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Role of tissue microenvironment resident adipocytes in colon cancer

Maria Tabuso, Shervanthi Homer-Vanniasinkam, Raghu Adya, Ramesh P Arasaradnam

Maria Tabuso, Ramesh P Arasaradnam, Department of Gastroenterology, University Hospitals Coventry and Warwickshire, Clifford Bridge Road, Coventry CV2 2DX, United Kingdom

Shervanthi Homer-Vanniasinkam, Raghu Adya, Ramesh P Arasaradnam, Warwick Medical School, University of Warwick, Coventry CV4 7AL, United Kingdom

Shervanthi Homer-Vanniasinkam, Leeds Institute of Cardiovascular and Metabolic Medicine, Faculty of Medicine and Health, University of Leeds, Leeds LS2 9JT, United Kingdom

Shervanthi Homer-Vanniasinkam, Division of Surgical and Interventional Sciences, UCL Medical School Building, 21 University Street, London WC1E 6AU, United Kingdom

Ramesh P Arasaradnam, Applied Biological and Experimental Sciences, University of Coventry, Coventry CV1 5FB, United Kingdom

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Correspondence to: Dr. Raghu Adya, Warwick Medical

School, University of Warwick, Coventry CV4 7AL, United Kingdom. r.adya@warwick.ac.uk
Telephone: +44-2476-573539

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Abstract

Colorectal cancer (CRC) is a multifactorial disease characterized by several genetic and epigenetic alterations occurring in epithelial cells. It is increasingly recognized that tumour progression is also regulated by tumour microenvironment (TME). The bidirectional cross-talk between tumour resident adipocytes and cancer cells within TME has been proposed as active contributor to carcinogenesis. Tumour resident adipocytes exhibit an activated phenotype characterized by increased secretion of pro-tumorigenic factors (angiogenic/inflammatory/immune) which contribute to cancer cell proliferation, invasion, neoangiogenesis, evasion of immune surveillance and therapy resistance. Furthermore, adipocytes represent a fuel rich source for increasing energy demand of rapidly proliferating tumour cells. Interestingly, a relationship between obesity and molecular variants in CRC has recently been identified. Whether adipose tissue promotes cancer progression in subsets of molecular phenotypes or whether local tissue adipocytes are involved in inactivation of tumour suppressor genes and/or activation of oncogenes still needs to be explored. This editorial highlights the major findings related to cross-talk between adipocytes and colon cancer cells and how local paracrine interactions may promote cancer progression. Furthermore, we provide future strategies in studying colonic TME which could provide insights in

bidirectional cross-talk mechanisms between adipocytes and colonic epithelial cells. This could enable to decipher critical signalling pathways of both early colonic carcinogenesis and cancer progression.

Key words: Tumour resident adipocytes; Dysfunctional adipocytes; Adipose tissue; Cancer cell-tumour resident adipocyte cross-talk; Colon cancer microenvironment

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Core tip: The tumor microenvironment (TME) has been implicated in cancer progression and chemoresistance. Adipocytes are active components of the TME. Bidirectional cross-talk between adipocytes and cancer cells has recently been postulated to actively contribute to tumor initiation and progression. This Editorial highlights the role of local paracrine interactions between adipocytes and colon cancer cells. Discovery of signalling pathways activated by tumor resident adipocytes in colon cancer will allow better understanding of carcinogenesis and provide potential therapeutic targets.

