the present study explored this role in the sling and circular muscle components of the cat LES. The BK_{Ca} responsiveness to the inhibitory neurotransmitter NO was significant only in the LES circular muscle. Furthermore, exogenous NO, whether provided by SNP or released by EFS, produced its inhibitory effect to relax LES

smooth muscle predominantly through other mechanisms than the activation of the BK_{Ca} channel. There may be a small endogenous source of NO that has a small inhibitory effect to slightly reduce resting tone in both muscles. These findings add to the other known physiological properties of the two muscles and their regional differences. Any NO-BK_{Ca} relaxant effect, if needed, would be directed primarily to facilitate relaxation of the high intrinsic myogenic tone of the LES circular muscle.

Since SNP produced a large and significant activation of the BK_{Ca} channel only in the circular smooth muscle cells, a difference in sensitivity of the BK_{Ca} to NO is one possible explanation for this disparity, assuming that the two muscles express a similar number of channels^[33]. This disparity could also reflect regional differences in K⁺ channel molecular constitution through expression of different regulatory subunits leading to channel isoforms with different channel activity and sensitivity to voltage and intracellular calcium^[15,34]. In the human esophagus there are at least four different splice variants of BK_{Ca} with differences in expression at various sites of the esophageal body and LES^[33].

It is generally accepted that *in vivo*, NO is the main neurotransmitter for active inhibition of the LES^[1-4]. The circular muscle with its high intrinsic tone relaxes predominantly due to the neural release of the inhibitory neurotransmitter NO as well as some non-NO inhibitory influence^[2,3,9,13,25,35-39]. The sling muscle with its tone maintained by cholinergic excitation^[21-24,26,40], presumably relaxes predominantly by switching off this excitatory influence, although NO can also relax the sling when contracted by cholinergic stimulation^[24,30].

The primary mechanism for NO-mediated relaxation is considered to be *via* a cyclic guanosine monophosphate (cGMP) pathway^[41-43]. There are several potential intracellular pathways whereby NO can lead to smooth muscle relaxation. Activation of the BK_{Ca} by NO could potentially occur directly or indirectly through interaction with a number of these mechanisms. For example, the PKG-pathways are accepted intracellular messengers that can lead ultimately to LES circular muscle relaxation in dog, opossum and cat^[44-51]. In addition, an involvement of the contractile proteins has been proposed in the sequence of events targeted by NO for LES relaxation in opossum^[14]. The BK_{Ca} channel integrates changes in both intracellular Ca²⁺ and membrane potential, and the cGMP pathway can in counter-part influence these latter parameters^[15]. Moreover, cross-talk between NO and the ion channel may be facilitated by a close spatial relationship between ion channels and NOS sequestered in or in the vicinity of caveolae, seen in dog LES circular muscle^[52-54]. Although it is unclear why endogenous NO had minimal effect on resting LES tone in this study of the cat, variability in the effect of different NO donors and NO states on gut smooth muscle physiology as well as species differences have also been reported and are likely contributing factors^[55]. The apparent species and smooth muscle differences open the door to further experimentation. Our aim in this paper was not

directed to a study of the potential intracellular pathways and mechanisms underlying these differences.

Although the BK_{Ca} current density is greater in the circular muscle, the channel plays a role in setting the RMP in both LES muscles^[16]. IbTX causes significant membrane depolarization in both LES muscles, as confirmed in the present study. This modulation of the RMP by the BK_{Ca} is also present in the opossum LES circular muscle^[17]. Our present experiments in isolated smooth muscle cells of the cat LES, show that this activation of the BK_{Ca} in setting the RMP involves at least an endogenous source of NO in both LES regions, consistent with findings in dog LES circular muscle^[9,18].

Although the BK_{Ca} contributes to setting the RMP, and^[16,17], in the current muscle strip studies, blockade of the BK_{Ca} with IbTX did not result in a significant increase in muscle tension in either muscle. This is unlike the esophageal body where both IbTX and tetra ethyl ammonium increase tonic and phasic contractility^[8]. In the opossum LES circular muscle, blockade of the BK_{Ca} results in an increase in tone when the RMP is raised to the point where ongoing spiking activity is produced. In those experiments, BK_{Ca} blockade changed the RMP only slightly (from -43.4 to -37.8 mV). The further transient depolarizations associated with the spike-like action potentials then are associated with calcium (Ca²⁺) entry through the L-type channel^[17]. The findings fit with the knowledge that Ca²⁺ is required for, and Ca²⁺ availability can alter LES circular smooth muscle motor response in dog and opossum^[7,14,18,56].

The lack of major effect of IbTX on resting tone in both cat LES muscles in the present experiments would occur if membrane depolarization is unable to sufficiently activate channels for entry of extracellular calcium for tension development, either directly or through the stimulation of spiking activity. In the LES muscles, L-type calcium channel activation is only seen at membrane potentials more positive than about -20 mV^[27,57], levels not reached in the sling (-42 mV) or in the circular muscle (-31 mV) with IbTX or other blockade of the BK_{Ca}^[16,17]. Furthermore, we have shown that resting tone in both muscles utilizes both intracellular and extracellular calcium sources, the circular muscle more dependent on an extracellular, and the sling on an intracellular source^[28]. In addition, the two muscles utilize different calcium entry portals, the circular muscle using the L-type and the sling a non L-type channel. Therefore, our findings in the cat indicate that if endogenous NO is acting on the BK_{Ca} to modulate resting tone, this modulation is mainly through an indirect action, and augmented by its other mechanisms of action^[7,14,15,18,49]. Furthermore, there appears to be a more complex relationship in the LES between active changes in membrane potential such as spiking activity or slow wave activity and levels of LES tone^[58-63]. Our present findings, taken together with findings in other species, indicate that changes in resting tone are likely dependent on Ca²⁺ entry associated with the active changes in membrane potential rather than entry with any small changes in the RMP^[17]. Further studies are required to assess which mechanisms and pathways are involved.

In terms of resting tone in the LES, our findings have potential functional significance. In the present *in vitro* study in the cat, TTX blockade of excitatory as well as inhibitory nerve activity failed to significantly affect basal tension in circular smooth muscle strips. This finding supports the concept that *in vivo*, intrinsic ongoing myogenic contraction underlies resting tone of the circular muscle. The intrinsic tone in the circular muscle is not significantly modulated by cholinergic excitation and/or nitrergic inhibition^[3,4]. On the other hand, cholinergic neural influence, normally vagally-driven, acts to augment and maintain the low intrinsic basal resting tone in the sling.

A better understanding of the asymmetries of both circular and sling LES muscles at the cellular level could give insights into the pathogenesis of and potential therapeutic usefulness for patients with motor disorders such as achalasia and gastroesophageal reflux disease. For instance, in human and cat, addition of atropine decreases the leftward resting LES pressure where the sling is located, with little effect in the other radial orientations^[22,40]. Although in the present cat experiments, exogenous NO causes smooth muscle relaxation of both the LES circular and sling smooth muscles, whether the BK_{Ca} channel is blocked or not, activation of the BK_{Ca} channel by NO provides another potential mechanism to augment the muscle relaxing effect of the cGMP pathway. Whether such a mechanism may become more important, for example in circumstances where NO release to produce relaxation of the circular muscle is decreased, as in patients with achalasia, requires further study^[64-68]. Hence, regional differences in muscle cell BK_{Ca} responsiveness to NO hold the potential for selective modulation of LES function in health and disease.

COMMENTS

Background

The lower esophageal sphincter (LES) is normally closed to prevent reflux of gastric acid, but opens with a swallow to allow passage of the bolus into the stomach. The LES has two main components, the circular and sling muscles. To maintain closure, the circular muscle has significant resting tone but is poorly responsive to neural cholinergic excitation. On the other hand, the sling has little resting tone, and its closing contraction is maintained by vagal release of acetylcholine. Opening of the LES with a swallow requires relaxation of the two muscles. Relaxation of the circular muscle is therefore dependent on active relaxation induced by an inhibitory neurotransmitter such as nitric oxide (NO), while relaxation of the sling can readily occur with vagal excitation turned off.

Research frontiers

Although NO can relax smooth muscle by a number of cellular mechanisms, in some species activation of the BK_{Ca} by NO is important for the modulation of resting tone and relaxation of the LES. The importance of this mechanism was assessed in isolated cells and in strips of muscle from the two muscle components of the cat LES. The cat esophagus is very similar to the human esophagus with a significant portion of the distal esophagus composed of smooth muscle.

Innovations and breakthroughs

The BK_{Ca} responded to the inhibitory neurotransmitter NO, administered as sodium nitroprusside (SNP), only in the LES circular muscle, the muscle with high resting tone. Furthermore and as opposed to other species, exogenous NO, whether provided by SNP or released by electrical field stimulation, provided its inhibitory effect to relax LES smooth muscle predominantly through other mechanisms than the activation of the BK_{Ca} channel.

Applications

Any NO-BK_{Ca} relaxant effect, if needed, would be directed primarily to facilitate

relaxation of the high intrinsic myogenic tone of the LES circular muscle. Such a mechanism may become more important, for example in circumstances where NO release to produce relaxation of the circular muscle is decreased, as in patients with achalasia. Since NO provided its inhibitory effect to relax LES smooth muscle predominantly through other mechanisms than the activation of the BK_{Ca} channel, further research should be directed to these other mechanisms. Such studies hold the potential for new therapeutic approaches applicable to the human esophagus.

Terminology

Achalasia: a condition where swallowing difficulty is due to obstruction of the esophagus at the LES resulting from failure of LES relaxation due to absence of the inhibitory neurotransmitter NO.

Peer review

This is a well-designed and interesting study that provides convincing evidence regarding the activation of large conductance potassium channels within the circular smooth muscle during administration of exogenous nitric oxide. The role of endogenous nitric oxide appears to be relatively less significant.

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Effects of meal size and proximal-distal segmentation on gastric activity

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assessed, twice, by scintigraphy. The test meal consisted of 60 or 180 mL of yogurt labeled with 64 MBq ^{99m}Tc-tin colloid. Anterior and posterior dynamic frames were simultaneously acquired for 18 min and all data were analyzed in MatLab. Three proximal-distal segmentations using regions of interest were adopted for both meals.

RESULTS: Intragastric distribution of the meal between the proximal and distal compartments was strongly influenced by the way in which the stomach was divided, showing greater proximal retention after the 180 mL. An important finding was that both dominant frequencies (1 and 3 cpm) were simultaneously recorded in the proximal and distal stomach; however, the power ratio of those dominant frequencies varied in agreement with the segmentation adopted and was independent of the meal size.

CONCLUSION: It was possible to simultaneously evaluate the static intragastric distribution and phasic contractility from the same recording using our scintigraphic approach.

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Key words: Gastric motility; Phasic contractions; Proximal stomach; Scintigraphy; Meal size

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Abstract

AIM: To evaluate the effects of meal size and three segmentations on intragastric distribution of the meal and gastric motility, by scintigraphy.

METHODS: Twelve healthy volunteers were randomly

INTRODUCTION

Scintigraphy is the gold standard for measuring gastric emptying and offers the advantage of completely characterizing the complex physiology of intragastric distribution of the meal (IDM) between the proximal and the distal regions^[1-3]. Quantification of intragastric distribution could help to define abnormal physiology and explain certain functional dyspeptic symptoms^[4], especially when global gastric emptying values are normal^[3]. Proximal-distal segmentation approaches employed to divide the stomach in proximal and distal regions remain a challenge for studying IDM; therefore, a validation is necessary before incorporating it into clinical practice^[3].

At least three segmentation approaches were adopted, based on the proximal stomach defined immediately after a meal, to divide the stomach into two equal areas and using the incisura^[1,2,4-7]. No previous studies have compared the results obtained by those three approaches, and several studies have perceived that there may be problems regarding the division of gastric segments into proximal and distal regions^[6,8]. In particular, the two gastric compartments might not be easily identifiable, the incisura may not be pronounced, and somewhat arbitrary definitions cannot be always applied^[8].

Apart from the well-known phasic motor activity of 3 cpm (cycle per minute) in the distal stomach, a phasic activity of 1 cpm was observed in dogs and humans using different techniques and appears to be concentrated in the proximal region^[9-12]. Thus, considering that each frequency of contraction can be associated with one gastric region, the dominant frequencies could facilitate the characterization of the proximal and distal regions of stomach. The functions of the stomach regions can vary according to the nutrient content^[7], and there is a clinical recommendation for consumption of smaller and more frequent meals to avoid postprandial symptoms in patients with common gastrointestinal disorders^[13,14]. However, there is little information about the effects of meal size on intragastric distribution, especially for semisolid small meals. The aim of this study was to evaluate the effects of meal size and three proximal-distal segmentations on intragastric distribution and gastric motor activity by scintigraphy.

MATERIALS AND METHODS

Subjects

Twelve healthy volunteers (three female and nine male) with a range of body mass indices of 18.5-24.9 kg/m² and an age of 25-45 years participated in the studies. None had a history of digestive disease or abdominal surgery. Informed written consent was obtained from each participant. The studies were performed in agreement with Declaration of Helsinki and the local Ethics Committee approved the protocol.

Study protocol

Each volunteer was evaluated twice on separate occasions for ingestion of 60 and 180 mL of a semisolid

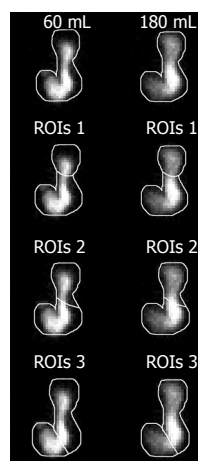


Figure 1 Scintigraphic images showing the composite images after meals with 60 mL and 180 mL, and the three proximal-distal image segmentations (ROIs) adopted. ROIs: Regions of interest.

test meal (yogurt containing 1 kcal/mL). The yogurt was chosen to simulate a small meal that is commonly ingested between large meals. *In vitro* tests were achieved to assure that the Tc-99m tin colloid and the yogurt were adequately blended^[12]. The studies were performed in the morning, after an overnight fast, in a randomized order, and were separated by an interval of one wk. Both test meal were labeled with 64 MBq ^{99m}Tc-tin colloid as a nonabsorbable carrier and consumed with the volunteers standing upright in front of the gamma camera.

A dual-head gamma camera (Sopha Vision, Model DST, Sophycamera; Medical Sopha Vision America, Twinsburg, OH, USA) equipped with a parallel-hole low-energy and high-resolution collimator was used. The gamma camera was set up to record activity around the 140-keV photopeak of ^{99m}Tc. A dynamic set of 1080 frames (1 frame/s) was acquired for 18 min and images were stored in a 64 × 64 matrix for further analysis. A geometric mean of the anterior and posterior gastric counts was determined for each time point and corrected for radionuclide decay^[3].

Data analysis

All digital images were analyzed in MatLab (Mathworks Inc., Natick, MA, USA). The total stomach was outlined in the composite image (summation of all images) with a cursor over the largest anterior gastric image obtained by ingestion of 180 mL. The outline for each 180 mL analysis was individually copied and fitted in the 60 mL image of the same subject. This outline was then subdivided into two regions of interest (ROIs) corresponding to the proximal and distal stomach, according to each method used (Figure 1): ROIs 1: The proximal stomach region was the “reservoir” area seen in all subjects in the first postprandial frames and the line used to divide proximal/distal stomach was drawn immediately below this region^[2,7,15]; ROIs 2: The image was divided into two equal areas, designated the proximal and distal stomach, by a mid-length separation in the longitudinal axis of the stomach^[1,4,16]; and ROIs 3: The proximal and distal regions of the stomach were separated by a fainter band of radioactivity coinciding with the angula; thus the stomach was arbitrarily divided by drawing a line across the incisura angularis^[5,6].

Intragastric distribution of the meal

The time for meal consumption was measured individually. Thus, a value considering 100% retention of the meal was dependent of the activity at the end of the lag phase (the frame before any activity appeared in the small intestine) and immediately after meal completion. Time zero started when the retention was 100%. For each region (total, proximal and distal stomach) activity time curves, expressed as percentages of activity in the total stomach with 100% of meal retention, were obtained.

The intragastric distribution of 60 and 180 mL were assessed from activity time curves derived from each region and considering the three proximal-distal segmentations by calculating the following parameters: (1) initial retention: the percentage of initial activity (%) contained in the total, proximal and distal stomach at time zero; (2) final retention: the percentage of final activity (%) contained in the total, proximal and distal stomach at 18 min; (3) proximal emptying half-time ($T_{1/2}$): expressed as the time (min) when the initial retention in the proximal stomach decreased by 50%; (4) maximal distal content: the highest activity value (%) in the distal stomach at any time point in the study; and (5) gastric emptying of the whole stomach (representing % retention over time) was obtained from time zero to 18 min.

Comparisons of the data for three proximal-distal segmentations were made for both meals employing area under curves (AUC) and statistical moment analysis. The AUC derived from the proximal or distal stomach was expressed as percentage of AUC obtained from total stomach. The statistical moment (minutes) was obtained through the temporal average from the proximal or distal distribution curve, normalized by AUC^[17]. This quantification allowed determination of a distribution time that could be associated with the midpoint of the proximal and distal distribution curves.

Contractility

Fast fourier transform (FFT) was employed to analyze phasic contractions in both gastric regions (proximal and distal) and for each type of proximal-distal segmentation. A bi-directional Butterworth band-pass filter with a cutoff frequency at 5-75 mHz (0.3-4.5 cpm) was applied.

Dominant frequencies were expressed as the frequency at which the highest FFT power spectrum was observed in the proximal and distal regions. Values were expressed as power ratios (%), determined by dividing the power of each dominant frequency by the total power (sum of both frequencies), and multiplying the results by 100 for each stomach region in all proximal-distal segmentations^[18].

Statistical analysis

Data were expressed as mean \pm SE. The hypothesis of a normal data distribution was confirmed using Shapiro-Wilk's test. Data obtained by meals of 60 and 180 mL were compared using Student's *t* test and *P*-values less than 0.05 were considered significant. Comparisons among types of proximal-distal segmentation were ana-

lyzed by one-way ANOVA and Tukey's test, with *P* < 0.05 considered significant.

RESULTS

Our data demonstrated a significant effect of meal size and the three proposed proximal-distal segmentations on intragastric meal distribution and gastric contractility. After ingestion, both meals were rapidly dispersed through the whole stomach with a minimal lag phase. There was no difference between the lag-phase for 60 mL (1.9 ± 0.2 min) and for 180 mL (2.3 ± 0.2 min).

The three proposed proximal-distal segmentations (ROIs) could be applied to all volunteers. Figure 2 shows the profile of IDM of the 60 and 180 mL meal over 18 min for the three types of proximal-distal segmentations. A redistribution of food from the proximal to the distal stomach according to employed segmentation was observed.

There was greater proximal retention after 180 mL ingestion compared to 60 mL, which was proportional to the increase in the area of proximal stomach generated by the type of proximal-distal segmentation. ROIs 1 presented a smaller proximal region and a fast redistribution of the meal for the distal area occurred. By contrast, in ROIs 3, the initial retention in the proximal region was greater than in the other proximal-distal segmentations and presented a slow redistribution of the meal for the distal area. ROIs 2 showed an intermediate pattern. Thus, there was a significant difference among the three ROIs segmentations employed (*P* < 0.05).

No difference was found in the percentage of the meal retained in the total stomach after 60 mL ($13.3\% \pm 3.0\%$) and 180 mL ($13.0\% \pm 2.4\%$) ingestion over 18 min. Table 1 compares the effect of meal size and proximal-distal segmentation in IDM parameters, reinforcing the relationship between proximal area and parameters of regional gastric emptying.

Table 2 presents the mean area under the curve and the statistical moment for the proximal and distal regions, for each of the three types of segmentation, after ingestion of the test meals. For the smaller meal, there was a significant difference among ROIs, while the increased meal size generated a difference only between ROIs 1 and 3.

Moment calculation demonstrated that the differences between the ROIs were only evident with 60 mL but not with 180 mL meal for the three types of proximal-distal segmentation. Comparison of moments for the distal region for both meals showed no significant differences. The statistical moment obtained in the proximal area was influenced by the type of segmentation adopted (Table 2), representing an option to quantify a time related to intragastric distribution.

An important finding was both dominant frequencies were recorded in the proximal stomach (16.0 ± 1.0 mHz or 1 cpm and 50.0 ± 2.0 mHz or 3 cpm) and in the distal stomach (16.0 ± 1.0 and 50.0 ± 1.0 mHz), independently of gastric segmentation. The dominant frequencies were

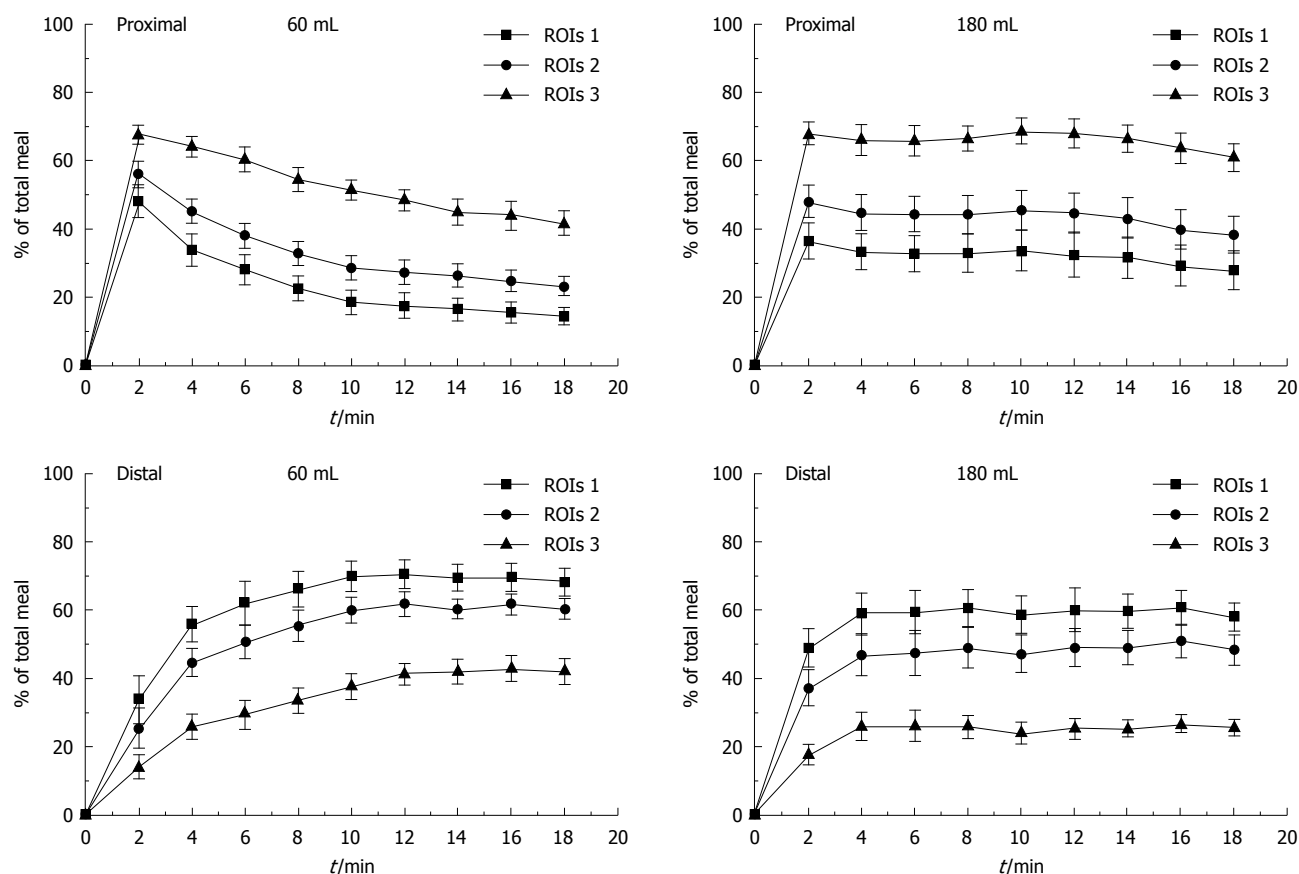


Figure 2 Proximal and distal stomach curves of intragastric distribution according to proximal-distal segmentation ROIs 1, ROIs 2 and ROIs 3 for meals with 60 and 180 mL. Data are expressed as mean \pm SE. ROIs: Regions of interest.

Table 1 Effect of meal volume and proximal-distal segmentation in intragastric distribution parameters (mean \pm SE)

	60 mL			180 mL		
	ROIs 1	ROIs 2	ROIs 3	ROIs 1	ROIs 2	ROIs 3
Total stomach						
Initial retention	100	100	100	100	100	100
Final retention	82.5 \pm 3.8	83.5 \pm 3.2	83.8 \pm 2.9	84.9 \pm 3.3	85.7 \pm 3.1	85.5 \pm 3.2
Proximal (%)						
Initial retention	39.8 \pm 6.8	51.7 \pm 6.0	73.2 \pm 3.8 ^{a,c}	38.6 \pm 6.4	52.3 \pm 6.4	75.2 \pm 4.3 ^{a,c}
Final retention	14.1 \pm 2.5	22.8 \pm 3.0	41.6 \pm 3.7 ^{a,c}	27.7 \pm 5.7 ^e	38.3 \pm 5.5 ^e	60.4 \pm 4.2 ^{a,c,i}
T1/2 (min)	4.2 \pm 0.6	7.0 \pm 0.8	> 18.0	11.0 \pm 2.2	> 18.0	> 18.0
Distal (%)						
Initial retention	60.1 \pm 6.7	48.1 \pm 6.0	26.6 \pm 3.8 ^{a,c}	61.4 \pm 6.4	47.6 \pm 6.4	24.7 \pm 4.3 ^{a,c}
Final retention	68.4 \pm 4.0	60.7 \pm 3.2	42.2 \pm 4.0 ^{a,c}	57.1 \pm 4.2	47.6 \pm 4.5 ^e	25.1 \pm 2.5 ^{a,c,i}
Maximum content	80.0 \pm 4.5	69.0 \pm 3.7	48.0 \pm 3.6 ^{a,c}	69.0 \pm 5.0	56.7 \pm 5.7	32.0 \pm 3.8 ^{a,c}

^a $P < 0.05$ vs ROIs 1; ^c $P < 0.05$ vs ROIs 2; ^a $P < 0.04$ vs 60 mL; ^e $P < 0.03$ vs 60 mL; ⁱ $P < 0.003$ vs 60 mL. ROIs: Regions of interest.

observed to overlap in the signal of proximal stomach at different time points (Figure 3). Power ratio calculations indicated that there was a rearrangement of the maximum power of each frequency (1 and 3 cpm) according to the segmentation type.

The difference is shown in the power spectrum of these frequencies according to the type gastric segmentation (Figure 4). A difference could be observed in the distal region between 60 and 180 mL: for 180 mL meal there was an increase of the 1 cpm and a decrease of the 3 cpm

in ROIs 2 and 3. There were no significant changes for ROIs 1.

DISCUSSION

The results showed that meal distribution in the human stomach differs according to the volume used (60 or 180 mL) and noticeably according to proximal-distal image segmentation used. Dynamic gastric scintigraphy was effective for determining two dominant frequencies (1 and

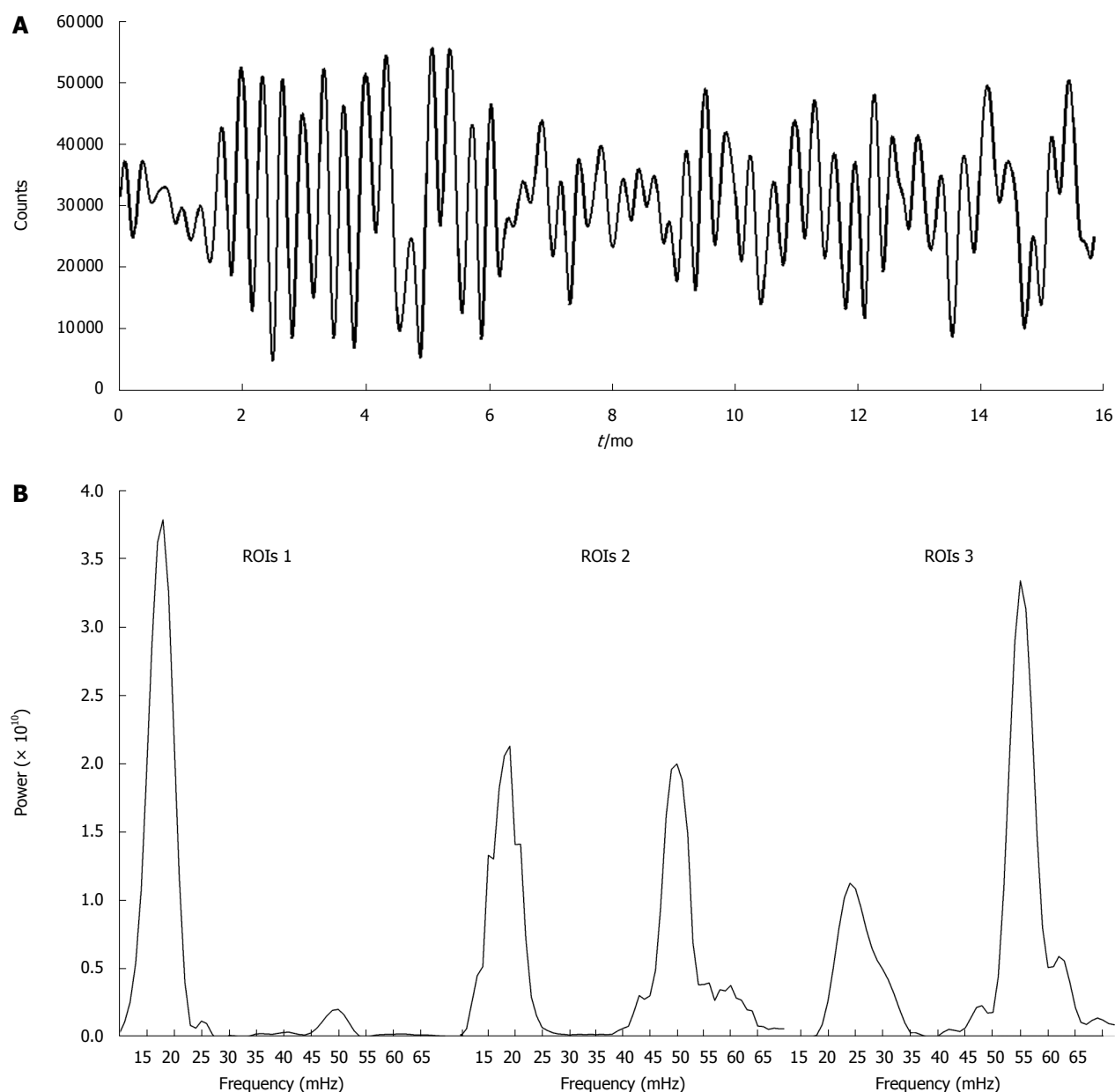


Figure 3 Both dominant frequencies (1 cpm or 17 mHz and 3 cpm or 50 mHz) can be observed overlapping in the signal of the proximal stomach (A), the power ratio of this signal indicated that there was a rearrangement of the maximum power of each of the frequencies (1 and 3 cpm) in agreement with the segmentation type B).

3 cpm), and the magnitude of these contractions in both the proximal and distal stomach.

It has been previously shown that volume and meal size influenced gastric emptying^[19,20], thus, it seems logical to advise patients to decrease the size and increase the frequency of meals during certain conditions, such as pregnancy, gastroesophageal reflux disease, and functional dyspepsia^[14]. Our meals were chosen to investigate IDM and motility patterns using semisolid small meals in order to establish a pattern in healthy volunteers. Thereafter, our approach can be applied in patients to verify IDM and gastric motor activity after the recommended consumption of a smaller meal.

The emphasis of most previous studies was on total gastric emptying^[19], despite the fact that regional gastric

emptying is more frequently abnormal than total gastric emptying^[2]. The partitioning of ingested meals between the proximal and distal stomach is related to the genesis of dyspeptic symptoms, such as early satiety, fullness, and nausea^[1,4,21]. The meal distribution within the area of the stomach might strongly influence the way in which the stomach is subdivided^[4]. Unfortunately, the methods used for defining these two areas are still controversial and poorly defined^[15]. Our data demonstrated a direct relationship between gastric compartment size and IDM. The choice of the segmentation technique should consider variations in the stomach shape^[16] and the objective of the study. For example, using ROIs 1 it is possible analyze details of the fundic accommodation process' whereas employing ROIs 2 both dominant frequencies

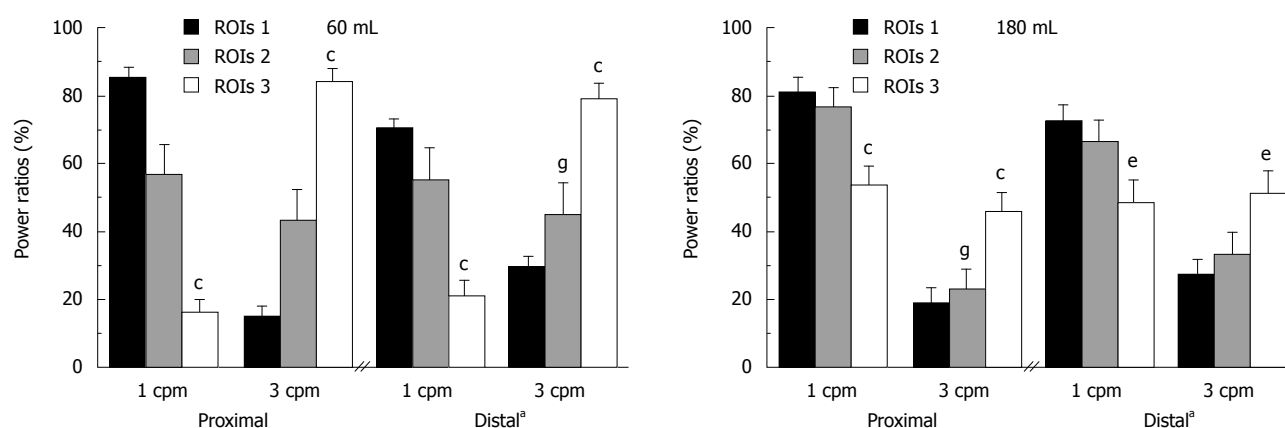


Figure 4 The power ratios showed the presence of both frequencies (1 cpm and 3 cpm) in the proximal and distal stomach, and that their intensities depend on the adopted proximal-distal segmentation and on the meal size ingested. ^a $P < 0.03$ between 60 and 180 mL for both frequencies in distal region, ^c $P < 0.005$ between ROIs 1 and ROIs 3, ^e $P < 0.05$ between ROIs 1 and ROIs 3, ^g $P < 0.05$ between ROIs 2 and ROIs 3. ROIs: Regions of interest.

Table 2 Mean area under the curve and time distribution by statistical moments for proximal and distal regions for the three proximal-distal segmentations, after ingestion of the test meal of 60 and 180 mL (mean \pm SE)

	Proximal			Distal		
	ROIs 1	ROIs 2	ROIs 3	ROIs 1	ROIs 2	ROIs 3
AUC (counts.s)						
60 mL	27.2 \pm 2.9	31.4 \pm 2.5	43.9 \pm 1.7 ^{a,c}	72.8 \pm 2.9	68.6 \pm 2.5	56.1 \pm 1.7 ^{a,c}
180 mL	35.3 \pm 4.1	39.4 \pm 3.2	49.0 \pm 3.8 ^a	64.7 \pm 4.1	60.6 \pm 3.2	51.0 \pm 3.8 ^a
P	< 0.09	< 0.03 ¹	< 0.1	< 0.09	< 0.03 ¹	< 0.1
Statistical moments (min)						
60 mL	6.9 \pm 0.3	7.7 \pm 0.2 ^c	8.5 \pm 0.1 ^{a,c}	10.4 \pm 0.3	10.5 \pm 0.3	10.7 \pm 0.2
180 mL	8.7 \pm 0.2	9.0 \pm 0.1	9.2 \pm 0.1	9.8 \pm 0.3	10.0 \pm 0.3	10.1 \pm 0.3
P	< 0.00001 ¹	< 0.00003 ¹	< 0.0006 ¹	< 0.2	< 0.1	< 0.07

¹60 mL vs 180 mL. ^a $P < 0.05$ vs ROIs 1, ^c $P < 0.05$ vs ROIs 2, ^e $P < 0.05$ vs ROIs 1. ROIs: Regions of interest.

can be equally observed, and ROIs 3 is useful to evaluate antral contractility.

Generally, twenty minutes after the ingestion of a larger meal, a gradual decrease in proximal stomach activity begins, with a corresponding increase in the distal stomach, indicating a redistribution of food from the proximal to the distal stomach^[1,4,5]. Hence, our IDM data were obtained during this initial stage, showing that the 60 mL meal quickly began to be redistributed to the distal region; whereas, using 180 mL, there was a slower redistribution from proximal to distal stomach in all segmentations adopted (Figure 2). In the curves obtained from ingestion of 180 mL was impossible calculate $T_{1/2}$ from the proximal stomach during the 18 minutes of recording, except for ROIs 1, reinforcing the retention in the proximal region. In the final retention values, there was a significant difference between three ROIs segmentation and both meals ingested (Table 1).

To quantify the IDM, we used the statistical moments, which have previously been utilized only in pharmaceutical approaches^[17], and the traditional AUC. The statistical moments and AUC provide complementary information about the data observed. Both quantification methods showed that proximal-distal segmentations have more effect for the 60 mL meal than for 180 mL. However,

there was no difference in the statistical moment between ROIs in the distal curve profile, whereas there was a significant difference in AUC. Distal accumulation time was defined previously by our group as the time elapsing from the meal ingestion until the activity reached 99% of the maximum value in the sigmoidal tracing over the distal stomach curves^[12]. The distribution time obtained by the statistical moment was very close to the distal accumulation time, but can be employed for any kind of curve, including proximal stomach curves.

It is important to emphasize that partitioning of ingested meals between the proximal and distal stomach is related to gastric accommodation. Abnormal IDM might be a consequence of disturbed proximal stomach accommodation^[4] in a considerable subset of patients with functional dyspepsia and might have a role in symptom production, such as early satiety and weight loss^[6]. Studies of dyspepsia have shown a preferential accumulation in the distal stomach, suggesting defective postprandial relation of the proximal stomach; however, it is difficult to draw general conclusions because each study employed a different proximal-distal partition. It would be interesting to provide data on the relationship between meal size, proximal-distal segmentation, IDM, and accommodation in this patient group. Information on the accommodation

process will have clinical value, especially for studying patients with dyspepsia and normal gastric emptying, and it may contribute directly to improved medical therapy^[22].

Fast Fourier transformation of our scintigraphic recordings defined two dominant frequencies in the distal as well as the proximal stomach, in all volunteers (Figure 3). Meal size did not affect the dominant frequencies of the contractions or their power, but there was a large difference in the power spectra of these frequencies, based on image segmentation. The power ratio of the proximal signal was rearranged for the maximum power of each frequency (1 and 3 cpm), according to segmentation type. Hence, there was a power gradient from ROIs 1 to ROIs 3, where 1 cpm decreases and 3 cpm increases in the human proximal stomach (Figures 3 and 4). This motor activity around 1 cpm on the proximal stomach^[9,10,23] has not been extensively documented in humans, although it has been correlated with functional dyspepsia^[9], mainly due to methodological issues, such as differences in barostat systems^[24] and/or filter parameters employed in data analysis^[12].

In summary, the results of research and/or diagnosis can be deeply influenced by the proximal-distal segmentation method adopted. Two dominant frequencies (1 and 3 cpm) can be simultaneously registered in the proximal and distal stomach, but the proximal-distal segmentation should be considered carefully to analyze their power spectra. The protocol developed in this study can be applied in patients with several disorders, with the advantages of simultaneous evaluation of IDM and gastric contractions.

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COMMENTS

Background

The stomach is composed of two distinct functional regions. The distal stomach is capable of generating 3 vigorous contractions per minute (cpm), which cause reduction in size of ingested particles and subsequent emptying. The proximal stomach is primarily concerned with storage of the ingested food, with a slight contraction activity around 1 cpm. During a meal, the stomach continuously adapts its size to the content by gradually relaxing its musculature, performing the so-called accommodation to distension. Intra-gastric distribution of the meal (IDM) between the proximal and distal stomach is related to the accommodation process, and is useful for defining abnormal physiology and for explaining certain functional disease symptoms.

Research frontiers

Proximal-distal segmentation approaches employed to divide the stomach into proximal and distal regions remain a challenge in the study of IDM. Unfortunately, the methods used for defining these two areas are still controversial and poorly defined. Validation is necessary before incorporating these methods into clinical practice. Two dominant frequencies (1 and 3 cpm) can be registered simultaneously in the proximal and distal stomach, but the proximal-distal segmentation should be considered carefully to analyze their power spectra. The motor activity around 1 cpm on the proximal stomach has been correlated

with functional dyspepsia. In this context, the frequency of contraction can be explored to elucidate certain disease patterns.

Innovations and breakthroughs

Scintigraphy already is the gold standard for measuring gastric emptying and offers the advantage of completely characterizing the complex physiology of IDM between stomach regions. New studies can be exploited to refine and extend its use in clinical practice. The functions of the stomach regions can vary according to the nutrient content and there is a clinical recommendation for consumption of smaller and more frequent meals to avoid postprandial symptoms in patients with common gastrointestinal disorders. However, there is little information about the effects of meal size on intra-gastric distribution, especially for semisolid small meals. In the area of functional disorders research, considerable effort is being expended on how to convert basic knowledge into benefits for patients' treatment. Therefore, in the present study we compared three kinds of segmentation in normal volunteers and showed that segmentation is remarkably important in the evaluation of IDM and gastric motility. This observation is particularly relevant when assessing patients.

Applications

Abnormal IDM might be a consequence of disturbed proximal stomach accommodation in a considerable subset of patients with functional dyspepsia and might have a role in symptom production, such as early satiety and weight loss. Studies of dyspepsia have shown a preferential accumulation in the distal stomach, suggesting defective postprandial accommodation in the proximal stomach; however, it is difficult to draw general conclusions, because each study employed a different proximal-distal partition. Thus, providing data on the relationship between meal size, proximal-distal segmentation, IDM, and accommodation in this patient group is very important. Information about the accommodation process will have clinical value, especially in the study of patients with dyspepsia and normal gastric emptying, and might directly influence medical therapy.

Terminology

IDM represents the distribution of gastric contents between the proximal and distal stomach during gastric emptying. Dyspepsia is a medical condition characterized by chronic or recurrent pain in the upper abdomen, bloating, and fullness.

Peer review

The authors evaluated the effects of meal size and three segmentations on intra-gastric distribution of meal and gastric motility by scintigraphy. This paper is an interesting report.

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PPIs are not associated with a lower incidence of portal-hypertension-related bleeding in cirrhosis

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Abstract

AIM: To determine if proton pump inhibitor use in cirrhotic patients with endoscopic findings of portal hypertension is associated with a lower frequency of gastrointestinal bleeding.

METHODS: Patients with cirrhosis and endoscopic findings related to portal hypertension, receiving or not receiving proton pump inhibitor (PPI) therapy, were included retrospectively. We assigned patients to two groups: group 1 patients underwent PPI therapy and group 2 patients did not undergo PPI therapy.

RESULTS: One hundred and five patients with a me-

dian age of 58 (26-87) years were included, 57 (54.3%) of which were women. Esophageal varices were found in 82 (78%) patients, portal hypertensive gastropathy in 72 (68.6%) patients, and gastric varices in 15 (14.3%) patients. PPI therapy was used in 45.5% of patients ($n = 48$). Seventeen (16.1%) patients presented with upper gastrointestinal bleeding; in 14/17 (82.3%) patients, bleeding was secondary to esophageal varices, and in 3/17 patients bleeding was attributed to portal hypertensive gastropathy. Bleeding related to portal hypertension according to PPI therapy occurred in 18.7% ($n = 9$) of group 1 and in 14% ($n = 8$) of group 2 (odds ratio: 0.83, 95% confidence interval: 0.5-1.3, $P = 0.51$).

CONCLUSION: Portal hypertension bleeding is not associated with PPI use. These findings do not support the prescription of PPIs in patients with chronic liver disease with no currently accepted indication.

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Key words: Drug prescription; Liver cirrhosis; Portal hypertension; Proton pump inhibitors; Upper gastrointestinal bleeding

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INTRODUCTION

Since their first clinical use, proton pump inhibitors (PPIs)

have provided benefits in the management of gastrointestinal diseases. This class of drugs is clearly indicated for the treatment of peptic ulcer disease, gastroesophageal reflux disease (GERD), and nonvariceal upper gastrointestinal bleeding, and for prophylaxis in selected users of nonsteroidal anti-inflammatory drugs (NSAIDs)^[1,2]. Unfortunately, the unnecessary prescription of PPIs has become an important problem, which increases economic costs in daily clinical practice^[3,4]. According to previous studies in the clinical context, only 12.3% of cirrhotic patients have an appropriate indication for the prescription of these drugs^[5]. Congestive gastropathy and esophageal and gastric varices are risk factors for the inappropriate use of PPIs^[5].

Few studies, other than pharmacological studies, have investigated the safety and utility of PPIs in cirrhotic populations^[6-9]. There are reports of possible hepatotoxicity associated with the use of PPIs in patients with chronic liver disease (CLD)^[9], but there have been no clinical or experimental trials on the adverse effects of PPIs in the treatment of acute or chronic complications in patients with cirrhosis and portal hypertension (PH). Some data on the possible use of PPIs for the long-term prophylaxis of variceal bleeding exist^[10], and a recent controlled trial by Zhoe *et al.*^[11] compared the efficacy of octreotide, vasopressin, and omeprazole for controlling acute bleeding associated with portal hypertensive gastropathy. However, more clinical evidence is required. The use of this class of drugs in cirrhotic patients seems more habit-related than evidence-based, ultimately leading to an increase in health costs.

In patients with cirrhosis and PH, upper gastrointestinal bleeding has an annual frequency of 25%-35%, and 80%-90% is of variceal origin. The mortality related to variceal bleeding is about 30% per episode, and is recurrent in 70% of patients after the first year^[12-15].

Considering the current paradigm of evidence-based medicine, the use of PPIs in patients with cirrhosis and endoscopic findings of PH is based only on expert opinion, with insufficient evidence to justify the use of these drugs as prophylaxis for variceal bleeding. The aim of this study was to determine whether the use of PPIs in patients with cirrhosis and endoscopic findings of PH (esophageal or gastric varices, or portal hypertensive gastropathy) is associated with a reduction in the frequency of gastrointestinal bleeding secondary to PH.

MATERIALS AND METHODS

We conducted a retrospective, observational, longitudinal, comparative study of outpatients with CLD and endoscopic evidence of PH, receiving or not receiving treatment with PPIs, between December 1, 2004 and January 1, 2006. The endoscopic data considered for PH were esophageal varices, gastric varices, and portal hypertensive gastropathy. The sample comprised a series of consecutive patients with clinical, biochemical, endoscopic, radiological, and/or histological signs of cirrhosis and PH who attended our gastroenterology and liver clinic. We included all patients over 18 years of age who had been reviewed on

at least two visits over the course of one year during the period of the study. All patients with incomplete electronic or paper charts, with no confirmatory endoscopic study at the time of the bleeding episode, were excluded from the study. These patients formed a subset of patients included in our previous work^[5]. Reasons for exclusion from the present study were absence of endoscopic evidence of PH ($n = 80$), and no previous endoscopy ($n = 28$).

The primary demographic and medical variables were age, sex, etiology of CLD, diagnosis of hepatocellular carcinoma, liver function tests, presence of ascites, encephalopathy, the model end-stage liver disease (MELD) score, and previous use of NSAIDs (at least five times per week during the last six months), cyclooxygenase-2 inhibitors, corticosteroids, anticoagulants, and aspirin. Any hospital stay associated with portal hypertensive bleeding was also recorded.

An endoscopic procedure was performed in all patients as an initial approach. Any patient with first endoscopy at the time of an episode of active bleeding was included. The primary endpoint of our study was the presence of portal hypertensive bleeding. We defined bleeding related to PH as any bleeding episode secondary to the rupture or erosion of esophageal or gastric varices and/or portal hypertensive gastropathy, manifested clinically as melena or hematemesis. All patients with suspected variceal bleeding during the period of the study were required to have an endoscopic procedure in the first 24 h after presentation. A regular diagnostic endoscope was initially used (GIF-100, GIF-130, GIF-140, or GIF-160; Olympus, Japan). The presence of esophageal or gastric varices, portal hypertensive gastropathy, red signs, and the size of the varices were recorded according to the Baveno IV consensus^[16]. Other variables assessed included nonliver-related findings such as esophagitis, hiatal hernia, erosive gastritis, and duodenal or gastric ulcer.

The use of PPIs and other medical prescriptions within the six months preceding the study were identified in the patients' records. We defined PPI users as those patients with cirrhosis who had taken 20 mg of omeprazole (or an equivalent dose of any other PPI) for at least eight weeks before the episode of portal hypertensive bleeding or initial evaluation (first considered visit). Confirmation of the patients' compliance with the PPI treatment was based on chart records. A diagnosis of GERD was made according to the definition: "a condition that develops when the reflux of stomach contents causes troublesome symptoms and/or complications"^[17]. Troublesome symptoms were defined by the patient as affecting his/her quality of life. The symptoms considered were heartburn, regurgitation, reflux-related chest pain, extraesophageal syndromes of GERD (laryngitis, cough, asthma) confirmed by their resolution with PPI therapy, pH monitoring, or endoscopic evidence of esophagitis, according to the Los Angeles classification (grades B, C, or D)^[17].

Statistical analysis

The results are expressed as distributions, absolute frequencies, relative frequencies, medians and ranges, or

means \pm SD. For comparison, patients were classified into two groups: patients who used PPIs and patients who did not use PPIs. The quantitative data were compared using the Student's *t*-test for variables with a normal distribution, and the Mann-Whitney *U* test for other variables. Differences between the proportions of categorical data were evaluated with Fisher's exact test when the number of expected subjects was less than five and otherwise with the χ^2 test. A multivariate logistic regression model was used to assess the independent association between PPI use and bleeding related to PH. A *P* value of < 0.05 was considered statistically significant.

Sample size calculation

According to data published by Hajime *et al*^[10] the frequencies of variceal bleeding in patients with and without PPI use were 10% and 52.4%, respectively (a difference of 42%). According to these data, to detect a difference of at least 42%, we required at least 25 patients for each group (group 1, patients with cirrhosis and PPI use; and group 2, patients with cirrhosis and no PPI use). All statistical analyses were conducted with SPSS statistical software (v. 12.0; SPSS Inc., Chicago, IL, USA).

RESULTS

We initially evaluated 135 patients. Thirty patients were excluded because of incomplete data, therefore, a total of 105 patients were included in the study. The characteristics of the included patients are shown in Table 1. The most frequent endoscopic finding was esophageal varices in 82 (78%) patients, 16 (19.5%) of whom were recorded as having large varices and/or red signs. Portal hypertensive gastropathy was found in 72 patients (68.6%) and gastric varices in 15 patients (14.3%). Of those patients with gastric varices, 13/15 (86.6%) also had esophageal varices. Other findings not related to CLD were erosive gastropathy in 14 patients (13.3%), hiatal hernia in eight patients (7.6%), duodenal ulcer (Forrest III) in three patients (2.9%), and gastric ulcer (Forrest III) in three patients (2.9%). Other comorbidities are shown in Table 1. There was a tendency [odds ratio (OR): 1.3, 95% confidence interval (CI): 0.72-2.6, *P* = 0.2] to non-portal hypertension-related bleeding episodes (*n* = 20; erosive gastropathy, duodenal ulcer, and gastric ulcer) in patients not using PPIs.

Forty-eight (45.5%) patients underwent PPI therapy. Most of these patients used omeprazole, although 10 used pantoprazole. During the period of evaluation, 16.1% (*n* = 17) presented with upper gastrointestinal bleeding related to PH, and in 82.3% of these patients (*n* = 14), this bleeding was secondary to esophageal varices, whereas in three patients it was attributable to portal hypertensive gastropathy. We recorded no episodes of bleeding secondary to gastric varices. When we analyzed the presence of variceal bleeding in patients classified according to their pattern of PPI use (group 1, patients using PPI, *n* = 48; and group 2, patients not using PPI, *n* = 57), the frequency was 18.7% (*n* = 9) in group 1 and 14% (*n* = 8) in group 2 (OR: 0.83, 95% CI: 0.5-1.3, *P* = 0.51). When we evaluated only those

Table 1 Characteristics of the patients included in the study classified by proton pump inhibitor use (mean \pm SD) *n* (%)

Variable	Patients using PPIs (<i>n</i> = 48)	Patients not using PPIs (<i>n</i> = 57)	<i>P</i> value
Age (yr)	56.1 \pm 13.8	57 \pm 12.4	0.71
MELD	12.8 \pm 6.3	11.5 \pm 5.4	0.25
CPT	8.3 \pm 1.8	7.2 \pm 2.2	0.55
Albumin (g/L)	28 \pm 0.6	32 \pm 1.5	0.16
Total bilirubin (mg/L)	27 \pm 3.7	24 \pm 3.3	0.62
ALT (UI/L)	47.2 \pm 22.6	50 \pm 40.5	0.66
Alkaline phosphatase (UI/L)	161.2 \pm 92.2	132.9 \pm 63.8	0.06
BMI	26.5 \pm 4.4	25.9 \pm 3.7	0.46
Sex, male	19 (40)	29 (51)	0.24
Etiology			
Viral hepatitis C	25 (52)	25 (44)	0.44
Alcohol	12 (25)	12 (21)	0.56
Cryptogenic	5 (10)	10 (18)	0.28
Autoimmune hepatitis	2 (4)	8 (14)	0.22
Other	4 (8)	2 (4)	0.26
Child-pugh-turcotte			
A	19 (40)	31 (54)	0.2
B	22 (46)	17 (30)	0.3
C	7 (15)	9 (16)	0.4
GERD	7 (15)	5 (9)	0.7
Gastric/esophageal varices	44 (92)	40 (70)	0.006
Large	6 (13)	3 (5)	0.1
Red signs	4 (8)	3 (5)	0.35
Responders to β -adrenergic blocker	13 (27)	11 (19)	0.34
NSAID	4 (8)	0 (0)	0.04
Antiplatelet agents use	7 (15)	5 (9)	0.1
Oral anticoagulation	1 (2)	1 (2)	0.9
Steroid use	3 (7)	2 (4)	0.37
Comorbidities			
Diabetes mellitus	19 (40)	28 (49)	0.43
Hypertension	9 (19)	14 (25)	0.63
High-level triglycerides	3 (6)	10 (18)	0.13

CPT: Child-pugh-turcotte class; GERD: Gastroesophageal reflux disease; MELD: Model for end stage liver disease; PPIs: Proton pump inhibitors; ALT: Alanine aminotransferase; NSAID: Non-Steroidal anti-inflammatory drugs; BMI: Body mass index (calculated as patient body weight divided by the square of their height expressed in kg/m²).

patients with upper gastrointestinal bleeding secondary to esophageal varices, we observed frequencies of 12.5% in group 1 and 14% in group 2 (OR: 1.07, 95% CI: 0.56-2.0, *P* = 0.81). A comparison of the characteristics of patients using PPIs and those not using PPIs is shown in Table 1.

The overall prevalence of GERD was 11.4% (*n* = 12), corresponding to 14.5% of group 1 (*n* = 7/48). Only seven (57.1%) patients with GERD received PPIs. Of the total number of patients with portal hypertensive bleeding, 11.7% (*n* = 2/17) had GERD. The presence of GERD was not statistically significantly associated with the presence of upper gastrointestinal bleeding (OR: 0.53, 95% CI: 0.15-1.8, *P* = 0.31). Univariate and multivariate analyses of the variables associated with gastrointestinal bleeding secondary to PH are shown in Table 2.

DISCUSSION

In this study, we observed that in patients with CLD and endoscopic evidence of PH, the presence of gastrointes-

Table 2 Univariate and multivariate analyses of risk factors associated with portal-hypertension related bleeding in cirrhotic patients

	B Coefficient	Standard error	Wald χ^2	OR (95% CI)	P value
Univariate					
Age ≥ 60	-	-	-	1.1 (0.37-3.5)	1
Sex, male	-	-	-	1.7 (0.5-5.2)	0.39
CPT C	-	-	-	1.6 (0.40-6.6)	0.36
GERD	-	-	-	0.53 (0.15-1.8)	0.31
MELD > 15	-	-	-	1.2 (0.35-4.4)	0.47
PPI use	-	-	-	0.83 (0.5-1.3)	0.51
LEV	-	-	-	12 (3-123)	< 0.001
Red signs	-	-	-	10 (2-58)	< 0.001
NSAID	-	-	-	0.86	0.55
Multivariate					
LEV	23.7	15.1	0	10 (4-110)	< 0.001
Red signs	22.0	14.2	0	9 (4-102)	< 0.001

CPT C: Child-pugh-turcotte class C; GERD: Gastroesophageal reflux disease; MELD: Model for end stage liver disease; PPI: Proton pump inhibitor; LEV: Large Esophageal varices; NSAID: Non-steroidal anti-inflammatory drugs; OR: Odds ratio; CI: Confidence interval.

tinal bleeding secondary to variceal or portal hypertensive gastropathy was not associated with the use of PPIs. This is a very important finding because it has been reported that the presence of PH on endoscopy is associated with an unacceptable, and according to our data, unnecessary prescription of PPIs in patients with cirrhosis^[5,18].

Soon after the introduction of PPIs into clinical practice, these drugs demonstrated their effectiveness in several gastrointestinal diseases. However, the overuse of this class of drugs has important economic implications. In patients with cirrhosis, many factors influence the appropriate prescription of PPIs^[5,18]. It was observed in previous studies that patients in the early stages of Child-Pugh-Turcotte classification, and with a low MELD score, were more likely to be prescribed PPIs appropriately than those in the more advanced stages of the disease or with endoscopic findings of PH^[5,18]. According to the scarce evidence available regarding the use of these drugs in the clinical context, it seems that physicians tend to consider the use of PPIs in cirrhotic patients as possibly beneficial for variceal bleeding. It has also been postulated in the past that gastroesophageal reflux may contribute to esophagitis and variceal bleeding in patients with CLD^[19]. In fact, there are few data on the use of PPIs in these patients, and these data are predominantly related to the pharmacological properties of the drugs^[20]. The findings of our study are consistent with those of other studies, which have reported that patients with PH, and especially those with portal hypertensive gastropathy, display increased bicarbonate production and an elevated gastric pH. The increased circulatory rate in these patients, the high gastric pH level, and the increased prevalence of hypochlorhydria are factors associated with lower pepsin activity^[21-25].

The main limitation of our study is its retrospective design. However, data concerning the association of portal hypertensive bleeding with the use of PPIs are scarce and

are based on only one study, published as an abstract^[10]. There is an absence of data from randomized trials, thus, prospective studies are still required to develop more reliable recommendations regarding the use of PPIs in this context. The diagnosis of PH in this study was based on esophageal varices, gastric varices, and hypertensive gastropathy, therefore, it is possible that some patients with a hepatic venous pressure gradient above 12 mmHg were overlooked. However, our study focused on patients with endoscopic findings related to PH.

In conclusion, our data support the hypothesis that the use of PPIs is not associated with upper gastrointestinal bleeding related to PH in cirrhotic patients. Therefore, these findings do not support the use of PPIs in patients with CLD and endoscopic evidence of PH without a currently accepted indication.

COMMENTS

Background

Gastrointestinal bleeding secondary to portal hypertension is a major complication in patients with cirrhosis, and proton pump inhibitors are frequently used to prevent it. These drugs have provided benefits in the management of many gastrointestinal disorders; unfortunately, the unnecessary prescription of these drugs has become an important problem, which increases costs in daily practice. Considering the current paradigm of evidence-based medicine, their use in patients with cirrhosis and portal hypertension is based only on expert opinion, with insufficient evidence to justify the use of these drugs as prophylaxes.

Research frontiers

Proton pump inhibitors are widely used among patients with chronic liver disease and endoscopic findings of portal hypertension with the aim of preventing bleeding, however, there is no appropriate evidence to support their use for this condition. In this study, the authors demonstrate that the use of proton pump inhibitors is not associated with a lower frequency of gastrointestinal bleeding in cirrhotic patients.

Innovations and breakthroughs

In this study, the authors observed that in patients with chronic liver disease and endoscopic evidence of portal hypertension, the presence of gastrointestinal bleeding secondary to variceal or portal hypertensive gastropathy was not associated with the use of proton pump inhibitors. This is a very important finding because it has been reported that the presence of portal hypertension on endoscopy is associated with an unacceptable, and according to the data, unnecessary prescription of proton pump inhibitors in patients with cirrhosis.

Applications

This study provided evidence on the use of proton pump inhibitors in patients with chronic liver disease and endoscopic findings of portal hypertension, and does not support their use without a currently accepted indication for their prescription in this group of patients.

Terminology

Proton pump inhibitors are a class of drugs that reduce the secretion of HCl in the stomach, consequently increasing gastric pH. The current principal accepted indications for these drugs include peptic ulcer disease and gastroesophageal reflux disease. Esophageal varices and hypertensive gastropathy are referred to as endoscopic findings of portal hypertension. Portal hypertension is the main cause of gastrointestinal bleeding in patients with cirrhosis, and its prevention is very important in clinical practice.

Peer review

This is a clear cut-off of the question of non-steroidal anti-inflammatory drugs with/without proton-pump inhibitors administration dilemma. The clinical problem is well addressed and presented, and the authors provide a rationale for their conclusions.

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Metabolomics of gastric cancer metastasis detected by gas chromatography and mass spectrometry

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Abstract

AIM: To elucidate the underlying mechanisms of metastasis and to identify the metabolomic markers of gastric cancer metastasis.

METHODS: Gastric tumors from metastatic and non-metastatic groups were used in this study. Metabolites and different metabolic patterns were analyzed by gas chromatography, mass spectrometry and principal components analysis (PCA), respectively. Differentiation performance was validated by the area under the curve (AUC) of receiver operating characteristic curves.

RESULTS: Twenty-nine metabolites were differentially expressed in animal models of human gastric cancer. Of the 29 metabolites, 20 were up-regulated and 9 were down-regulated in metastasis group compared to non-metastasis group. PCA models from the metabolite profiles could differentiate the metastatic from the non-metastatic specimens with an AUC value of 1.0. These

metabolites were mainly involved in several metabolic pathways, including glycolysis (lactic acid, alanine), serine metabolism (serine, phosphoserine), proline metabolism (proline), glutamic acid metabolism, tricarboxylic acid cycle (succinate, malic acid), nucleotide metabolism (pyrimidine), fatty acid metabolism (docosanoic acid, and octadecanoic acid), and methylation(glycine). The serine and proline metabolisms were highlighted during the progression of metastasis.

CONCLUSION: Proline and serine metabolisms play an important role in metastasis. The metabolic profiling of tumor tissue can provide new biomarkers for the treatment of gastric cancer metastasis.

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Key words: Gastric cancer; Metastasis; Metabolite; Metabolomics; Gas chromatography and mass spectrometry

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INTRODUCTION

Gastric cancer is one of the most common malignancies and the second cause of cancer-related death worldwide and in most Asian countries, such as China^[1,2]. So far, surgical resection remains the only curative treatment option^[3]. However, because of its asymptomatic properties,

most patients are frequently misdiagnosed until local and distant metastases occur, leading to a poor prognosis of gastric cancer patients with a 5-year survival rate of less than 30%. Most gastric cancer-related deaths occur as a result of metastasis. Metastatic recurrence is the main obstacle to the improvement of therapy for gastric cancer. No effective treatment modality is available for this deadly disease at present. Similarly, almost no prophylactic therapies can block dissemination of gastric cancer cells and prevent its metastasis. Currently, histological staging of gastric cancer is mainly based on the depth of its invasion and metastasis, both of which are considered the most important indicators of recurrence and prognosis of gastric cancer after curative resection. It has been shown that some gene candidates, such as cell adhesion molecules, are involved in the process of gastric cancer metastasis^[3-5]. However, no routine molecule markers for predicting gastric cancer metastasis and prognosis are available because of their high variability in expression levels.

The prognosis of patients with advanced gastric cancer remains very poor because the molecular mechanism underlying its metastasis is not fully understood^[3,5]. Gastric cancer metastasis, which is a complex and multistep process, involves release, migration and penetration of its cells through the vessel walls, arrest of its cells in microcirculation of distant organs and their subsequent migration and growth at the metastatic sites. To get a better insight into the mechanism of such a process, metastasis and non-metastasis animal models of gastric cancer were established using the human gastric cancer cell line SGC-7901. Because of the same genetic backgrounds, the animal is a suitable comparative system for studying the molecular changes in gastric cancer metastasis. At present, most investigations are focused on the identification of altered genes and proteins that play an important role in gastric cancer progression^[6-9]. However, only a few reports are available on the identification of key metabolites characterizing gastric cancer metastasis^[6,10,11]. Metabolomics, an omic science in systems biology, is the comprehensive and simultaneous profiling of metabolic changes occurring in living systems in response to genetic, environmental, or lifestyle factors^[11]. The missing link between genotype and phenotype can be established, which may provide information about gastric cancer that is complementary to genomics and proteomics analysis, thus improving our understanding of the pathogenic mechanisms and metabolic phenotype of gastric cancer. It has been recently shown that metabolomic method has great potentials in identifying the new diagnostic markers and therapeutic targets for different cancers, such as breast, prostate, pancreatic, liver, colon and gastric cancers^[12-18], suggesting that metabolic alterations play a role in the biology of cancer. A more recent metabolomic analysis showed that increased sarcosine synthesis is an important metabolic change during prostate cancer progression^[6]. However, to our knowledge, there is no metabolomic study on metastasis of gastric cancer^[6,10].

In this study, metabolomic difference in metastasis and non-metastasis models of gastric cancer was detected by gas chromatography (GC) and mass spectrometry

(MS), respectively, in accordance with our hypothesis that there were metabolite clusters associated with metastasis of gastric cancer.

MATERIALS AND METHODS

Chemicals

Tetrahydrofuran (THF), N-methyl-N-t-butyltrimethylsilyltrifluoro-acetamide (MBDSTFA) and chromatographic pure were purchased from Sigma Chemical Co. (St Louis, MO, USA). Pyridine, sodium hydroxide, chloroform, anhydrous ethanol, and anhydrous sodium sulfate, purchased from China National Pharmaceutical Group Corporation (Shanghai, China), were of analytical grade. Vacuum dryer was the product of Shanghai NOTED Technologies (China). Distilled water was obtained from the Milli-Q System (Millipore, MA, USA).

Animals

Male mice with severe combined immune deficiency (SCID) at the age of 6 wk, weighing 20-25 g, were obtained from Shanghai Experimental Animal Center, Chinese Academy of Sciences (China). The mice were housed under specific pathogen-free conditions with free access to food and drinking water. Experiment was performed in accordance with the Chinese National Guidelines for the Care and Use of Laboratory Animals and the relative ethical regulations of our university.

Animal treatment

Twenty-two SCID mice were randomly divided into metastasis group ($n = 8$), non-metastasis group ($n = 8$), and control group ($n = 6$). An animal model of metastasis was induced by orthotopic implantation of histologically intact tissue of human gastric carcinoma as previously described^[19] with some minor modifications. Human gastric cancer SGC-7901 (Shanghai Cancer Institute), a poorly-differentiated adenocarcinoma line, was originally derived from a primary tumor and maintained by passage in subcutis of nude mice. Tumors were removed aseptically with necrotic tissues cleaved away. The adjacent healthy tissues were cut into pieces (about 3 mm × 4 mm) in Hank's balanced salt solution, weighed and adjusted to 100 mg. After the SCID mice were anesthetized with 4.3% trichloraldehyde hydrate, an incision was made through the left upper abdominal pararectal line of mice in metastasis group with the peritoneal cavity carefully exposed. A part of the serosal membrane in middle of the greater curvature of stomach was mechanically injured using scissors. One hundred mg of tumor pieces was fixed at each injured site on the serosal membrane surface. After the stomach was returned to the peritoneal cavity, the abdominal wall and skin were closed. After the SCID mice were anesthetized with 4.3% trichloraldehyde hydrate, an incision was made in the left outer of mice in non-metastasis group. Then, 100 mg of tumor tissue pieces was implanted into the subcutis (ectopic implantation) of mice. Meanwhile, the mice in non-metastasis group underwent the same ortho-

topic operation as those in metastasis group but with no tumor implantation into the gastric wall. The mice that underwent the same procedure with no tumor implantation served as a control group. All animals tolerated the surgical procedure well with no anesthesia-related death.

Sample collection and pathological examination

After anesthesia, all mice were sacrificed and subjected to autopsy. Tumors growing at the orthotopic or subcutaneous sites were harvested. Half of each tumor was snap-frozen at -80°C , while the other half was fixed in 4% formalin and embedded in paraffin. Tissues were collected from lymph nodes and all organs, fixed in 4% formalin, and processed for routine paraffin embedding after careful macroscopic examination. The tissue was cut into 4 μm -thick sections which were stained with hematoxylin and eosin, and evaluated histologically for metastasis of lymph nodes, liver or other organs by microscopy.

Sample pretreatment and derivation

One mL still water and one mL anhydrous ethanol were added to each 100 mg tissue sample. Gastric carcinoma tissue samples were ground with still water and anhydrous ethanol (1:1, v/v). The mixture was ultrasonicated at 4°C for 30 min and vortexed for 2 min. The tissue sample (0.1 g/mL) was centrifuged at $18000 \times g$ (10000 r/min) for 10 min. The aqueous layer was adjusted to pH 7.8 with 0.1 mol/L potassium phosphate buffer solution, and 1.5 mL supernatant was obtained from each sample. The collected supernatant (500 μL) was evaporated to dryness at 50°C for 24 h in a vacuum dryer. Then 150 μL tetrahydrofuran was added to each of the dried tissue extracts, vortex-mixed for 10 min and evaporated to complete dryness in a nitrogen evaporator. MBDSTFA (100 μL) was added to each sample and derived at 60°C for 30 min. The samples were vortexed for 30 s after derivation, for GC and MS analysis.

GC and MS analysis

Each of the samples was injected into an Agilent 6980 GC system equipped with a HP5MS capillary column (30 mm \times 0.25 mm, i.d., 0.25 μm), and a quadrupole mass spectrometric detector (Agilent Technologies, Palo Alto, CA, USA). Helium was used as the carrier gas with a constant flow rate of 1.0 mL/min. One μL sample was injected into the Agilent 6980 GC system at 280°C . The column temperature was initially kept at 100°C for 3 min, and then elevated to 220°C at an increasing rate of 10°C per min, followed by 10°C per min to 280°C for 5 min. Both of the interface and ion source temperature were 200°C . MS was conducted in an electron impact ionization mode at 70 eV. Mass data were obtained in a full scan mode from m/z 100 to 600. Total ion chromatograms (TIC) and fragmentation patterns of GC were acquired using the GC/MSD ChemStation Software (Agilent Technologies, Palo Alto, CA, USA). Compounds were identified by comparing the mass spectrum with a standard mass spectrum in the National Institute of Standards and Technology (NIST) Mass Spectra Library. Peaks with a similarity index more than

70% were the assigned compound names, while those with a similarity index less than 70% were considered unknown metabolites^[16]. Chromatograms were subjected to noise reduction prior to peak area integration. Any known artificial peaks due to derivatization of column bleed and BSTFA should be excluded from the data set. The resulting three-dimensional matrix included sample information, peak retention time, and peak area.

Statistical analysis

Data, normalized by dividing the sum of all peak areas in the sample (1 mg) before multivariate analysis, were expressed as mean \pm SD and introduced into SPSS16.0 for Windows. Metabolite levels were compared by independent *t* test for the detection of significant differences in metastasis and non-metastasis groups. $P < 0.05$ was considered statistically significant. Principal components analysis (PCA) was performed to differentiate the metabolic patterns in metastasis and non metastasis groups. The differentiation performance was validated by the area under the curve (AUC) of receiver operating characteristic (ROC) curves. Similarly, metabolomic differences in metastasis group, non-metastasis group and control group were analyzed by *t* test and PCA.

RESULTS

The mean weight of mice in three groups was 23.81 ± 0.16 , 23.87 ± 0.19 and 23.98 ± 0.19 g, respectively. Microscopy showed localized poorly-differentiated adenocarcinoma in all mice of non-metastasis and metastasis groups at the implanted sites. The average tumor weight was 4.28 ± 0.20 g in non-metastasis group and 4.30 ± 0.3 g in metastasis group, indicating that the tumor grows at a similar rate in the subcutis or stomach. However, the metastatic rate was significantly different when the tumor was implanted into the ectopic or orthotopic sites. Two thirds of the mice bearing an orthotopic tumor developed metastatic tumors in the region of lymph nodes, half in liver and one fourth in other organs. In contrast, no tumor metastasis was observed in the non-metastasis group after ectopic implantation. Macroscopic and histological examination showed no gastric cancer in the mice without tumor implantation. These results indicate that GC and MS are of a high reproducibility in the retention time of metabolites. Of the 152 signals detected in the tissue samples, some were not consistently found in other samples or could not be assigned to the unique metabolites because the abundance was too low. Fifty-eight signals could be identified by comparing a standard mass chromatogram with that in the NIST Library (Table 1). According to the NIST Mass Spectra Database, most of the chromatograms were identified as endogenous metabolites, such as amino acids, organic acids, inorganic acids, fatty acids, and pyrimidines, which were involved in several metabolic pathways, including glycolysis (lactic acid, alaline), serine metabolism (serine, phosphoserine), proline metabolism (proline), glutamic acid metabolism, glutamine metabolism, tricarboxylic acid (TCA) cycle (succinate), nucleotide metabolism (pyrimidine), fatty acid

Table 1 Tissue metabolites in mice of control, non-metastasis and metastasis groups

Peak No.	Percentage	RT (min) metabolites	Match
1	6.1128	Propanoic acid	87
2	7.9894	Acetic acid	97
3	9.8780	Silanol	93
4	10.9850	Lactic acid	88
5	11.4610	Propanedioic acid	96
6	12.1110	2-methylglutaconic acid	80
7	14.9138	Butanoic acid	90
8	15.4200	L-Alanine	89
9	15.9640	Glycine	92
10	16.4966	Urea	93
11	16.7655	Acetamide	81
12	17.5162	1, 2-Butanediol	93
13	17.9103	L-methioninamide	82
14	18.1660	L-Valine	80
15	18.3168	Ethanol	73
16	18.6734	5-Nonanol	74
17	19.1113	D, L-leucine	98
18	19.3427	Pyrimidine	93
19	19.4178	Dimethylglycine	96
20	19.7869	L-isoleucine	86
21	20.4186	Succinate	85
22	21.0066	Propanamide	87
23	21.5133	Butanedioic acid	81
24	21.8323	2-butenic acid	95
25	23.0521	Hexanethioic acid	89
26	23.8090	Malic acid	92
27	24.3032	L-Proline	91
28	24.4971	Pyrrolidine	72
29	24.5909	L-Methionine	83
30	25.0350	L-Serine	97
31	25.5230	L-Threonine	84
32	25.9421	Silanol	95
33	26.2298	1, 3-propanediamine	91
34	26.4800	L-phenylalanine	96
35	26.5676	Tetrame-thylenediamine	83
36	27.1681	Hexanoic acid	97
37	27.3933	L-Aspartic acid	90
38	27.9563	Glutamine	94
39	28.1940	Phosphoserine	98
40	28.3942	Mannofuranose	83
41	28.7319	L-Glutamic acid	84
42	28.8383	Hypoxanthine	91
43	29.5264	Glucofuranose	75
44	29.7953	L-Lysine	95
45	30.0330	2-furancarboxylic acid	99
46	30.7462	Glucose	97
47	31.6532	L-histidine	87
48	31.7845	L-arginine	89
49	32.0472	L-Tyrosine	92
50	32.1598	D-ribofuranose	83
51	32.4726	Decanoic acid	81
52	32.6540	Hexanedioic acid	95
53	33.5861	5-pyrimidinecarboxylic acid	89
54	34.6932	Myo-inositol	94
55	34.9560	Hexadecanoic acid	90
56	35.0435	Docosanoic acid	92
57	35.3250	Octadecanoic acid	79
58	35.4439	Maltose	92

metabolism (docosanoic acid, and octadecanoic acid), and methylation(glycine).

The GC and MS data about tissue metabolites in metastasis and non-metastasis groups were analyzed by

Table 2 Different metabolites identified in metastasis and non-metastasis groups (mean \pm SD)

No.	Metabolites	Metastasis group (n = 8)	Non-metastasis group (n = 8)	P	Fold
1	Lactate	4.194 \pm 0.595	2.662 \pm 0.218	0.000	1.58
2	Propanedioic	0.058 \pm 0.004	0.0324 \pm 0.002	0.000	1.80
3	Alanine	2.643 \pm 0.145	1.903 \pm 0.195	0.000	1.39
4	Glycine	0.762 \pm 0.054	0.456 \pm 0.172	0.001	1.67
5	Valine	1.036 \pm 0.113	0.650 \pm 0.047	0.000	1.59
6	Leucine	2.447 \pm 0.306	1.756 \pm 0.204	0.000	1.39
7	Pyrimidine	0.214 \pm 0.030	0.166 \pm 0.031	0.008	1.29
8	Dimethylglycine	0.372 \pm 0.047	0.263 \pm 0.020	0.000	1.42
9	Isoleucine	0.772 \pm 0.087	0.992 \pm 0.104	0.000	-1.29
10	Succinate	0.0794 \pm 0.006	0.123 \pm 0.018	0.000	-1.56
11	Propanamide	0.101 \pm 0.007	0.147 \pm 0.006	0.000	-1.45
12	Butanedioic	0.050 \pm 0.008	0.073 \pm 0.015	0.004	-1.46
13	Malic acid	3.053 \pm 0.348	1.788 \pm 0.116	0.000	1.71
14	Proline	1.173 \pm 0.093	0.479 \pm 0.072	0.000	2.45
15	Pyrrolidine	0.105 \pm 0.016	0.067 \pm 0.0083	0.000	1.57
16	Methionine	1.169 \pm 0.099	1.518 \pm 0.101	0.000	-1.30
17	Serine	1.742 \pm 0.108	1.175 \pm 0.074	0.000	1.48
18	Threonine	0.538 \pm 0.040	0.632 \pm 0.063	0.003	-1.17
19	Aspartic	5.870 \pm 0.485	3.839 \pm 0.453	0.000	1.53
20	Glutamine	0.090 \pm 0.010	0.153 \pm 0.016	0.000	-1.71
21	Phosphoserine	0.169 \pm 0.0096	0.109 \pm 0.012	0.000	1.56
22	Glutamate	3.533 \pm 0.310	2.727 \pm 0.200	0.000	1.30
23	Hypoxanthine	1.673 \pm 0.150	1.841 \pm 0.138	0.035	-1.10
24	Lysine	2.304 \pm 0.137	1.808 \pm 0.114	0.000	1.27
25	Glucose	1.338 \pm 0.118	1.956 \pm 0.142	0.000	-1.46
26	Arginine	1.342 \pm 0.178	0.987 \pm 0.088	0.000	1.36
27	Inositol	0.409 \pm 0.030	0.314 \pm 0.022	0.000	1.30
28	Docosanoic	0.123 \pm 0.012	0.076 \pm 0.016	0.000	1.61
29	Octadecanoic	0.756 \pm 0.103	0.555 \pm 0.134	0.005	1.36

P-value was calculated by student t test. $P < 0.05$ was considered statistically significant. Fold change with a positive value indicates a relatively higher level of metabolites while a negative value indicates a relatively lower level in metastasis group than in non-metastasis group.

Student's *t* test. Marker metabolites selected by Student's *t* test are presented in Table 2. Among these metabolites, proline was the most up-regulated tissue metabolite in metastasis group, which was 2.45-fold higher than that in non-metastasis group. Glutamine was the most down-regulated tissue metabolite in the metastasis group, which was 1.71-fold lower than that in the non-metastasis group. The lactic acid, L-alanine, L-valine, leucine, malic acid, L-aspartic acid, serine, phosphoserine, dimethylglycine, glycine, L-glutamic acid, L-lysine, myo-inositol, propanedioic acid, docosanoic acid, octadecanoic acid, arginine, pyrroline, and pyrimidine were significantly up-regulated, while the glucose, succinate, L-isoleucine, L-methionine, propanamide, L-threonine acid, and butanedioic acid were remarkably down-regulated in the metastasis group compared to the non-metastasis group. The main metabolic pathways associated with metastasis of gastric cancer included glycolysis (lactic acid, alanine), serine metabolism (serine, phosphoserine), proline metabolism (proline), tricarboxylic acid (TCA) cycle (succinate, malic acid), fatty acid metabolism (docosanoic acid, and octadecanoic acid), and methylation(glycine).

The levels of lactic acid, propanedioic acid, L-alanine,

L-valine, glycine, leucine, malic acid, pyrroline, serine, L-proline, L-methionine, L-threonine, L-aspartic acid, phosphoserine, L-glutamic acid, L-lysine, arginine, myo-inositol, docosanoic acid, and octadecanoic acid were higher in non-metastasis group and metastasis group than in control group, while the levels of glucose, L-isoleucine, succinate, and glutamine were lower in control group than in non-metastasis group and metastasis group.

PCA was used to identify the metabolic patterns associated with gastric cancer metastasis. The PCA scores of non-metastasis group and metastasis group were scattered into two different regions. ROC analysis, which was performed using the values determined by the first two components of the PCA model, confirmed the robustness of the PCA model. The sensitivity and specificity trade-offs were summarized for each variable with the AUC. The AUC value for the PCA model was 1.00, demonstrating a good differential value for metastasis of gastric cancer.

The PCA model was also validated by ROC analysis (AUC = 1.00), showing that the growth of human gastric cancer in mice was of distinct metabolic properties compared to that in the host.

DISCUSSION

Metastasis is one of the leading causes for poor prognosis of patients with gastric cancer^[20]. The approaches used in tumor staging and prognosis assessment have a large number of limitations^[21,22]. Recent advances in the field of metabolomics have provided the new opportunities to identify novel diagnostic markers and therapeutic targets for gastric cancer^[6,11]. It is very important to establish a relevant model of cancer metastasis. Cell lines are often used as experimental models to investigate the genetic and biochemical alterations in tumor cells^[23]. However, these models have limitations due to the lack of interactions between tumor cells and host^[24]. Our previous study showed that human gastric cancer growing at orthotopic sites produces metastasis, and does not result in metastasis in SCID mice when it is implanted into the ectopic sites^[19]. It has been shown that a mouse gastric cancer model develops metastasis similar to human tumor^[19], which is in favor of research on gastric cancer metastasis. Consistent with our results, an orthotopically implanted pancreatic cancer model is a prerequisite for identifying a differential gene expression pattern unique to metastatic human pancreatic cancer^[23].

In the present study, a highly metastasis model was successfully induced by orthotopic implantation of histologically intact tissue of SGC-7901 human gastric carcinoma while a non-metastasis model was established by subcutaneous implantation of the same cancer tissue in SCID mice. Tissue samples from gastric and subcutaneous tumors were derived from the same human gastric cancer with the same genetic background. The main difference in the two tumor models was their metastatic abilities. Therefore, metabolic analysis of these samples is valuable for the elucidation of metastatic mechanisms. To our knowledge, this is the first metabolomic investigation of gastric cancer metastasis in animal models. The compara-

tive metabolomic strategy was used to identify the marker metabolites which were differentially expressed in animal models of human gastric cancer with distinct metastatic phenotypes. Of the 29 differential metabolites detected in the tumor models, 20 were up-regulated and 9 were down-regulated in the metastasis group compared to those in the non-metastasis group, indicating that these metabolites are involved in several metabolic pathways.

The lactic acid level was higher while the glucose level was lower in metastasis group than in non-metastasis group. Lactic acid is the end product of glycolysis. Increased glucose uptake and consumption are frequently observed in many cancer cells even under normoxic conditions, known as the Warburg effect. It has been demonstrated that the lactic acid level is increased in various metastatic cancers, including renal, uterine cervix, head and neck, colorectal cancers^[25-27]. Moreover, the high lactate level in tumor tissue is associated with its metastasis and poor prognosis^[27]. In fact, increased lactic acid produced by tumor cells can result in acid-mediated matrix degradation, T cell inactivation, up-regulation of VEGF and HIF-1 alpha, and enhancement of cell motility, thus providing favorable conditions for metastatic spread^[25,27]. Therefore, high lactic acid levels reflect an increased energy demand for tumor progression.

In the present study, GC and MS showed that proline was the most up-regulated tissue metabolite, indicating that increased proline in metastatic gastric cancer tissue may be correlated with the increased turnover of extracellular matrix in metastatic cancer cells. Tumor cells need increased degradation of collagen during the process of invasion and metastasis, thus producing a large amount of proline. Pyrroline-5-carboxylic (P5C) is the precursor of proline and also its degradation product. Proline oxidase, also known as proline dehydrogenase, catalyzes the first step of proline to P5C in mitochondria and the latter is converted to proline by the cytosolic P5C reductase. It has been demonstrated that proline oxidase can be induced by p53 due to genotoxic stress and initiates apoptosis by the mitochondrial and death receptor pathways^[28]. Proline is a stress substrate and matrix metalloproteinases can degrade collagen in the extracellular matrix. So far little attention has been paid to the correlation between proline metabolism and tumor progression. It has been shown that proline consumption is increased in patients with metastatic renal cancer^[29]. Increased proline biosynthesis has been recently observed in metastatic breast cancer cell lines^[30]. Furthermore, proline metabolism is linked with arginine and glutamate metabolism, TCA cycle and pentose phosphate pathway (PPP) due to P5C^[28], suggesting that the significantly up-regulated metabolism of proline is highly correlated with cancer metastasis.

In this study, serine metabolism was involved in the metastatic process of gastric cancer, showing alterations in the pathway. The serine and phosphoserine increased 1.48-fold and 1.56-fold, respectively in metastatic tumors. It was reported that all the three genes involving the serine biosynthesis pathway, phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1),

and phosphoserine phosphatase (PSPH) are up-regulated in highly metastatic breast cancer cells^[31], which is agreement with our results. Although most amino acids are increased in gastric cancer and colorectal cancer tissue compared with their adjacent normal tissue^[17], little is known about the alterations related to metastatic behaviors. In the present study, the levels of leucine, valine, glutamate and lysine were higher while the levels of methionine and threonine were lower in metastatic specimens, indicating that the demand for energy is increased in metastatic progression. Glycine can derive from serine synthesized by the glycolytic intermediate phosphoglycerate and its elevated levels may be associated with glycolysis. Sarcosine, a methylation derivative of the amino acid, glycine, is related to prostate cancer progression^[6]. Dimethylglycine is another methylation product of glycine and how it is up-regulated remains unclear.

The disturbed TCA cycle, observed in a large number of tumors including gastric cancer, is considered to be related to carcinogenesis^[17]. In this study, the TCA cycle intermediates such as succinate and malic acid were remarkably perturbed in metastatic specimens, suggesting that enhanced glycolysis contributes to metastatic progression.

In this study, the expression patterns of metastatic and non-metastatic human carcinoma were compared by metabolomic analysis, and several important metabolic pathways associated with metastasis of gastric cancer were identified. Of note, proline and serine metabolism were highlighted in this study. Further functional and clinical sample analysis of the metabolic pathways is needed to demonstrate their role in gastric cancer metastasis. The metabolic pathways may be exploited as biomarkers for gastric cancer progression.

COMMENTS

Background

Gastric cancer is the second cause of cancer-related death worldwide, and its metastasis is one of the leading causes of cancer-related death. The molecular mechanisms underlying gastric cancer metastasis are still not fully understood. Recent metabolomic studies have shown that metabolic alterations play a role in the biology of gastric cancer. The metabolic profiling of tumor tissue is used to elucidate the underlying mechanisms and identify the metabolomic markers of gastric cancer metastasis for improving its diagnostic and therapeutic strategies.

Research frontiers

Metabolomics, an OMIC science in systems biology, is the comprehensive and simultaneous profiling of metabolic changes occurring in living systems in response to genetic, environmental, or lifestyle factors. Recently, metabolomic method has shown great potentials in identifying the new diagnostic markers and therapeutic targets for cancers. However, metabolomic study on gastric cancer metastasis remains scarce.

Innovations and breakthroughs

Recently, most investigations have been focused on identifying the altered genes and proteins that play a role in cancer progression. In this study, the expression patterns of metastatic and non-metastatic human carcinoma were compared by metabolomic analysis, and several important metabolic pathways associated with metastasis of gastric cancer were identified. This is the first report on metabolomic investigation of gastric cancer metastasis.

Applications

The results of this study indicate that the metabolic pathways can be exploited as biomarkers for gastric cancer progression, which can be used in diagnosis and treatment of gastric cancer.

Terminology

Metabolomics, an OMIC science in systems biology, is the comprehensive and simultaneous profiling of metabolic changes occurring in living systems in response to genetic, environmental and lifestyle factors. Gas chromatography (GC) and mass spectrometry (MS) have been widely applied in metabolomic investigation because of their high sensitivity, peak resolution and reproducibility.

Peer review

To elucidate the underlying mechanisms of metastasis and identify metabolomic markers of gastric cancer metastasis, the authors performed GC/MS to identify the metabolomic difference in metastatic and non-metastatic lesions. The results indicate that proline and serine metabolism play an important role in gastric cancer metastasis, and that metabolic profiling of tumor tissue can provide new biomarkers for the treatment of gastric cancer metastasis.

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Impact of *KRAS* mutation and PTEN expression on cetuximab-treated colorectal cancer

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We then selected 61 patients treated with cetuximab, either in combination with chemotherapy, or alone as a second-line or third-line regimen to assess whether *KRAS* mutation or PTEN protein expression is associated with the response and the survival time of mCRC patients treated with cetuximab.

RESULTS: *KRAS* mutation was found in 30 (33.3%) tumor samples from the 90 patients, and positive PTEN expression was detected in 58 (64.4%) of the 90 patients. Among the 61 patients who were treated with cetuximab as a second-line or third-line regimen, the resistance to cetuximab was found in 22 patients with *KRAS* mutation and in 39 patients without *KRAS* mutation, with a response rate of 4.5% and 46.1% respectively ($P = 0.001$), a shorter median progression-free survival (PFS) time of 14 ± 1.3 wk and 32 ± 2.5 wk respectively ($P < 0.001$), a median overall survival (OS) time of 11 ± 1.2 mo and 19 ± 1.8 mo respectively ($P < 0.001$), as well as in 24 patients with negative PTEN expression and in 37 patients with positive PTEN expression respectively ($P < 0.001$), with a responsive rate of 4.2% and 48.6% respectively, a shorter median PFS survival time of 17 ± 2.0 wk and 28 ± 1.9 wk respectively ($P = 0.07$), and a median OS time of 11 ± 1.3 mo and 18 ± 1.9 mo respectively ($P = 0.004$). Combined *KRAS* mutation and PTEN expression analysis showed that the PFS and OS time of patients with two favorable prognostic factors were longer than those of patients with one favorable prognostic factor or no favorable prognostic factor ($P < 0.001$).

CONCLUSION: *KRAS* mutation and PTEN protein expression are significantly correlated with the response rate and survival time of Chinese mCRC patients treated with cetuximab.

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Key words: Cetuximab; Metastatic colorectal cancer; *KRAS* mutation; Phosphatase and tensin protein expression

Abstract

AIM: To investigate the prognostic value of *KRAS* mutation, and phosphatase and tensin (PTEN) expression in Chinese metastatic colorectal cancer metastatic colorectal cancer (mCRC) patients treated with cetuximab.

METHODS: Ninety Chinese mCRC patients treated with cetuximab were evaluated for *KRAS* mutation and PTEN protein expression by DNA sequencing of codons 12 and 13 and immunohistochemistry, respectively.

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INTRODUCTION

The incidence of colorectal cancer (CRC) has been increasing in the past decades and CRC is the third-leading cause of cancer-related deaths in China. During the past few years, several new biological agents have been evaluated in metastatic colorectal cancer (mCRC) with a remarkable anti-mCRC activity. Epidermal growth factor receptor (EGFR), one of the most promising targets, can activate the proliferation and prolong the survival time of cancer cells through the Ras/Raf/mitogen-activated protein kinase (MEK)/EPH receptor B2 (ERK) pathway or the phosphoinositide-3-kinase (PI3K)/PTEN/AKT pathway^[1].

Cetuximab (Erbix[®], Merck KGaA, Darmstadt, Germany), a chimeric mouse/human antibody against the extracellular domain of EGFR, has a single-agent activity in mCRC refractory to irinotecan, oxaliplatin and fluoropyrimidines, and restores chemosensitivity in irinotecan-refractory mCRC patients^[2-4]. However, only a small number of patients can benefit from cetuximab. The response rate to the combined cetuximab and irinotecan is about 23%^[2]. Immunohistochemical studies showed that EGFR protein expression in CRC patients is not a useful predictor for the response to cetuximab^[5,6].

Recent reports are available on the EGFR pathways, such as *KRAS*/BRAF/MAPKs, and on their potential correlation with cetuximab activity. *KRAS* somatic mutation occurs in approximately 40% of CRC patients. The negative predictive value of *KRAS* mutation has been confirmed in CRYSTAL study of first-line fluorouracil, leucovorin, and irinotecan (FOLFIRI) with or without cetuximab, demonstrating that only the patients with *KRAS* wild-type mutations benefit from cetuximab treatment^[7-9].

Increasing interest in anti-EGFR therapy has been focused on another EGFR pathway, and PI3K/AKT/PTEN. PTEN encodes phosphatase with phosphatidylinositol-3, 4, 5-triphosphate (PIP-3) produced by the activity of PI3K as its major substrate. Loss of PTEN function increases PIP-3 concentration, and subsequent AKT hyperphosphorylation stimulates the proliferation of cancer cells^[10].

It was reported that PTEN protein expression and *KRAS* mutation can predict the outcome of mCRC patients treated with cetuximab plus irinotecan, and negative PTEN expression in mCRC patients can predict the resistance to cetuximab plus irinotecan. Combined PTEN

expression and *KRAS* mutational analysis can help to identify a subgroup of mCRC patients who have a greater chance of benefiting from EGFR inhibition^[11].

KRAS and PTEN are the important molecular determinants of the EGFR downstream signal pathway and play an important role in anti-EGFR therapy in Western countries. However, little is known about the correlation between *KRAS* mutation and PTEN protein expression with the activity of anti-EGFR mono-antibody in Asian populations. This retrospective study was to evaluate the prognostic value of EGFR downstream cascade members, *KRAS* and PTEN, in Chinese mCRC patients treated with cetuximab plus chemotherapy.

MATERIALS AND METHODS

Patients

We retrospectively assessed 90 mCRC patients (59 males and 31 females with a median age of 53.0 ± 13.9 years) treated with cetuximab in Sun Yat-Sen University Cancer Center and Beijing Cancer Hospital from June 2000 to August 2008. The patients had histologically proven colorectal adenocarcinoma and the tumor response to cetuximab treatment was evaluable. Tissue samples of primary colorectal tumor were taken. *KRAS* mutation and PTEN protein expression in the patients were analyzed. Of the 90 patients, 3 received cetuximab monotherapy, 58 received cetuximab in combination with irinotecan-based chemotherapy, and 29 received cetuximab in combination with oxaliplatin-based chemotherapy. Cetuximab was administered as the first- fourth lines of treatment in 29, 23, 28, and 10 patients, respectively (Table 1). Paraffin-embedded tumor tissue samples from 100 mCRC patients (69 males and 31 females with a mean age of 50.5 ± 12.1 years) not treated with cetuximab were used for gene analysis. Furthermore, *KRAS* mutation in these patients was analyzed to confirm the mutation frequency of *KRAS*.

Skin toxic effects were assessed according to the National Cancer Institute Common Toxicity Criteria, version 2. Tumor response to cetuximab was evaluated by computerized tomodensitometry according to the response evaluation criteria in solid tumors (RECIST) and classified as complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). Patients with CR or PR and SD or PD were classified as responders and non-responders, respectively, for the analyses.

Nucleotide sequence analysis

KRAS mutation was analyzed by extracting genomic DNA from the paraffin-embedded tissue sections with a QIAamp DNA mini kit (Qiagen, Berlin, Germany) according to its manufacturer's instructions. Exon 1 of the *KRAS* gene (GenBank, L00045, nt 102 to 235) was then directly PCR-amplified in a thermal cycler. The sequences of primers used for *KRAS* analysis (codons 12 and 13) are identical to those used in a previous study^[12]. PCR condi-

Table 1 Data about patients enrolled in this study

Characteristics	Patients, <i>n</i> (%)
Sex	
Male	59 (65.6)
Female	31 (34.4)
Age (yr)	
Median	53
Range	23-75
Tumor site	
Colon	39 (43.3)
Rectal	51 (56.7)
Combined chemotherapy	
Irinotecan-based	58 (64.4)
Oxaliplatin-based	29 (32.2)
Monotherapy	3 (3.3)
Cetuximab use	
First line	29 (32.2)
Second line	23 (25.6)
Third line	28 (31.1)
Fourth line or more	10 (11.1)
Response status	
Complete response	3 (3.3)
Partial response	31 (34.4)
Stable disease	35 (38.9)
Progressive disease	21 (23.3)

tions were as follows: 1 cycle at 95°C for 9 min, 45 cycles at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 1 min, followed by 1 cycle at 72°C for 5 min. After confirmed by agarose electrophoresis and ethidium bromide staining, the PCR products were purified and automatically sequenced with an ABI PRISM 3730 (Applied Biosystems, California, USA), then analyzed with Chromas software version 2.0 (Gene Codes Corporation, USA). All sequencing reactions were performed in both forward and reverse directions, by independent PCR.

PTEN protein expression

PTEN protein expression in 3-mm thick tissue sections was evaluated using the anti-PTEN clone 6H2.1 (Millipore Company, Massachusetts, USA). The sections were deparaffinized and hydrated by passing through xylene and a graded series of ethanol. Endogenous peroxidase activity of the sections was blocked by incubating them in 0.3% hydrogen peroxide for 20 min. Antigen was retrieved for 30 min at 98°C in a 0.01 mol/L sodium citrate buffer (pH 6.4) in a microwave oven. After blocked for 30 min in 0.75% normal goat serum, the sections were incubated with 6H2.1 at a dilution of 1:100 overnight at 4°C, washed in PBS, and then incubated with biotinylated goat anti-mouse IgG followed by avidin peroxidase using a Vectastain ABC elite kit (Vector Laboratories, California, USA). The reaction products were counterstained with hematoxylin, and the sections were evaluated under a light microscope. PTEN protein expression was detected mainly in cytoplasm, although nuclear signals were occasionally observed as previously reported^[13]. The intensity of reaction was assessed as a score of either 1+, 2+ or 3+, and the percentage of positive cells was classified into three groups

(0%-25%, 25%-50% and > 50% of cells) and assigned to 1, 2 or 3 points, respectively. Tumors producing more than 4 points were considered PTEN-positive tumors according to the two values for the products. The evaluation was performed without knowledge of the clinical data or the results of other analyses.

Statistical analysis

Fisher's exact test was used to calculate the *P* values for KRAS mutation, PTEN expression, skin toxicity, and response to cetuximab. PFS time was calculated as the period of time from the first day of cetuximab treatment to the date of tumor progression, the date of death due to any factor, or the date of last follow-up. OS time was calculated as the period of time from the first day of cetuximab treatment until death due to any factor, or until the date of last follow-up. Cox proportional hazards regression model was used in survival analysis. PFS curves for PTEN expression and KRAS mutation were plotted using the Kaplan-Meier method, and the difference in biomarkers was evaluated using the log-rank test. Analysis was carried out with the SPSS software version 16.0 (SPSS Company, USA). *P* < 0.05 was considered statistically significant.

RESULTS

The clinical characteristics of mCRC patients treated with cetuximab are summarized in Table 1. The median follow-up time was 13.5 mo. Of the 90 patients, 34 (37.8%) had a response to cetuximab plus chemotherapy. The median PFS and OS time was 22 wk (range 8-129 wk), and 11 mo (range 2-48 mo), respectively. KRAS mutation was found in 30 (33.3%) tumor tissue samples from the 90 patients. Of the 30 tumor tissue samples, 25 and 5 were the single amino acid substitutions in codons 12 and 13, respectively. The KRAS mutations on codon 12 predominantly involved the second base of the codon, with the presence of GaT mutation (GGT-GaT, Gly-Asp, G12D), GtT mutation (GGT-GtT, Gly-Val, G12D), aGT mutation (GGT-aGT, Gly-Ser, G12C), and tGT mutation (GGT-tGT, Gly-Cys, G12C), in 16 (53.3%), 6 (20.0%), 2 (6.7%), and 1 (3.3%) out of the 30 patients, respectively. The KRAS mutations on codon 13 corresponded to the transition G-a at the second base of the codon (GGC-GaC, Gly-Asp, G13D). Positive and negative PTEN expression was detected in 58 (64.4%) and 32 (36.6%) of the 90 patients, respectively (Figure 1).

Of the 100 mCRC patients used for confirmation of KRAS mutation, 29 (29%) displayed KRAS mutation on codons 12 and 13, which was not significantly different from that in the 90 patients (*P* = 0.213). The KRAS mutations from GGT to GaT (Gly-Asp) and GtT (Gly-Val) on codon 12 accounted for 58.6% (17 of 29) and 20.7% (6 of 29) of the specified mutations, respectively. Mutations from GGC to GaC (Gly-Asp) occurred in 20.7% (6 of 29) of KRAS mutations on codon 13. The total mutation rate was 31% in 190 patients evaluated for KRAS mutations.

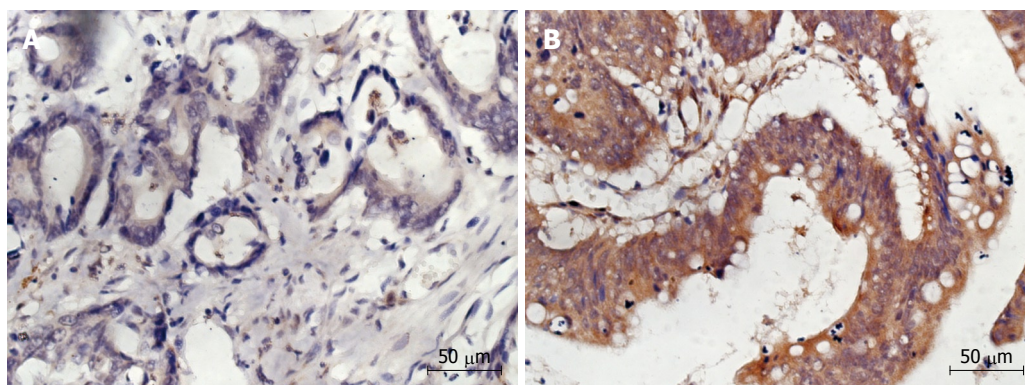


Figure 1 Immunohistochemistry showing phosphatase and tensin protein expression. A: Patients with absent phosphatase and tensin (PTEN) expression (HE stain, × 400); B: Patients showing positive PTEN expression (HE stain, × 400).

Table 2 Correlation of *KRAS* gene status and phosphatase and tensin protein expression with clinical response to cetuximab in previously treated colorectal cancer patients *n* (%)

	<i>KRAS</i>			PTEN		
	Mutation	No mutation	<i>P</i>	Positive	Negative	<i>P</i>
CR	0 (0)	2 (5.1)	0.001	2 (5.4)	0 (0)	0.001
PR	1 (4.5)	16 (41.0)		16 (43.2)	1 (4.2)	
SD	7 (31.8)	17 (43.6)		10 (27.0)	14 (58.3)	
PD	14 (63.6)	4 (10.3)		9 (24.3)	9 (37.5)	
Total	22 (100)	39 (100)		37 (100)	24 (100)	

CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; PTEN: Phosphatase and tensin.

We attempted to assess whether the *KRAS* mutation, PTEN protein expression or skin toxicity is associated with the clinical response of mCRC to cetuximab. Sixty-one patients treated with cetuximab plus chemotherapy as a second-, third-, or greater-line regimen were enrolled for analysis. The *KRAS* mutation was detected in 22 patients (36.1%). One of the 22 patients with *KRAS* mutation responded to cetuximab, and 18 of the 39 patients without *KRAS* mutation responded to cetuximab, with a response rate of 4.5% and 46.1%, respectively ($P = 0.001$). Positive PTEN expression was detected in 37 (60.7%) out of the 61 patients. Eighteen of the 37 patients with normal PTEN expression and one of the 24 patients with negative PTEN expression responded to cetuximab, with a response rate of 48.6% and 4.2%, respectively ($P = 0.001$). The absence of *KRAS* mutation and the presence of PTEN protein expression were significantly associated with a high response rate to cetuximab (Table 2). Meanwhile, combined *KRAS* mutation status and PTEN expression analysis showed that 24 (39.3%) of the 61 patients had no *KRAS* mutation and positive PTEN expression, with a remarkably higher effective rate than other patients (70.8% *vs* 5.4%, $P < 0.001$). Fisher's exact test also showed that the skin toxicity was significantly associated with a high response rate to cetuximab ($P < 0.001$).

Furthermore, we assessed whether *KRAS* mutation,

PTEN protein expression, or skin toxicity is associated with the PFS and OS time in the 61 patients. Univariate analysis of PFS time showed that *KRAS* mutation was significantly associated with a short PFS time ($P < 0.001$). The median PFS time of mCRC patients without and with *KRAS* mutation was 32 ± 2.5 wk and 14 ± 1.3 wk, respectively. The PFS time was longer in mCRC patients with positive PTEN protein expression than in those with negative PTEN protein expression (28 ± 1.9 wk *vs* 17 ± 2.0 wk, $P = 0.07$) (Figure 2). No difference was found in the median PFS time between the patients with and without skin toxicity (27 ± 2.9 wk *vs* 18 ± 1.7 wk, $P = 0.113$). The median OS time of mCRC patients without *KRAS* mutation was significantly longer than that of those with *KRAS* mutation (19 ± 1.8 mo *vs* 11 ± 1.2 mo, $P < 0.001$). The median OS time of mCRC patients with positive PTEN expression was significantly longer than that of those with negative PTEN expression (18 ± 1.9 mo *vs* 11 ± 1.3 mo, $P = 0.004$) (Figure 3). The median OS time of mCRC patients with skin toxicity was longer than that of those without skin toxicity (17 ± 1.5 mo *vs* 11 ± 1.0 mo, $P = 0.025$). Multivariate analysis of the 61 patients showed that both *KRAS* mutation and PTEN protein expression were closely related with a shorter OS time ($P < 0.001$). No correlation was found between skin toxicity and *KRAS* mutation or PTEN protein expression.

In this study, the absence of *KRAS* mutation and positive PTEN expression were found to be two favorable prognostic factors for mCRC patients. Combined *KRAS* mutation and PTEN expression analysis showed that the median PFS time of mCRC patients with the two favorable prognostic factors was longer than that of those with only one favorable prognostic factor or with no favorable prognostic factor (32 ± 2.5 wk *vs* 17 ± 1.9 wk and 11 ± 1.8 wk, $P = 0.001$) (Figure 4). The median OS time of these three groups of mCRC patients was 22 ± 2.3 mo, 11 ± 1.5 mo, and 6 ± 1.0 mo, respectively ($P < 0.001$).

DISCUSSION

KRAS serves as a mediator for the extracellular ligand

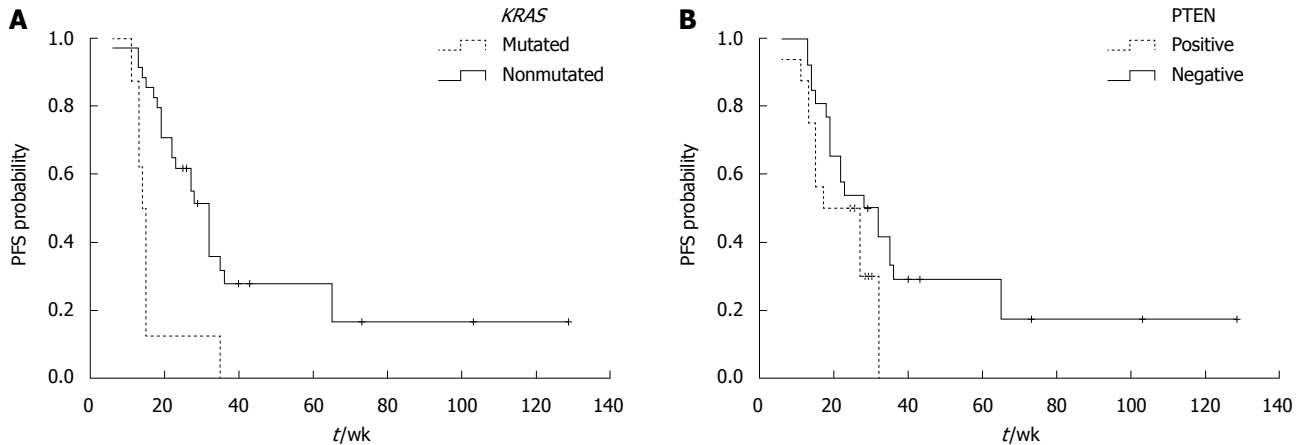


Figure 2 Progression-free survival time of patients with or without *KRAS* mutation (A) and phosphatase and tensin protein expression (B). A: $P < 0.001$; B: $P = 0.07$. PFS: Progression-free survival.

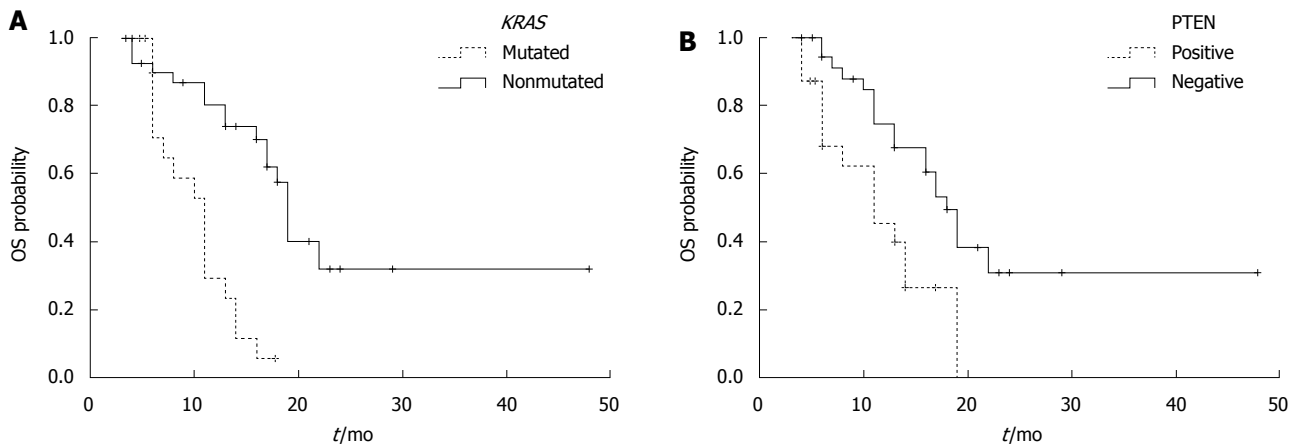


Figure 3 Overall survival time of patients with or without *KRAS* mutation (A) and phosphatase and tensin protein expression and skin toxicity (B). A: $P < 0.001$; B: $P = 0.004$. OS: Overall survival.

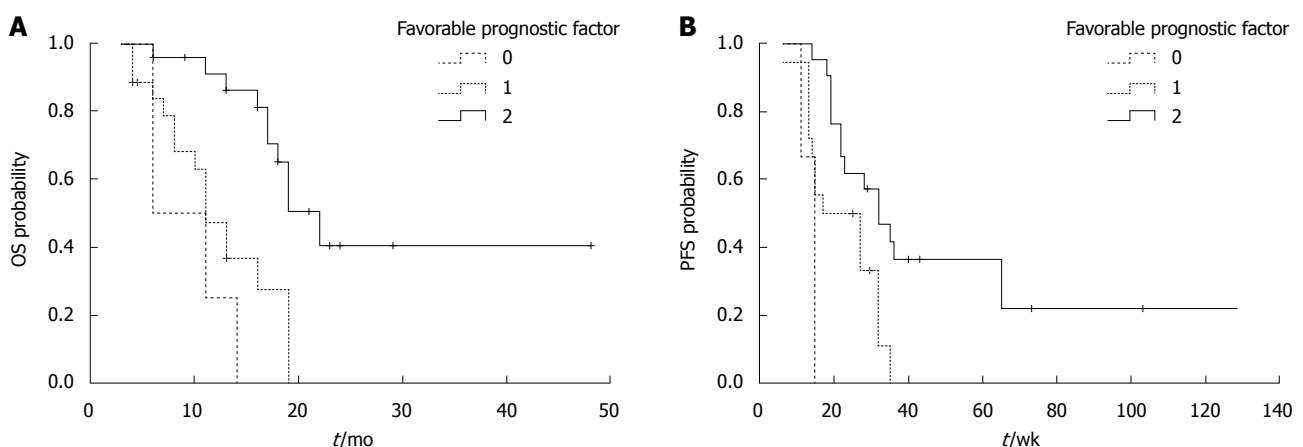


Figure 4 Overall survival and progression-free survival time of patients with or without favorable prognostic factors. A: $P < 0.001$; B: $P = 0.001$. 0: *KRAS* mutation and loss of phosphatase and tensin (PTEN); 1: Either no *KRAS* mutation or normal PTEN expression; 2: No *KRAS* mutation and normal PTEN expression. OS: Overall survival; PFS: Progression-free survival.

binding and intracellular transduction signals from EGFR to nuclei. RASCAL study evaluated the *KRAS* mutational in 2721 CRC patients from 22 centers and demonstrated

that *KRAS* mutation is closely related with the progression and outcome of CRC^[14]. In the present study, *KRAS* mutation was observed in about 40% (20%-50%) of spo-

radic CRC patients. Up to 90% of activating *KRAS* mutations were detected at codons 12 and 13, less than 70% of activating *KRAS* mutations were frequently detected at codon 12, and 70% of activating *KRAS* mutations were detected at codon 13. It has been shown that the most frequent types of *KRAS* mutation in CRC patients are GGT-GaT (Gly-Asp) and GGT-GtT (Gly-Val) transitions at codon 12^[15]. In our study, 31% of *KRAS* mutations occurred in 190 CRC patients, with most specified *KRAS* mutations found at the second base of codon 12. The most common *KRAS* mutation at codon 12 was GGT to GaT. These findings are consistent with those in a previous study^[15], indicating that the frequency or type of *KRAS* mutations is not different in Chinese and Western CRC patients.

It has been demonstrated that the benefit of cetuximab treatment in combination with first-line chemotherapy is restricted to CRC patients with *KRAS* wild-type mutations^[9,16]. The relation between *KRAS* mutation and response to anti-EGFR therapy has also been intensively studied^[11,13,17]. Lièvre *et al.*^[17] found that CRC patients with *KRAS* mutations are resistant to cetuximab therapy and have an unfavorable prognosis. It was reported that the PFS and OS time are shorter in CRC patients with *KRAS* mutations than in those with wild-type *KRAS* mutations^[7,8]. In our study, *KRAS* mutation was found to be a powerful predictor for the resistance to cetuximab, the response rate of CRC patients with *KRAS* and *KRAS* wild-type mutations was 4.5% and 46.1%, respectively. The PFS and OS time of CRC patients with *KRAS* mutations was shorter than that of those without *KRAS* mutations. It has also been shown that treatment with tyrosine kinase inhibitors is not effective for non small cell lung cancer patients with *KRAS* mutations^[18,19], indicating that *KRAS* mutations play a fundamental role in the EGFR pathway, thus rendering EGFR inhibitors ineffective^[20].

In our study, the response rate of CRC patients with *KRAS* wild-type mutations was only 46.1%, indicating that there must be other unidentified genetic determinants of resistance to cetuximab therapy for CRC. The PI3K/PTEN/AKT pathway is on the other side of the two EGFR pathways. PTEN is a tumor suppressor protein that regulates the PI3K/AKT signal transduction. Its loss is associated with intrinsic activation of the AKT pathway and confers resistance to inhibitors of the HER family^[21]. Thomas and Grandis demonstrated that PTEN is lost in 30% of sporadic CRC patients^[22]. PTEN protein expression may be another molecular predictor for the response to cetuximab. Frattini *et al.*^[13] reported that loss of PTEN protein expression is associated with the lack of response to cetuximab. Sartore-Bianchi *et al.*^[10] showed that loss of PTEN protein expression is associated not only with the lack of objective tumor response, but also with a shorter OS time of mCRC patients treated with cetuximab. Loupakis *et al.*^[11] revealed that combined PTEN expression and *KRAS* mutation analysis helps identify a subgroup of mCRC patients who have a greater chance of benefiting from EGFR inhibition. In our study, posi-

tive PTEN expression was detected in 64.4% of mCRC patients, which is consistent with previous reports^[10-13]. In this study, the response to cetuximab was significantly correlated with PTEN protein expression. The PFS and OS time of mCRC patients with negative PTEN protein expression was shorter than that of those with positive PTEN expression. The response rate of the 24 mCRC patients with no *KRAS* mutation and positive PTEN expression was substantially higher than that of those with *KRAS* mutation and positive PTEN expression. Combined *KRAS* mutation and PTEN protein expression analysis showed that the PFS and OS time of mCRC patients with two favorable prognostic factors was longer than that of those with one favorable prognostic factor or no favorable prognostic factor, indicating that a comprehensive analysis of *KRAS* mutation and PTEN protein expression is a better predictor for the clinical outcome of mCRC patients treated with cetuximab, which requires further confirmation in a prospective series.

It has been shown that skin toxicity is significantly associated with the response to cetuximab and OS time of mCRC patients^[2,5,23,24], which is consistent with the findings in our study. In our study, the response rate of mCRC patients with skin toxicity was higher than that of those without skin toxicity, and the OS time of mCRC patients with skin toxicity was also longer than that of those without skin toxicity. However, univariate analysis showed that skin toxicity was only associated with OS time, while multivariate analysis showed that *KRAS* mutation and PTEN protein expression were the significant risk factors for OS time, indicating that skin toxicity alone is insufficient to predict the outcome of mCRC patients treated with cetuximab. Moreover, *KRAS* mutation and PTEN protein expression was detected before cetuximab treatment and can thus be included in the algorithm of treatment decision^[17].

To our knowledge, this is the first study on *KRAS* mutation and PTEN protein expression in Chinese mCRC patients. Other markers were also identified in our study, which can be used to select mCRC patients who are likely to benefit from cetuximab treatment, showing that *KRAS* mutation and PTEN protein expression in Chinese mCRC patients are similar to those in other populations. In this study, skin toxicity was insufficient to predict the outcome of mCRC patients treated with cetuximab, and *KRAS* mutation and PTEN protein expression were significantly associated with the response rate to cetuximab and survival time of these patients.

In conclusion, combined *KRAS* mutation and PTEN protein expression analysis is a better predictor for the clinical outcome of mCRC patients treated with cetuximab. Prospective studies with a large number of patients are required to further confirm the results of our study.

COMMENTS

Background

The incidence of colorectal cancer (CRC) has been increasing in past decades

and CRC is presently the third-leading cause of cancer-related deaths in China. During the past few years, several new biological agents have been evaluated in metastatic colorectal cancer (mCRC) with remarkable clinical activity. Cetuximab is an important biological agent used in treatment of mCRC, but it is effective only in a subset of mCRC patients.

Research frontiers

Studies have shown that KRAS mutation and phosphatase and tensin (PTEN) protein expression are associated with the response to cetuximab and may have a prognostic value. However, the situation in Asian patients is unknown. The authors evaluated the prognostic value of KRAS mutation and PTEN protein expression in Chinese mCRC patients treated with cetuximab plus chemotherapy.

Innovations and breakthroughs

To the authors' knowledge, this is the first study on KRAS mutation and PTEN protein expression in Chinese mCRC patients. The results of this study show that KRAS mutation and PTEN protein expression in Chinese mCRC patients are significantly correlated with the response rate and survival time of patients treated with cetuximab. A comprehensive analysis of KRAS mutation and PTEN protein expression is a better predictor for the clinical outcome of patients treated with cetuximab.

Applications

KRAS mutation and PTEN protein expression can be used to select Chinese mCRC patients who are likely to benefit from cetuximab treatment.

Terminology

Epidermal growth factor receptor (EGFR), one of the most promising targets, can activate the proliferation and prolong the survival time of cancer cells through the Ras/Raf/mitogen-activated protein kinase (MEK)/EPH receptor B2 (ERK) or the phosphoinositide-3-kinase (PI3K)/ PTEN /AKT pathway. KRAS serves as a mediator for the extracellular ligand binding and intracellular signal transduction from EGFR to nuclei. PTEN is a tumor suppressor protein that regulates the PI3K/AKT signal transduction. Its loss is associated with the intrinsic activation of the AKT pathway.

Peer review

The manuscript describes the impact of KRAS mutation and PTEN protein expression, either alone or in combination, on cetuximab-treated Chinese colorectal cancer patients. The manuscript targets a topic that is of scientific and clinical interest.

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Irinotecan-involved regimens for advanced gastric cancer: A pooled-analysis of clinical trials

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1.12, 95% CI: 0.92-1.36, $P = 0.266$) and ORR [risk ratio (RR) = 1.23, 95% CI: 0.71-2.14, $P = 0.458$]. However, the CPT-11-containing combination chemotherapy was significantly advantageous over the non CPT-11-containing combination chemotherapy for TTF (HR = 1.35, 95% CI: 1.12-1.64, $P = 0.002$). Grade 3/4 haematological toxicity (thrombocytopenia: RR = 0.20, 95% CI: 0.09-0.48; $P < 0.001$) and gastrointestinal toxicity (diarrhea: RR = 4.09, 95% CI: 2.42-6.93, $P < 0.001$) were lower in patients with advanced gastric cancer after CPT-11-containing combination chemotherapy than after non CPT-11-containing combination chemotherapy.

CONCLUSION: CPT-11-containing combination chemotherapy is advantageous over non CPT-11-containing combination chemotherapy for TTF with no significant toxicity. CPT-11-containing combination chemotherapy can be used in treatment of advanced gastric cancer.

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Key words: Meta-analysis; Advanced gastric cancer; Chemotherapy

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Abstract

AIM: To assess the efficiency and toxicities of irinotecan (CPT-11)-involved regimens in patients with advanced gastric cancer.

METHODS: Randomized phases II and III clinical trials on chemotherapy for advanced gastric cancer were searched from MEDLINE, EMBASE, Cochrane Controlled Trials Register, and EBSCO. Relevant abstracts were manually searched. A total of 657 patients were analyzed for their overall response rate (ORR), time to treatment failure (TTF), overall survival (OS) rate, and toxicities. Overall survival rate, reported as hazard ratio (HR) with 95% CI, was used as the primary outcome measure.

RESULTS: Four randomized controlled trials on chemotherapy for advanced gastric cancer were detected. The CPT-11-containing combination chemotherapy was not significantly advantageous over the non CPT-11-containing combination chemotherapy for OS rate (HR =

INTRODUCTION

Although the incidence of gastric cancer has been sharply declined during the second half of the 20th century, it remains the second leading cause of cancer-related death in the world^[1]. The morbidity and mortality rate of gastric

cancer increase with age. The most effective treatment for gastric cancer is radical gastrectomy. A substantial number of patients, however, eventually die of recurrence after curative resection. Although systemic chemotherapy can improve the quality of life in patients with gastric cancer^[2], the outcome of patients with unresectable gastric cancer is still extremely poor with a median survival time of 3-5 mo after the best supportive care^[2-4].

Randomized clinical trial data demonstrate that the survival rate and quality of life are better in patients with advanced gastric cancer after chemotherapy than in those after the best supportive care^[5]. Over the years, a number of single-agent chemotherapy trials have confirmed that gastric cancer is a relatively “chemosensitive” disease^[6-9]. It is, therefore, necessary to investigate different combination chemotherapies, both in phase II and randomized phase III trial settings.

First line chemotherapy usually consists of different combination regimens with 5-fluorouracil (5-FU) and cisplatin, including FP (5-FU and cisplatin) and ECF (epirubicin, cisplatin, and 5-FU). It has been shown that the response rate and progression-free survival rate are better for patients with gastric cancer after FP therapy than after 5-FU or other combination therapies^[10,11]. The additional survival advantage yielded by these combination therapies appears to be marginal. However, no standard regimen has yet been established. Thus, it is necessary to develop new agents and combination regimens to achieve greater survival benefits in advanced or recurrent unresectable gastric cancer. Since 2005, combination chemotherapy for advanced gastric cancer has been focused on the integration of other chemotherapy agents, including docetaxel, irinotecan, oxaliplatin, capecitabine, and S-1.

Irinotecan (CPT-11) is a water-soluble camptothecin derivative. CPT-11 and its active metabolite (SN-38) bind reversibly to the topoisomerase I-DNA complex and induce cancer cell death by preventing relegation of single-strand DNA breaks^[12,13]. It has been shown that CPT-11 acts as a single agent in oesophago-gastric cancer. It was reported that the overall response rate of advanced gastric cancer patients to chemotherapy is 16%-20%^[14,15]. It has been reported that CPT-11 in combination with leucovorin/5-FU (ILF)^[16] or cisplatin (IP)^[17] exhibits its antitumor activity against advanced gastric cancer.

Several phases II and III randomized trials are available on CPT-11-containing or non CPT-11-containing combination chemotherapy for advanced gastric cancer^[18-21]. The meta-analysis in this study was to compare the two therapies by evaluating their clinical efficiency and toxicities.

MATERIALS AND METHODS

Literature search

Trials were searched from MEDLINE, OLD MEDLINE, CancerLit, EMBASE, and ISI Web of Science, incorporating Science Citation Index, Technology Proceedings, and Current Contents Databases as far back as they go. References of selected articles and previous systematic reviews were also searched for any other relevant

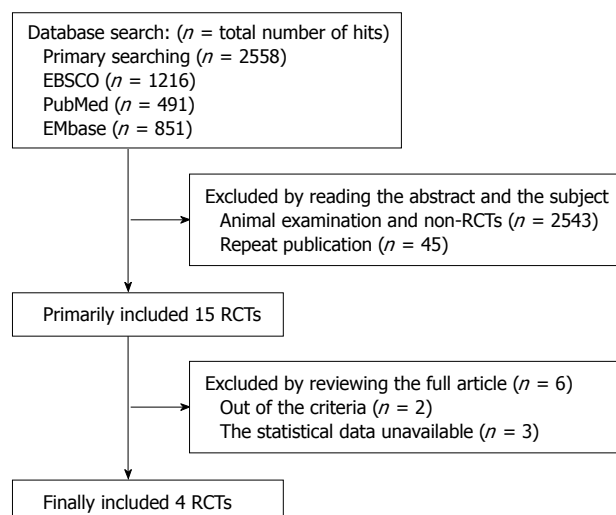


Figure 1 Article search and riddling progression. RCTs: Randomized controlled trials.

trials (Figure 1). The search strategy included the following key words variably combined: metastatic, metastasis, gastric, cancer, CPT-11, randomized, trial.

Two independent reviewers assessed the eligibility of the searched abstracts. If the eligibility of the abstract was unclear, the full article was retrieved for clarification. Any disagreements were solved by discussion. The selection criteria included study design (randomized or controlled trials), participants (patients with histologically confirmed advanced or recurrent adenocarcinoma, including diffuse type, intestinal type of the stomach or gastroesophageal junction). The exclusion criteria were nonrandomized trials, animal examination, single-arm phase II trial, or adequate statistical analysis with information missed. Care was taken to include only primary data or data that superseded earlier works. The deadline for trial inclusion was November 6, 2009.

Statistical methods

Overall survival rate was used as the primary outcome measure. Secondary outcome measures evaluated were overall response rate (ORR: number of partial and complete responses) and toxicities (published by the authors with the most frequently reported events analyzed). Hazard ratio (HR) and 95% CI as relevant effect measures were estimated directly or indirectly from the given data. Appropriate data, such as log-rank test *P* value, were extracted for the estimation of the log HR and its variance as previously described^[22,23]. Summary statistical data were extracted from the published trials according to the standard methods for survival end points, with HR and CI as preferred sources for estimation, and log-rank *P* value/event count as a second choice^[22]. Standard techniques for meta-analysis^[24] were used to calculate the pooled estimates. All analyses were conducted using the Stata software version 8.2 (Stata Corp LP, College Station, TX). All tests were two sided. Fixed-(primarily) and random-effect model methodology was applied. All reported *P* values

Table 1 Trials comparing irinotecan-containing and nonirinotecan-containing combination chemotherapies, treatment schedule and quality of each trial

Study	Regimen	Patients (n)	RR (%)	Survival (mo)	
				Progression free	Overall
Bouché <i>et al</i> ^[18]	FU	45	13.0	3.2	6.8
	CF	44	27.0	4.9	9.5
	IF	45	40.0	6.9	11.3
Moehler <i>et al</i> ^[19]	ILF	56	42.9	4.5	10.8
	ELF	58	24.1	2.3	8.3
Dank <i>et al</i> ^[20]	IF	170	31.8		9.0
	CF	163	25.8		8.7
Nakashima <i>et al</i> ^[21]	IP	44	47.0	5.7	14.8
	SP	32	80.0	7.8	15.6

FU: Fluorouracil; CF: Cisplatin and FU; IF: Irinotecan and FU; RR: Response rate; ILF: Irinotecan, leucovorin and FU; ELF: Etoposide, leucovorin and FU; IP: Irinotecan and cisplatin; SP: S-1 and cisplatin.

resulted from two-sided versions of the respective tests.

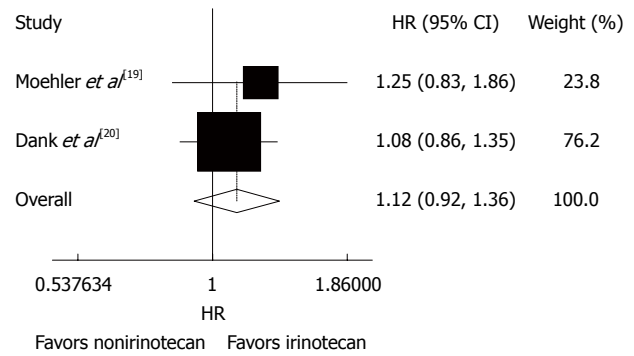
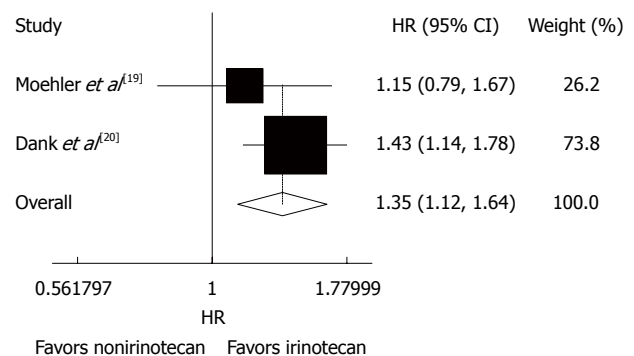
In consideration of possible heterogeneity across the studies, a statistical test for heterogeneity was performed as previously described^[25]. $P < 0.05$ was considered statistically significant for the heterogeneity. A fixed effect approach was adopted unless there was evidence for significant unexplained heterogeneity, in which a random effect approach was used. In the absence of heterogeneity, the two methods provided identical results, because the fixed-effect model using the Mantel-Haenszel's method assumes that studies are sampled from populations with the same effect size, making an adjustment to the study weights according to the in-study variance, whereas the random-effect model using the DerSimonian and Laird's method assumes that studies are taken from populations with varying effect sizes, calculating the study weights both from in-study and between-study variances, considering the extent of variation or heterogeneity. Funnel plots and Egger's linear regression test were used to show the potential publication bias in diagnosis of advanced gastric cancer^[26].

RESULTS

The results of 4 randomized phases II and III trials, including 2 Europe randomized phase II trials^[18,19], 1 Europe randomized phase III trial^[20], and 1 Japanese study^[21], that have been published or presented at major international meetings, were included in this analysis. These studies included 657 patients with metastatic gastric cancer, of whom 315 (48%) received the CPT-11-containing combination chemotherapy. Treatment schedule and quality of each trial were evaluated (Table 1).

Overall survival time

The overall survival rate was reported in the 4 trials^[18-21], during which 315 patients received CPT-11-containing combination chemotherapy and 342 patients received non CPT-11-containing combination chemotherapy. However,

**Figure 2** Overall survival rate of patients with advanced gastric cancer after irinotecan-containing and nonirinotecan-containing combination chemotherapies. HR: Hazard ratio.**Figure 3** Time to treatment failure of patients with advanced gastric cancer after irinotecan-containing and nonirinotecan-containing combination chemotherapies. HR: Hazard ratio.

only 2 trials reported the HR. The other trials showed that the overall survival time of patients with gastric cancer was 11.3-14.8 mo after CPT-11-containing combination chemotherapy and 6.8-15.6 mo after non CPT-11-containing combination chemotherapy. No striking inter-study heterogeneity was found ($P = 0.535$, $I^2 = 0.0\%$) in the 4 trials. Meta-analysis of the pooled data demonstrated that the overall risk of death was not different between the two chemotherapies (HR = 1.12, 95% CI: 0.92-1.36, $P = 0.266$, Figure 2).

Time to treatment failure

Two trials^[19,20] analyzed the impact of time to treatment failure (TTF) with no striking inter-study heterogeneity ($P = 0.327$, $I^2 = 0.0\%$). The fixed-effect pooled estimation for TTF showed comparable results (HR = 1.35, 95% CI: 1.12-1.64, $P = 0.002$, Figure 3), suggesting that the outcome is significantly better in patients with advanced gastric cancer after CPT-11-containing combination chemotherapy than in those after non CPT-11-containing combination chemotherapy.

Overall response rate

Risk ratio (RR) was reported in the 4 trials^[18-21]. The overall response rate (ORR) of patients with advanced gastric cancer was 13%-80% to CPT-11-containing combination che-

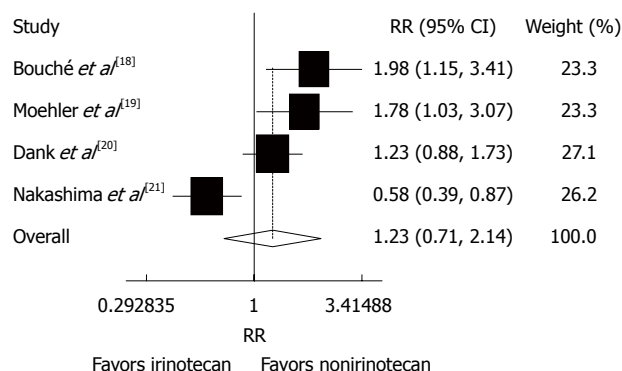


Figure 4 Overall response rate of patients with advanced gastric cancer after irinotecan-containing and nonirinotecan-containing combination chemotherapies. RR: Risk ratio.

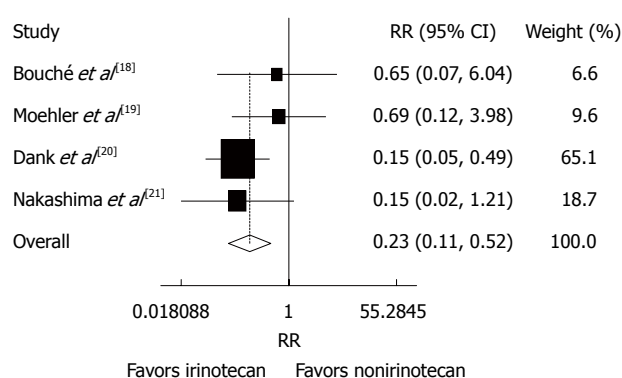


Figure 5 Grade 3/4 haematological toxicities (thrombocytopenia) in patients with advanced gastric cancer after irinotecan-containing and nonirinotecan-containing combination chemotherapies. RR: Risk ratio.

motherapy and 22%-47% to the non CPT-11-containing combination chemotherapy. A significant heterogeneity was observed ($P = 0.000$, $I^2 = 83.3\%$). The random-effect pooled estimate including 657 patients evaluated for ORR showed that the RR of CPT-11-containing combination chemotherapy was increased (RR = 1.23, 95% CI: 0.71-2.14, $P = 0.458$, Figure 4).

Toxicities

Reported toxicities were analyzed in all trials. The incidence of grade 3/4 thrombocytopenia (RR = 0.23, 95% CI: 0.11-0.52, $P < 0.001$) and diarrhea (RR = 4.00, 95% CI: 2.38-6.71, $P < 0.001$) was lower in patients after CPT-11-containing combination chemotherapy than in those after non CPT-11-containing combination chemotherapy (Figures 5 and 6). The incidence of other grade 3/4 haematological toxicities, such as neutropenia (RR = 0.60, 95% CI: 0.28-1.31, $P = 0.201$), febrile neutropenia (RR = 0.64, 95% CI: 0.37-1.10, $P = 0.108$) and leucopenia (RR = 0.85, 95% CI: 0.59-1.23, $P = 0.388$), as well as other grade 3/4 gastrointestinal toxicities, such as nausea (RR = 0.84, 95% CI: 0.46-1.54, $P = 0.582$), vomiting (RR = 0.83, 95% CI: 0.44-1.56, $P = 0.556$), anorexia (RR = 0.66, 95% CI: 0.31-1.41, $P = 0.278$) was similar in patients with advanced gastric cancer after CPT-11-containing and non CPT-

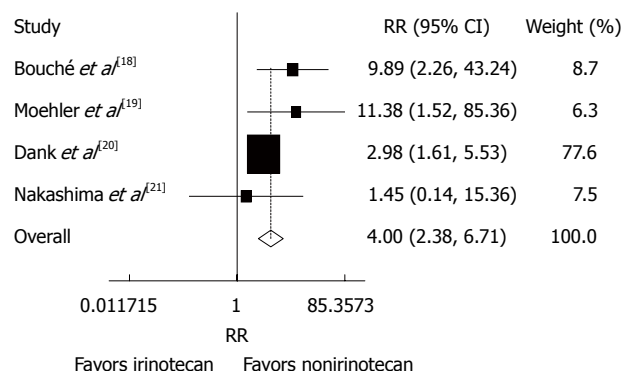


Figure 6 Grade 3/4 gastrointestinal toxicities (diarrhea) in patients with advanced gastric cancer after irinotecan-containing and nonirinotecan-containing combination chemotherapies. RR: Risk ratio.

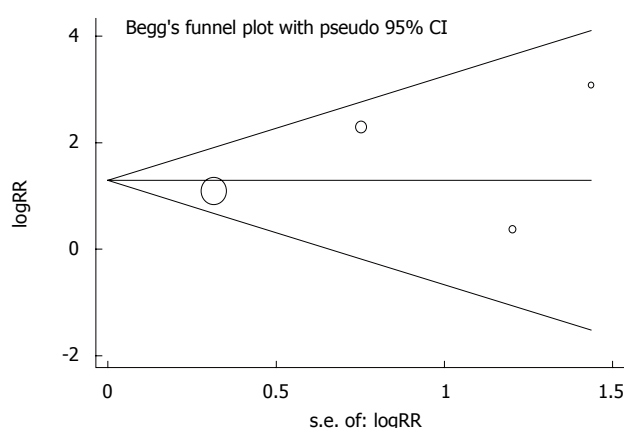


Figure 7 Funnel plots and Egger's linear regression test showing the potential publication bias in diagnosis of advanced gastric cancer^[22]. RR: Risk ratio.

11-containing combination therapies. No evidence for heterogeneity was found except for neutropenia and alopecia.

Publication bias

Evidence of publication bias was detected by plotting funnel plots of HR. Studies were plotted in order of decreasing variance of the log HR. Visual inspection of the funnel plots with respect to the 3 end points (OS rate, TTF and ORR) did not reveal any hint of publication bias (Figure 7). Funnel plots for all comparisons could not identify relevant publication bias, although the number of included studies was relatively small.

DISCUSSION

During the last decade, advances in treatment of patients with advanced gastric cancer have been achieved as a result of the integration of novel, effective agents into treatment algorithms. The availability of CPT-11 further enriches the options for combination therapy, because CPT-11 is effective and tolerable in patients with advanced gastric cancer.

The present systematic review revealed the major findings in ongoing debatable questions. We wonder whether

CPT-11-containing combination chemotherapy is better than non CPT-11-containing combination chemotherapy for advanced gastric cancer. One of the earlier systematic reviews on 3 randomized controlled trials (RCTs) of chemotherapy for advanced gastric cancer concluded that there is no convincing evidence that demonstrates a significant benefit in overall survival rate of patients after CPT-11-containing combination chemotherapy^[27]. The resulting HR for the overall survival rate was 0.88% (95% CI: 0.73-1.06), which is in favor of CPT-11-containing combination chemotherapy. However, the study^[23] did not assess other outcomes or toxicities. In our analysis, the 2 trials that assessed TTF showed that the overall summary estimate favored the CPT-11-containing combination chemotherapy with no significant inter-trial heterogeneity.

Most reported end points covered in the RCTs were searched and the most appropriate statistical methods for meta-analysis of time-to-event data extracted from published reports were used in our study. However, the quality of life was not stressed in patients with advanced gastric cancer due to the different methods used in reporting their quality of life. Although the data about response rate and adverse events were pooled to permit a clinically relevant analysis, these parameters varied. The response rate was reported according to the clinical parameters, WHO and RECIST criteria, whereas the CTC, WHO and ECOG scales were used in analysis of toxicity data.

In conclusion, there is insufficient evidence that the overall survival rate, overall response rate are better for patients with advanced gastric cancer after CPT-11-containing combination chemotherapy than after non CPT-11-containing combination chemotherapy. CPT-11-containing combination chemotherapy is advantageous over non CPT-11-containing combination chemotherapy for TTF and grade 3/4 thrombocytopenia.

Irrespective of the positive impact of presently available chemotherapy, the prognosis of patients with advanced gastric cancer remains poor, with a median survival time of 7-10 mo. Further RCTs are needed to assess which CPT-11 combination chemotherapy is least toxic.

COMMENTS

Background

The morbidity and mortality of gastric cancer increase with age. It has been shown that irinotecan (CPT-11) acts as a single agent in oesophago-gastric cancer. The aim of this meta-analysis was to assess the efficiency and toxicities of CPT-11 involved regimens in patients with advanced gastric cancer.

Research frontiers

Several phases II and III randomized trials have been reported comparing CPT-11-containing or non CPT-11-containing combination chemotherapy for advanced gastric cancer. The authors wonder whether CPT-11-containing combination chemotherapy is better than non CPT-11-containing combination chemotherapy for advanced gastric cancer.

Innovations and breakthroughs

This systematic review revealed the major findings in ongoing debatable questions. One of the earlier systematic reviews of 3 randomized controlled trials of chemotherapy for advanced gastric cancer concluded that there is no convincing evidence that demonstrates that the overall survival rate of patients with advanced gastric cancer is higher after CPT-11-containing combination chemotherapy than after non CPT-11-containing combination chemotherapy. However, this study did

not assess other outcomes or toxicities. In the authors' analyses, 4 randomized phases II and III trials were included. A total of 657 patients were analyzed for their overall response rate, time to treatment failure (TTF), overall survival rate and toxicities. The two trials that assessed TTF showed that the overall summary estimate favored CPT-11-containing combination chemotherapy.

Applications

The findings in the study suggest that CPT-11 based combination chemotherapy is a candidate regimen for advanced gastric cancer. Further studies are needed to compare common chemotherapy with/without target therap.

Peer review

The authors in their meta-analysis showed that CPT-11-containing combination chemotherapy was advantageous over non CPT-11-containing combination chemotherapy for TTF, overall survival rate, overall response rate and toxicity. The availability of CPT-11 further enriches the options for combination therapy, because CPT-11 is effective and tolerable in patients with advanced gastric cancer. Further randomized control trials are needed to assess which chemotherapy provides favorable overall survival rate with less toxicity. Moreover, target therapy agents should be taken into consideration to see if they can achieve better clinical benefits in patients with advanced gastric cancer.

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Hepatic blood inflow occlusion without hemihepatic artery control in treatment of hepatocellular carcinoma

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Abstract

AIM: To investigate the clinical significance of hepatic blood inflow occlusion without hemihepatic artery control (BIOwHAC) in the treatment of hepatocellular carcinoma (HCC).

METHODS: Fifty-nine patients with HCC were divided into 3 groups based on the technique used for achieving hepatic vascular occlusion: group 1, vascular occlusion was achieved by the Pringle maneuver ($n = 20$); group 2, by hemihepatic vascular occlusion (HVO) ($n = 20$); and group 3, by BIOwHAC ($n = 19$). We compared the procedures among the three groups in term of operation time, intraoperative bleeding, postoperative liver function, postoperative complications, and length of hospital stay.

RESULTS: There were no statistically significant differences ($P > 0.05$) in age, sex, pathological diagnosis, preoperative Child's disease grade, hepatic function,

and tumor size among the three groups. No intraoperative complications or deaths occurred, and there were no significant intergroup differences ($P > 0.05$) in intraoperative bleeding, hepatic function change 3 and 7 d after operation, the incidence of complications, and length of hospital stay. BIOwHAC and Pringle maneuver required a significantly shorter operation time than HVO; the difference in the serum alanine aminotransferase or aspartate aminotransferase levels before and 1 d after operation was more significant in the BIOwHAC and HVO groups than in the Pringle maneuver group ($P < 0.05$).

CONCLUSION: BIOwHAC is convenient and safe; this technique causes slight hepatic ischemia-reperfusion injury similar to HVO.

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Key words: Hepatic blood inflow occlusion without hemihepatic artery control; Hepatocellular carcinoma; Intraoperative bleeding; Ischemia-reperfusion injury

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INTRODUCTION

Intraoperative bleeding occurs most frequently during hepatic resection. Bleeding-associated blood transfusions increase the postoperative complications and mortality rate^[1], especially in cases complicated with hepatocirrho-

sis^[2]. Therefore, control of intraoperative bleeding is a prerequisite for reducing the number of cases requiring blood transfusions during liver resection. The common approaches to reduce blood transfusion include lowering the intraoperative central venous pressure^[3-5], and using cut-ultrasound aspiration and microwave thermocoagulation in liver surgery^[6,7], and hepatic inflow occlusion with or without outflow control. Hepatic inflow control (with or without outflow control) plays an important role in constructing a bloodless surgical field. However, hepatic ischemia-reperfusion injury (HIRI) occurs in all hepatic vascular occlusions to some extent. The normal liver receives 70%-75% of its blood supply from the portal vein and 40%-60% of its oxygen supply from arterial blood. Considering the differences between the distribution and oxygen content of the blood in the portal vein and that in the hepatic artery, we performed hepatic blood inflow occlusion without hemihepatic artery control (BIOwHAC)^[8] to minimize HIRI by a modified surgical procedure. During BIOwHAC, the proper hepatic artery was surgically exposed, after which its left and right branches were separated. For the right and left hemihepatic occlusions, catheters were advanced and bypassed the portal vein, bile duct, and the respective branches of the hepatic artery before being tightened.

We designed a retrospective case-control study to compare BIOwHAC, Pringle maneuver and hemihepatic vascular occlusion (HVO) in terms of operation time, intraoperative bleeding, and postoperative liver function, and postoperative complications. We also assessed the merits and demerits of these three approaches.

MATERIALS AND METHODS

Inclusion criteria

Between March 2005 and January 2009, 162 patients were treated at our hospital for liver neoplasms. Of these, 59 patients who met with the following criteria were included in this trial: availability of complete data, presence of pathologically diagnosed hepatocellular carcinoma (HCC), cancers confined to half of the liver and being suitable for hepatic portal anatomical vascular occlusion, and resectable tumors. Patients who underwent pericardial devascularization, splenectomy, gastroenterostomy and biliointestinal anastomosis were excluded.

Vascular occlusion procedures

Of the 59 patients, 20 underwent Pringle maneuver, 20 underwent HVO, and 19 underwent BIOwHAC. The patients in the Pringle maneuver group underwent the Pringle's occlusion by the conventional method at the hepatoduodenal ligament using urethral catheters. Patients in the HVO group underwent vascular occlusions *via* two approaches. (1) The catheters were introduced into the confluence of the left and right hepatic ducts along the common hepatic duct without dissecting the hepatoduodenal ligament; the tangential clamp was placed at the superior

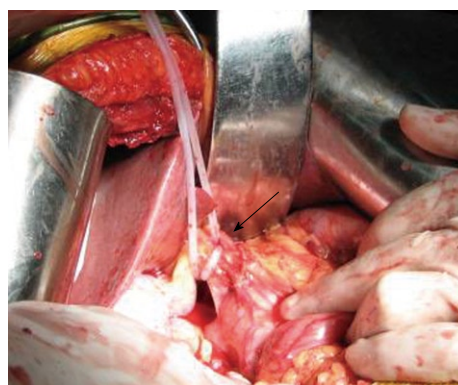


Figure 1 Application of hepatic blood inflow occlusion without hemihepatic artery control (arrow shows non-occlusive left hepatic artery).

hepatic capsule or inferior transverse fissura ligamenti teretis and then the internal liver parenchyma and the external Glisson sheath were bluntly separated with care; if no resistance occurred at that time, the urethral catheter was exited at the portal vein bifurcation-caudate lobe junction towards the posterior hepatoduodenal ligament and then insufflated to achieve right hemihepatic occlusion; one end of the catheter was inserted through the lesser omentum foramen to the hepatogastric ligament and insufflated to achieve left hemihepatic occlusion; and (2) The affected side of the hepatic artery and the portal vein were separated to occlude them simultaneously or individually. Patients in the BIOwHAC group underwent BIOwHAC as follows: the proper hepatic artery was surgically exposed and the left and right branches of the proper hepatic artery were separated; for right hemihepatic occlusion, the catheter bypassed the portal vein, bile duct, and the right hepatic artery and was tightened; for left hemihepatic occlusion, the catheter bypassed the portal vein, bile duct, and the left hepatic artery and was tightened (Figure 1).

Data collection

Complete background information, including age, sex, liver function, Child's disease grade, level of serum markers of hepatitis, and α -fetoprotein levels was collected from all the patients.

Computed tomography (CT) and magnetic resonance imaging (MRI) images and operative record were carefully previewed and information regarding the size of HCC, hepatic vascular occlusion approach, occlusion time, type of liver resection, and operation time were precisely recorded.

The levels of albumin, total bilirubin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were recorded 1, 3, and 7 d after operation to determine the deviations from preoperative values.

Patients were observed for postoperative complications such as bleeding from hepatic section, biliary fistula, subphrenic abscess, responsive pleural effusion, hepatic encephalopathy, pulmonary infection, hemorrhage from

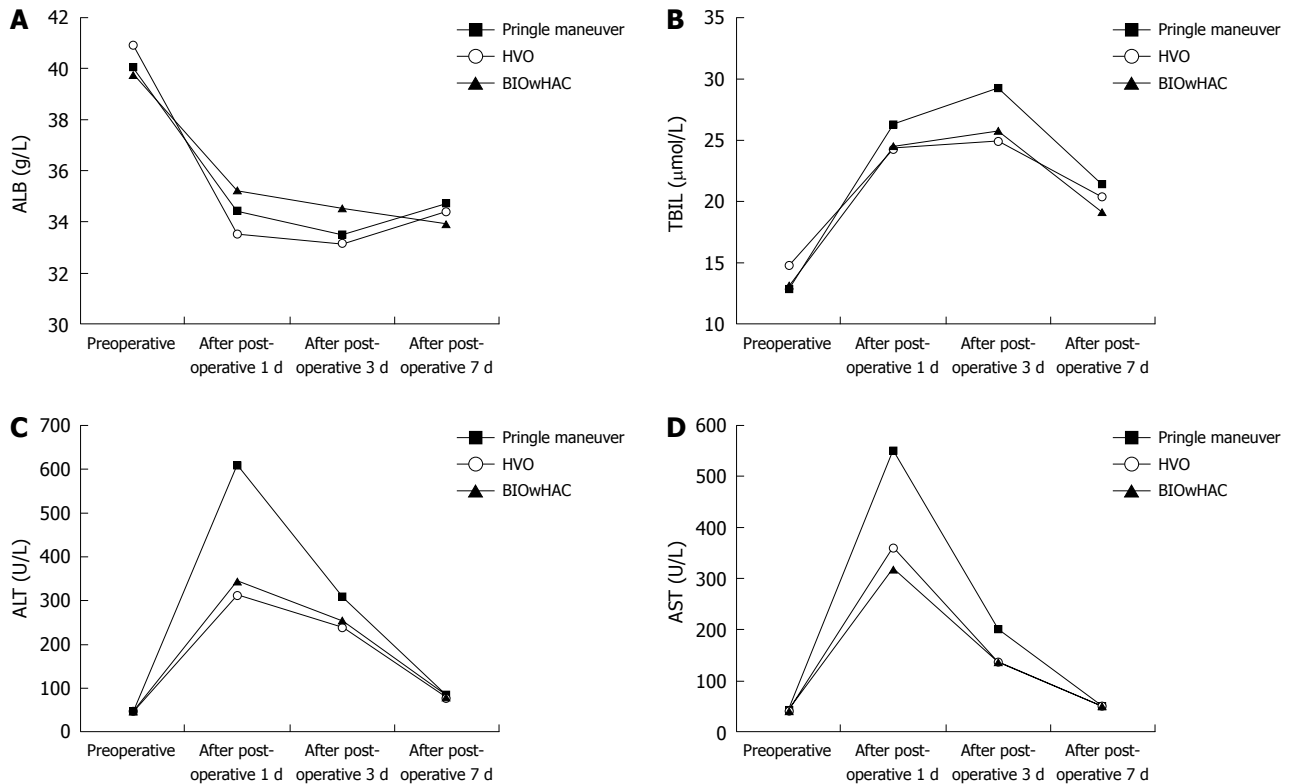


Figure 2 Preoperative and postoperative albumin concentrations (A), total bilirubin levels (B), serum alanine aminotransferase levels (C) and aspartate aminotransferase levels (D) in the 3 approaches for achieving occlusions. HVO: Hemihepatic vascular occlusion; BIOwHAC: Hepatic blood inflow occlusion without hemihepatic artery control; ALB: Albumin; TBIL: Total bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

Table 1 Patients' background information¹ (mean ± SD)

Groups	Pringle maneuver (n = 20)	HVO (n = 20)	BIOwHAC (n = 19)
Age (yr)	54.35 ± 10.03	54.20 ± 11.34	54.26 ± 7.05
Gender (M:F)	16:4	13:7	14:5
Pathologic diagnosis	HCC	HCC	HCC
Complication	10	9	10
hepatocirrhosis (cases)			
Hepatitis virus positive (cases)	18	18	17
Increase in AFP (cases)	12	11	10
Child's disease grade (cases, A:B)	19:1	19:1	18:1
Serum albumin (g/L)	40.04 ± 3.42	40.87 ± 3.12	39.78 ± 2.71
Serum bilirubin (μmol/L)	12.86 ± 5.60	14.68 ± 3.37	13.08 ± 7.81
Serum ALT (U/L)	45.22 ± 32.99	45.04 ± 63.01	47.63 ± 40.48
Serum AST (U/L)	39.43 ± 17.20	40.72 ± 21.57	41.56 ± 41.01

¹There is no significant intergroup difference ($P > 0.05$). BIOwHAC: Hepatic blood inflow occlusion without hemihepatic artery control; HVO: Hemihepatic vascular occlusion; AFP: α -fetoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

the digestive tract, and long-term (> 30 d) hepatic dysfunction.

Statistical analysis

Data were expressed as mean ± SD and variance analysis was performed; χ^2 test was performed for countable data. $P < 0.05$ indicates significant difference.

RESULTS

Background information

Patients' information on age, sex, preoperative Child's disease grade, pathologic diagnosis, and preoperative hepatic function are listed in Table 1; and the information about tumor size and type of liver resection is shown in Table 2.

Intraoperative information

In all the patients, the procedures were performed without intraoperative complications and deaths. Each group underwent vascular occlusion only once, and there were no statistically significant intergroup differences ($P > 0.05$) in occlusion time, type of liver resection, bleeding and blood transfusions (Table 2). BIOwHAC and Pringle maneuver required significantly shorter operation time than HVO (151.84 ± 41.77 min and 158.50 ± 43.77 min, respectively *vs* 219.25 ± 58.09 min, $P < 0.05$), but the time required for BIOwHAC was almost equivalent to that for Pringle maneuver ($P > 0.05$).

Postoperative hepatic function variation

Serum albumin level was reduced in all the patients 1 and 3 d after operation, but increased after 7 d, and no significant intergroup difference was observed (Figure 2A). The total serum bilirubin level increased 1 and 3 d after operation, but decreased after 7 d, with no significant intergroup differences (Figure 2B). In addition, there was an increase in serum ALT and AST levels 1 d after operation

Table 2 Intraoperative status of patients (mean \pm SD)

Groups	Pringle maneuver (<i>n</i> = 20)	HVO (<i>n</i> = 20)	BIOwHAC (<i>n</i> = 19)
Right hemiliver (cases)	2	3	1
Left hemiliver (cases)	1	2	1
S ₂ + S ₃ (cases)	3	5	3
S ₆ + S ₇ (cases)	2	2	2
S ₅ + S ₆ (cases)	2	2	1
S ₅ + S ₈ (cases)	1	2	1
S ₆ (cases)	2	2	3
S ₇ (cases)	3	1	2
S ₅ (cases)	2	1	2
S ₄ (cases)	2	0	2
S ₈ (cases)	0	0	2
Tumor size (cm)	6.33 \pm 3.39	7.60 \pm 4.03 ^b	6.11 \pm 3.18 ^{b,c}
Operation time (min)	158.50 \pm 43.77 ^a	219.25 \pm 58.09	151.84 \pm 41.77 ^a
Occlusion time (min)	20.60 \pm 4.91	25.70 \pm 8.29 ^b	18.94 \pm 5.13 ^{b,c}
Occlusion frequency (time)	1	1	1
Bleeding volumes (mL)	700.00 \pm 163.32	1017.5 \pm 663.57 ^b	789.47 \pm 683.04 ^{b,c}
Blood transfusion (cases)	8	11	9
Transfusion volumes (mL)	775.00 \pm 679.81	709.09 \pm 317.66 ^b	766.66 \pm 580.94 ^{b,c}

^a*P* < 0.05 *vs* HVO; ^b*P* > 0.05 *vs* pringle maneuver; ^c*P* > 0.05 *vs* HVO. BIOwHAC: Hepatic blood inflow occlusion without hemihepatic artery control; HVO: Hemihepatic vascular occlusion.

Table 3 Postoperative complications and length of hospital stay (mean \pm SD)

Groups	Pringle maneuver (<i>n</i> = 20)	HVO (<i>n</i> = 20)	BIOwHAC (<i>n</i> = 19)
Postoperative complications (cases)	4	5	4
Responsive pleural effusion (cases)	3	2	3
Pulmonary infection (cases)	1	3	1
Hepatic section bleeding (cases)	0	0	0
Bile leakage (cases)	0	0	0
Subphrenic abscess (cases)	0	0	0
Hepatic encephalopathy (cases)	0	0	0
Hemorrhage of digestive tract (cases)	0	0	0
Long-term liver dysfunction (cases)	0	0	0
Perioperative deaths (cases)	0	0	0
Length of hospital stay (d)	21.75 \pm 4.32	22.95 \pm 5.30	21.47 \pm 9.36

BIOwHAC: Hepatic blood inflow occlusion without hemihepatic artery control; HVO: Hemihepatic vascular occlusion

but a decrease after 7 d. Patients in the Pringle maneuver group had more significant variation in the serum ALT and AST levels (*P* < 0.05) before and 1 d after operation than the BIOwHAC and HVO groups, but there was no significant difference between BIOwHAC and HVO groups (*P* > 0.05). No significant intergroup differences were noted in the serum ALT and AST levels (*P* > 0.05) 3 and 7 d after operation (Figure 2C and D).

Postoperative complications and length of hospital stay

The most common postoperative complication in this study was pleural effusion with pulmonary infection, occurring in 4 cases (20%) of the Pringle maneuver group, 5 cases (25%) of the HVO group, and 4 cases (21.1%)

of the BIOwHAC group. No significant intergroup differences were noted in length of hospital stay among the three groups (Table 3).

DISCUSSION

Owing to the recent improvement in the liver resection technique intraoperative hepatic vascular occlusion and perioperative management, surgical complications and mortality rate have been considerably reduced^[8-10]. Pringle maneuver is the commonly used method for hepatic vascular occlusion, which enables effective control of bleeding. Multiple approaches for hepatic vascular occlusions have been developed; however the Pringle maneuver is more popular because it can be used in various types of liver resection^[11,12]. Man *et al*^[13] reported that the Pringle maneuver is superior to non-vascular occlusion, but can lead to obvious HIRI and is time consuming, especially in cases complicated with liver cirrhosis^[14]. In 1987, Makuuchi performed HVOs through portal venous branch occlusion and hepatic arterial branch occlusion^[15]. HVO helps conserve the contralateral hepatic vascular inflow and facilitates the hepatic operation and mildly affects postoperative liver function in favor of patients with liver cancer and hepatocirrhosis^[15]. However, HVO must be performed by surgeons who are proficient in portal vein surgery so as to avoid incident damage to the interior conduit of the Glisson sheath, hemorrhage, and biliary fistula. If there are communicating branches between the non-occluded and the occluded hemilivers, hepatic bleeding may be quite severe^[16].

Although no significant intergroup differences were observed in the changes of the serum AST and ALT levels 3 and 7 d after operation, patients in the HVO and BIOwHAC groups had significantly lower AST and ALT levels than those in the Pringle maneuver group 1 d after

operation. This implies that HVO and BIOwHAC are superior to Pringle maneuver in minimizing the HIRI. In HVO, the vascular occlusion is continuous; but in the Pringle maneuver the circulation is maintained with occlusion for 15-20 min and reperfusion for 5 min, this interrupted occlusion gives rise to several problems^[3]. In our study, the patients underwent only a single vascular occlusion, which is the reason why no significant inter-group differences were seen in intraoperative bleeding and in the number of cases requiring blood transfusions.

In humans, the confluence of the left and right hepatic ducts lies superiorly, the portal bifurcation is inferior to this bifurcation, and below is the proper hepatic arterial bifurcation. Therefore, it is convenient and safe to intraoperatively expose the proper hepatic artery and separate its left and right branches. Thus, surgeons can occlude the portal vein, bile duct, and occlusive-side artery branch in a single operation. BIOwHAC is convenient and safe as compared to Pringle maneuver. Our results showed that HVO required significantly longer operation time than BIOwHAC and Pringle maneuver (219.25 ± 58.09 min *vs* 151.84 ± 41.77 min and 158.50 ± 43.77 min) due to the net operation of vascular occlusion for 20-30 min. In addition, resection of the cholecyst is required in the right HVO, but not in BIOwHAC, which can satisfy the wish of the patients who want to conserve the cholecyst.

In the normal liver, 70%-75% of blood supply comes from the portal vein and 40%-60% of oxygen supply from arterial blood. Portal vein can not supply all parts of the liver with oxygen, and when the portal vein is completely occluded, the level of oxygen consumption in the liver remains the same as that before occlusion. This indicates that the hepatic artery alone is sufficient to meet the oxygen demand of the liver^[17]. Our results showed that the patients in the BIOwHAC group had significantly lower serum AST and ALT levels than those in the Pringle maneuver group 1 d after operation, but no significant differences from that in the HVO group. This finding shows that hemihepatic artery conservation can provide enough oxygen to meet the demand of the intact hemiliver. In addition, no significant intergroup difference was noted in the postoperative serum albumin levels, which may be attributed to postoperative exogenous supplements.

Our results indicated no significant intergroup differences in the intraoperative bleeding and blood transfusion, liver function 3 and 7 d after operation, incidence of postoperative complications, and length of hospital stay. We recommend BIOwHAC because it is a convenient and safe technique similar to the Pringle maneuver and can protect the liver function injury as effectively as HVO.

In conclusion, among the various kinds of approaches for hepatic vascular occlusion available currently, the most suitable one should be selected on the basis of comprehensive preoperative examinations (CT or MRI imaging and liver function tests), intraoperative examinations (pathological examination and lesion location),

examinations for invasion in the hepatic vein and inferior vena cava, cardiovascular status, as well as the experience and skill of surgeons and anesthesiologists.

COMMENTS

Background

Intraoperative bleeding occurs most commonly during hepatic resection. Hepatic inflow control plays an important role in constructing a bloodless surgical field.

Research frontiers

There are various techniques of hepatic vascular control, including Pringle maneuver, hemihepatic vascular occlusion (HVO), total hepatic vascular exclusion, liver hanging maneuver, *etc.*

Innovations and breakthroughs

Based on the differences between the distribution and oxygen content of the blood in the portal vein and that in the hepatic artery, hepatic blood inflow occlusion without hemihepatic artery control (BIOwHAC) was performed in this study.

Applications

BIOwHAC is convenient and safe similar to Pringle maneuver; this technique causes as slight hepatic ischemia reperfusion injury as HVO.

Terminology

BIOwHAC: the proper hepatic artery was surgically exposed, after which its left and right branches were separated. For the right and left hemihepatic occlusions, catheters were advanced and bypassed the portal vein, bile duct, and the respective branches of the hepatic artery before being tightened.

Peer review

This is a retrospective analysis on a single center series of different methods of inflow hepatic occlusion during liver resection for liver cancer. The study is well written and the methodology is correct.

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Human papillomavirus DNA and P16^{INK4A} expression in concurrent esophageal and gastric cardia cancers

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Abstract

AIM: To investigate the relationship between human papillomavirus (HPV) infection and concurrent esophagus and gastric cardia cancer from the same patient (CC) and examine the significance of P16^{INK4A} protein expression.

METHODS: Polymerase chain reaction was used to de-

tect the presence of HPV type16 (HPV16). The expression of P16^{INK4A} protein was detected using immunohistochemistry.

RESULTS: Among the CC specimens, HPV16-DNA was found in eight cases of esophageal squamous cell carcinoma (ESCC) and five cases of gastric cardia adenocarcinoma (GCA), respectively (47% vs 29%), and two of both ESCC and GCA. P16^{INK4A} was highly expressed in both ESCC and GCA. In the HPV-associated positive CC, higher P16^{INK4A} expression was observed in the GCA than in the ESCC (75% vs 25%, $P < 0.05$).

CONCLUSION: HPV16 as a correlated risk factor may play an important role in the development of ESCC and GCA. P16^{INK4A} may be a screening index in the HPV-associated carcinoma of gastric cardia.

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Key words: Esophageal squamous cell carcinoma; Gastric cardia adenocarcinoma; Human papillomavirus; Polymerase chain reaction; Immunohistochemistry

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INTRODUCTION

Esophageal carcinoma (EC) is one of the most common

malignant diseases worldwide. Linzhou (formerly known as Linxian) in Henan Province, northern China, has been recognized as the highest prevalence area of esophageal squamous cell carcinoma (ESCC) in the world^[1]. In epidemiology, gastric cardia adenocarcinoma (GCA) in China is characterized by its striking geographic distribution and its concurrence with ESCC^[2]. Similar phenomenon can be also observed in other esophageal cancer incidence areas worldwide^[3]. At present, ESCC and GCA still are the main cause of tumor-related deaths in this area. Another interesting feature at the high prevalence area is that the primary cancers of the esophagus and gastric cardia are concurrent in the same patient in Henan, we named them concurrent carcinoma of the esophagus and gastric cardia in the same patient (CC), which is not uncommon in this area (0.4%-2.5%)^[4]. The special pattern suggested that similar risk factors and mechanism might be involved in these two cancers.

Human papillomavirus (HPV) as one of the important tumor-related viruses has been firmly recognized in cervical cancer with HPV-DNA detected in > 99% specimens^[5]. However, its oncogenic role in other tumors still remains controversial. As to its role in ESCC, it was firstly mentioned by Syrjänen twenty years ago^[6]. Since then, many reports regarding this topic have been published, but the HPV infection rate in ESCC varied from zero to 90%^[7,8] and in GCA from zero to 68%^[9,10], depending on the specimens obtained from low- or high-risk areas and the methods used in each study^[11]. In order to further investigate the prevalence of HPV infection in upper digestive tract tumor, the samples with concurrent ESCC and GCA from the same patient were tested for the existence of HPV type16 (HPV16)-DNA.

HPVs are small DNA viruses that can be classified as either high-risk or low-risk types. HPV-16 and 18 is most common in high-risk group, especially type 16 which is considered a risk factor for EC at a high prevalence area in Henan^[8,12]. The E6 and E7 oncoproteins of the high-risk HPV types can efficiently destroy the cell cycle regulation and apoptotic pathways by binding to a number of host cell proteins, such as P16^{INK4A} protein, which is an inhibitor of cyclin-dependent kinase. The HPV oncoproteins are able to alter the cell cycle and leaves them vulnerable to other genetic changes, ultimately resulting in malignant transformation^[13,14]. However, the reports about HPV infection involved in the carcinogenesis of gastric cardia are very limited, and there has been no related study comparing the HPV detection rate and expression of P16^{INK4A} protein in CC tissues. In our study, we investigated the HPV infection and changes of P16^{INK4A} protein in the same concurrent cancer patient in the high-risk area of EC in Henan to understand the mechanism of esophagus/cardia carcinogenesis in this area, and further illustrate whether the two carcinomas have the similar pathogenesis.

MATERIALS AND METHODS

Clinical samples and diagnostic criteria of CC

A total of 23 cases of concurrent ESCC and GCA were

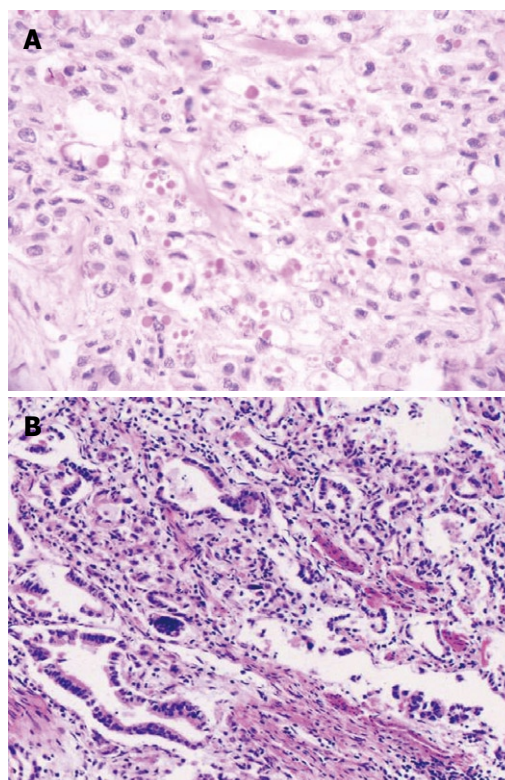


Figure 1 Hematoxylin and eosin staining in the concurrent esophageal and gastric cardia cancers tissue specimens. A: Esophageal squamous cell carcinoma tissue ($\times 100$); B: Gastric cardia adenocarcinoma tissue ($\times 100$).

obtained. Among them, DNAs from 17 ESCC and their corresponding GCA tissues were extracted from paraffin-embedded samples by conventional phenol-chloroform procedure. Six cases were from Linzhou Center Hospital, eight from Linzhou Yaocun Esophageal Cancer Hospital and three from Anyang City Cancer Hospital from September 2005 to June 2008. All the hospitals are located in the high incidence region of ESCC in Henan. There were twelve men and five women with an average age of 58 years. None of the patients received chemo- and radio-therapy before surgery.

The samples were immediately fixed by 10% formalin, dehydrated and paraffin-embedded, followed by pathological diagnosis and immunochemical analysis. The diagnosis of the CC was based on the following criteria: (1) All the tumors in the esophagus and cardia in the same patient are malignant; (2) All the tumors have defined pathological modality, i.e. concurrent esophageal squamous cell carcinomas and gastric cardia adenocarcinomas; and (3) None the tumors are metastatic (Figure 1). This study was approved by the Institutional Review Board of the School of Medicine, Zhengzhou University, China.

DNA extraction

The methods were used as described by Greer *et al.*^[15]. Briefly, each formalin-fixed and paraffin-embedded sample was cut into 10 μm thick sections, 5-10 slides were deparaffinized in xylene and graded alcohol, then the lysis buffer (300 mmol/L NaCl; 50 mmol/L Tris HCl pH 8.0; 0.2% SDS) was added into the tube with proteinase K

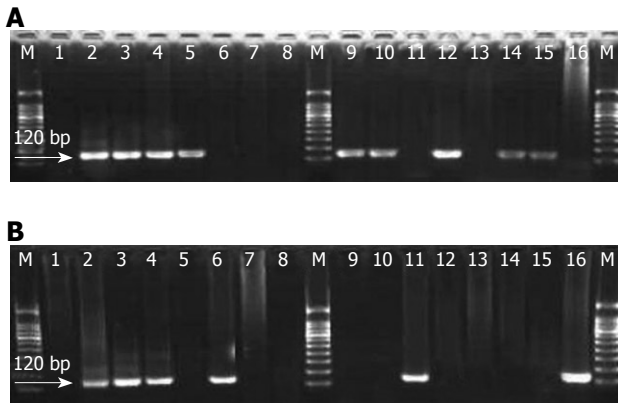


Figure 2 Amplification of human papillomavirus type 16-E6 gene fragment in the concurrent esophageal and gastric cardia cancers tissues. Polymerase chain reaction products were run in 3.0% agarose gel; Lane M: Molecular marker (100 bp ladder); Lane 1: Double water (negative control); Lane 2: Plasmid with human papillomavirus type 16 (HPV16)-E6 120 bp (positive control); Lanes 3-16: Represent positive and negative cases. A: HPV16-E6 gene fragment amplification in esophageal squamous cell carcinoma tissues; B: HPV16-E6 gene fragment in the corresponding gastric cardia adenocarcinoma tissue amplification.

(200 mg/L), and the solution was incubated at 55°C overnight until it became clear. DNA was then extracted using conventional phenol-chloroform procedure, precipitated with cold alcohol and dissolved in ion-free water, and the concentration was determined based on its optical density. Quality of the extracted DNA was tested by polymerase chain reaction (PCR) with β -actin primer: 5'-TCACCCA-CACTGTGCCCATC-3' and 5'-GAACCGCTCATT-GCCAATGG-3'. The DNA which was β -actin gene amplification positive was used to detect the presence of HPV16-DNA.

PCR and P16^{INK4A} protein immunostaining

The usable DNA went through PCR amplification using type-specific primer: 5'-TCAAAAGCCACTGT-GTCCTG-3' and 5'-CGTGTTCCTTGATGATCTGCA-3' targeting HPV16-E6 gene. Recombinant plasmid DNA HPV16^{pBR322} as positive control was obtained from Professor You-Lin Qiao, the Cancer Research Institute, Chinese Academy of Medical Sciences and double water was used as negative control in PCR. Reactions were set in 25 μ L 1 \times PCR buffer containing 10 mmol/L Tris HCl, pH8.4, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 4 mmol/L dNTP and 1.5 U Taq DNA polymerase (Promega). PCR conditions were as follows: an initial denaturation at 95°C for 5 min followed by 5 cycles with cycling profile at 95°C denaturing for 1 min, at 55°C annealing for 1 min, and at 72°C prolonging for 1 min, and then at 95°C denaturing for 20 s, at 55°C annealing for 30 s and at 72°C prolonging for 30 s with 45 cycles^[8]. The amplified products were revealed by electrophoresis on 3.0% agarose gels (Promega) containing 0.5% μ g/mL of ethidium bromide in 1 \times TAE buffer at 100 volts for 30 min. Samples were considered positive if a band of 120 bp was observed under the ultraviolet light (Figure 2). All the experiments from DNA extraction to HPV gene amplification were carried out at the Virus Research Insti-

tute, Chinese Academy of Preventive Medicine in Beijing, China by the same technician with the same protocol as established previously in Dr. Yi Zeng's laboratory. Positive control (plasmid with HPV16^{pBR322} DNA fragment) and negative control (double water) were set in each HPV-DNA amplification process. Each experimental step was taken following the previously established rules.

For immunohistochemistry, sections of paraffin-embedded tissue with a thickness of 3 μ m were deparaffinized by passage through xylene. After the endogenous peroxidase activity was blocked with 0.3% H₂O₂, the slides were then rehydrated with 0.01 mol/L sodium phosphate/citrate buffer at pH 8.0 and heated in 0.01 mol/L-citrate buffer at pH 6.0, 95°C for 30 min to retrieve the antigen. After rinsed in 0.01 mol/L phosphate-buffered saline (PBS) at pH 7.4, nonspecific antibody binding was reduced by incubating the sections with 10% fetal bovine serum in PBS for 30 min. The sections were incubated overnight at 4°C with a mouse monoclonal antibody of p16^{INK4A} protein (1:200 dilution, PharMingen International). After washing thoroughly with PBS, the slides were incubated with biotinylated horse anti-mouse IgG for 30 min followed by 1:100 dilution of the Avidin-Biotin-Peroxidase Complex (Vectastain elite ABC kit, Vector Laboratories, Burlingame, CA) for an additional 30 min. The peroxidase signal was visualized by treatment with DAB substrate-chromogen system (DAKO) for 8 min. Finally, the sections were stained lightly with hematoxylin. In statistical analysis, those having less than 10% cells stained positive were classified as negative and the others were regarded as positive cases^[16] (Figure 3).

Statistical analysis

Fisher's exact test was used to examine the association between HPV status and each clinicopathological factor including p16 expression. All the *P* values presented in the present study were two-sided. Experimental data were analyzed by statistical software SPSS 13.0.

RESULTS

The results of the PCR showed that, in the 17 CC, the detection rate of HPV16 in the ESCC was 47% (8/17), and 29% (5/17) in GCA. There was no significant difference between the two groups (*P* > 0.05). Two positive and six negative cases of ESCC and GCA were found in the HPV16-DNA simultaneously, illustrating the high consistency of HPV involved in the two kinds of tumors (47%) (Table 1).

In the 17 cases with CC, the positive ratio of P16^{INK4A} protein expression was 41% (7/17) in ESCC and 59% (10/17) in GCA, respectively. Twelve patients showed P16^{INK4A} protein changes (71%), among them, six patients were P16^{INK4A} immunoreaction positive (35%) in ESCC and GCA simultaneously.

According to the Fisher's Exact test, there was no significant difference between HPV infection and P16^{INK4A} protein expression in the ESCC and GCA specimens (*P* > 0.05). However, among the eight HPV positive ESCC

Table 1 Esophageal squamous cell carcinoma and gastric cardia adenocarcinoma in the same patient: Clinical and pathological features

Sample No.	Age (yr)	Gender	ESCC				GCA			
			Differentiation	Stage	HPV	P16	Differentiation	Stage	HPV	P16
1	57	Male	M	T1N0M0	+	-	M	T3N0M0	+	+
2	60	Male	M	T1N0M0	+	+	L	T2N0M0	+	+
3	62	Female	H	T3N0M0	+	-	M	T2N0M0	-	+
4	62	Male	M	T2N0M0	-	+	H	T2N0M0	+	-
5	50	Male	M	T1N0M0	-	+	M	T3N0M0	-	+
6	68	Male	M	T1N0M0	-	-	H	T3N0M0	-	-
7	71	Male	M	T3N0M0	+	-	L	T3N0M0	-	-
8	61	Female	M	T2N0M0	+	-	M	T1N1M0	-	+
9	51	Male	H	T3N0M0	-	+	L	T3N0M0	+	+
10	48	Male	M	T1N0M0	+	-	M	T3N0M0	-	-
11	57	Female	M	T3N0M0	-	-	L	T3N0M0	-	-
12	55	Male	H	T2N0M1	+	+	M	T3N1M0	-	+
13	57	Female	L	T3N0M0	+	-	M	T1N0M0	-	+
14	67	Male	M	T2N0M0	-	+	L	T1N0M0	+	+
15	64	Male	M	T3N0M0	-	+	L	T2N0M0	-	+
16	51	Male	M	T1N0M0	-	-	L	T3N1M0	-	+
17	52	Female	H	T1N0M0	-	-	L	T3N0M0	-	-

ESCC: Esophageal squamous cell carcinoma; GCA: Gastric cardia adenocarcinoma; HPV: Human papillomavirus; M: Moderately differentiated; H: Highly differentiated; L: Low differentiated.

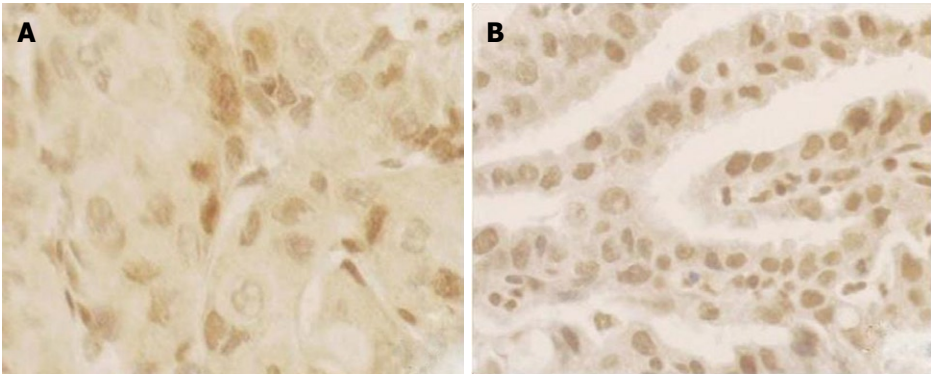


Figure 3 P16^{INK4A} protein expression in the concurrent esophageal and gastric cardia cancers tissues by immunohistochemical staining Avidin-Biotin-Peroxidase Complex method (× 200). A: P16^{INK4A} protein expression in esophageal squamous cell carcinoma; B: Expression of P16^{INK4A} protein in gastric cardia adenocarcinoma.

specimens, only two expressed P16^{INK4A}, while four expressed P16^{INK4A} in the HPV positive GCA tissues.

DISCUSSION

The present study demonstrates that both ESCC and GCA tissues from the same patient had HPV16-DNA infection, with an incidence of 47% and 29%, respectively. And HPV16 E6-DNA was observed in both ESCC and GCA tissues in two patients simultaneously. These results suggest that HPV16 might participate in the carcinogenesis of the ESCC and GCA.

In the Linzhou area in Henan, almost half of the ESCC patients have HPV16 infection, with a higher incidence than in GCA (47% *vs* 29%), but without a significant difference (*P* > 0.05). However, there were no correlations between HPV16 infection and gender, age, tumor size, depth of penetration, differentiation, lymph node metastasis and TNM stage (all *P* > 0.05). The dif-

ference and the mechanism of the affinity specificity of HPV16 to squamous epithelium and styloid glandular epithelium are still not clear^[17]. Esophageal epithelium and cardia epithelium are under the same internal environment and heredity of the same organism, as well as exposure to the same environment and carcinogenic agents, which may be associated with the co-infection of the two different epithelial tissues for HPV. Recently, it has been reported that colonic epithelium and colon carcinoma have HPV infections^[18-20], and further studies are still needed to confirm the biological significance and mechanisms for HPV invading the body.

Over the past 20 years, many reports regarding HPV infection in EC have been published, and the reported HPV detection rate in the literature varied largely. To explain these marked differences, different region, sampling methods, demographic and ethnic factor, disease status, and sensitivity of detection methods have been cited as potential causes of this inconsistency^[11,21]. In the present

study, the same method was used to analyze the two kinds of neoplasms with different histological type in the same patient, which greatly reduced the disparity of the methodology and/or population.

CC is an ideal model for illustrating the environmental influence on both ESCC and GCA with the similar genobackground and comparability of environmental agents. The present study will deepen the understanding on the mechanism of esophageal/cardia carcinoma in this area. Although these results indicating the presence of HPV-DNA in esophagus and gastric cardia carcinoma tissues, suggest a possible role for HPV in upper digestive tract tumors, further studies are necessary for establishing a definite causative role.

P16^{INK4A} gene is an important member of P53-Rb system, and its product P16^{INK4A} protein can prevent the cell to enter S-phase from G1-phase, and suppress cell proliferation, through inhibition of the phosphorylation of the retinoblastoma (pRb)^[22]. In this cascade regulation, pRb could negatively inhibit the expression of P16^{INK4A} protein.

The studies of cervical cancer showed that the HPV-E6/E7 protein combined with pRb is deactivated, and removes the negative inhibition of the expression of P16^{INK4A} protein, which causes the over-expression of P16^{INK4A}. In the present study, 7 (41%) cases showed the expression of P16^{INK4A} protein in ESCC, and 10 (59%) in GCA, which is similar to the results in the adenocarcinoma of the uterine cervix by Ansari-Lari *et al.*^[23]. In the study of HPV-associated cervical cancer, expression of P16^{INK4A} protein is higher in the adenocarcinoma than in the squamous cell carcinoma, and combined detection of HPV infection and P16^{INK4A} protein expression would be helpful to the diagnosis of the primary adenocarcinoma of the uterine cervix^[24]. In the present study, 2 of the 8 HPV-positive ESCC cases expressed P16^{INK4A}, while 4 of 5 HPV-positive GCA cases expressed P16^{INK4A}. These results suggested that in the HPV-associated CC, expression of P16^{INK4A} protein in GCA is higher than in ESCC. It is obvious that it would be of great significance to further understand the molecular discrepancy of the HPV16 positive and negative patients, and explore the exact mechanisms of the role of HPV in the carcinogenesis of esophagus and cardia.

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COMMENTS

Background

Esophageal cancer (EC) is one of the most common malignant diseases, with a remarkable geographical distribution and poor prognosis. The five-year survival rate is only 10%. However, the five-year survival rate for the patients with the early EC is more than 90%. More than 85% of the EC patients are diagnosed

at the late stage due to lack of early specific symptoms and unknown etiological factors, and the carcinogenesis remains the leading cause of late diagnosis for EC. Therefore, the current challenges in EC research are to obtain a better understanding of the exact etiological factors and molecular alteration in the esophageal carcinogenesis process to establish the strategies for prevention and early diagnosis of those with high risks.

Research frontiers

It has been well recognized that esophageal carcinogenesis is a progressive process involving multi-factors and multistage: tobacco, alcohol, fungal toxins, nutritional deficiencies, as well as infectious agents, are related to esophageal carcinogenesis. Among the infectious agents, human papillomavirus (HPV), a major cause of carcinoma of the cervix uteri throughout the world, is strongly implicated in the etiology of EC. HPV-16 and-18 are the most frequent genotypes, especially type-16 which is considered to be a risk factor for EC in the high prevalence area in Henan. On the other hand, oncoproteins of the high-risk HPV types can efficiently destroy the cell cycle and apoptotic pathways by binding to a number of host cell proteins, ultimately resulting in malignant transformation.

Innovations and breakthroughs

In this study, the authors found the HPV-16 DNA in 47% of esophageal squamous cell carcinomas (ESCC) and 29% of gastric cardiac adenocarcinomas (GCA) in concurrent cancers in the same patients (CC). Interestingly, HPV-16 DNA was detected in two cases of ESCC and GCA tissues simultaneously. These results suggested that HPV16 might participate in the carcinogenesis of the ESCC and GCA, and the two carcinomas might have similar risk of carcinogenesis. P16^{INK4A} was highly expressed in both ESCC and GCA tissues. In the HPV-associated positive CC, higher P16^{INK4A} expression was found in GCA than in ESCC (75% vs 25%, $P < 0.05$).

Applications

By knowing the prevalence and molecular alteration of high-risk HPV-associated EC, this study may contribute to the future strategies for prevention and early diagnosis of HPV-related malignancies, through the development of effective vaccines and biomarkers.

Terminology

HPVs are DNA viruses that infect basal skin and mucosal cells, and categorized according to their cervical oncogenicity-based risks.

Peer review

The manuscript is interesting, presenting data of HPV DNA detection in the concurrent esophageal squamous cell carcinoma and cardia adenocarcinoma and expression of p16 protein. Given the truth of these data, this would be a piece of evidence for involvement of HPV in esophageal carcinogenesis in man.

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Conference

January 28-30
Hong Kong, China
The 1st International Congress on
Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal
Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at
The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on
Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of
Gastroenterology & Endoscopy
Meeting

March 09-12
Brussels, Belgium
30th International Symposium on
Intensive Care and Emergency
Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian
National Association for Study of
the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on
Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian

Pacific Association for the Study of
the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in
Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology
and Hepatology Conference, EGH
2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic
Surgery

April 14-18
Vienna, Austria
The International Liver Congress™
2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress
of surgery and the 5th Croatian
Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual
Meeting

May 06-08
Munich, Germany
The Power of Programming:
International Conference on
Developmental Origins of Health
and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and
Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical
Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on
Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in
the Research of Probiotics and
Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver
Transplantation Society ILTS Annual
International Congress

June 20-23
Mannheim, Germany
16th World Congress for
Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific
Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on
Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's
Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory
Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference
on Antimicrobial Agents and
Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on
Controversies in Gastroenterology &
Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International
Symposium of Society of
Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of
Gastroenterology Annual Scientific
Meeting

October 23-27
Barcelona, Spain
18th United European
Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's
61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the
Management of Inflammatory Bowel
Disease

December 02-04
San Francisco, CA, United States
The Medical Management of HIV/
AIDS



Instructions to authors

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

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The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systematically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensus and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

Name of journal

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Figures should be numbered as 1, 2, 3, etc., and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: <http://www.wjgnet.com/1007-9327/13/4520.pdf>; <http://www.wjgnet.com/1007-9327/13/4554.pdf>; <http://www.wjgnet.com/1007-9327/13/4891.pdf>; <http://www.wjgnet.com/1007-9327/13/4986.pdf>; <http://www.wjgnet.com/1007-9327/13/4498.pdf>. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use

Instructions to authors

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Data that are not statistically significant should not be noted. ^a $P < 0.05$, ^b $P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, ^c $P < 0.05$ and ^d $P < 0.01$ are used. A third series of P values can be expressed as ^e $P < 0.05$ and ^f $P < 0.01$. Other notes in tables or under illustrations should be expressed as ¹F, ²F, ³F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, ▲, △, etc., in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

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Format

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- 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 Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

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

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.00000035706.28494.09]

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]

Volume with supplement

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

Issue with no volume

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

No volume or issue

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

Books

Personal author(s)

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

Chapter in a book (list all authors)

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

Author(s) and editor(s)

- 12 **Breedlove GK,** Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

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

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

Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as ν (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h; blood glucose concentration, *c* (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 $24.5 \mu\text{g/L}$; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kho I*, *Kpn I*, etc.

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