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INTRODUCTION

Colorectal carcinogenesis is multifactorial involving interactions between genetic mutations (*APC*, *TP53*, *PI3K*, *KRAS*, *BRAF*, *PTEN*), microsatellite instability, chromosomal instability, epigenetic alterations (locus-specific CpG island methylation, global DNA hypomethylation)^[1] and environmental factors (obesity, diabetes, metabolic syndrome, intestinal microbiome)^[2]. Moreover the importance of “field cancerisation” has been highlighted in terms of cancer development in the macroscopically normal colon^[3]. Epidemiologic studies support an association between high BMI and colorectal cancer (CRC) incidence and mortality^[4]. Adipose tissue has been recognised as a major endocrine organ, secreting adipokines (leptin, adiponectin, visfatin), growth factors and immune/inflammatory/angiogenic factors [tumour necrosis factor- α (TNF- α), interleukin-6 (IL-6), IL-8 and vascular-endothelial-growth-factor (VEGF)] involved in the regulation of systemic energy and metabolic homeostasis. Altered adipose tissue secretion of such factors has been implicated in dysregulation of inflammatory and immune responses leading not only to systemic low-grade inflammation and metabolic dysfunction, but also to local tissue inflammation

contributing to cancer progression^[5].

The tumour microenvironment (TME) comprising stromal cells (adipocytes, macrophages, fibroblasts, monocytes, neutrophils, infiltrating immune T-cells and B-cells) and extracellular matrix is a fertile soil for cancer cells. Increasing evidence suggests that paracrine bi-directional cancer cell-stromal cell cross-talk is a key driver not only of local inflammation, but also activation and recruitment of immune cells, neo-angiogenesis and extracellular matrix remodelling. This in turn contributes to cancer cell proliferation, invasion and migration^[6].

This Editorial highlights the contribution of adipose tissue secreted factors in colon cancer progression with a focus on bidirectional cross-talk between adipocytes and colon cancer cells.

ANATOMIC AND MOLECULAR RELATIONSHIP BETWEEN ADIPOCYTES AND CRC

The mesentery, constituted by loose connective tissue and adipocytes, is the primitive envelope of the colon. Therefore, the colon lies in close proximity to adipose tissue depots. Submucosal distribution of adipocytes has also been reported^[7], although the presence and proportion of submucosal fat in normal colonic wall is not well known. Increased submucosal fat deposition is a known characteristic of inflammatory bowel disease^[8]. However, this has also been described in subjects without intestinal pathology^[9]. Understanding normal colonic wall histology is essential in order to appreciate the close spatial relationship between adipocytes and colonic epithelial cells, progenitor cells of CRC.

Recently, an interesting area of research termed “molecular pathologic epidemiology” (MPE) has detected epigenetic modifications associated with obesity, providing insights into the relationship between obesity and molecular variants in CRC^[10]. It has been proposed that microenvironment-derived signals trigger heritable genetic changes within cancer cells, contributing to tumour evolution^[11]. Studies in breast cancer, suggest that bidirectional interactions induce sequential epigenetic modifications in both cancer and stromal cells with progression from in situ ductal carcinoma to invasive carcinoma^[12]. Epigenetic modifications induced by tumour resident adipocytes in colon cancer cells have not been reported, although MPE studies have identified a relationship between obesity and molecular variants in CRC^[10].

DYSFUNCTIONAL ADIPOCYTES AND CANCER

The main component of adipose tissue is white adipose tissue (WAT). Expansion of WAT is consequence of

Table 1 Role of adipocyte secreted factors and metabolites and signalling pathways

AT secreted factors	Function	Signalling pathway	Ref.
TNF- α	Pro-inflammatory, cell proliferation, anti-apoptotic, angiogenic	PI3K, NF- κ B	Pikarsky <i>et al</i> ^[17] , 2004 Huang <i>et al</i> ^[18] , 2009 Viatour <i>et al</i> ^[19] , 2005
IL-6	Pro-inflammatory, cell proliferation and anti-apoptotic	JAK/STAT3	Hodge DR <i>et al</i> ^[20] , 2005
Leptin	Promotion of cell survival, proliferation, differentiation, pro-inflammatory	JAK/STAT, PI3K, MAPK	Hefetz-Sela <i>et al</i> ^[22] , 2013 Hoda <i>et al</i> ^[23] , 2007
Adiponectin	Anti-inflammatory, anti-proliferative and pro-apoptotic effect	Inhibition of PI3K, AMPK/mTOR, JAK/STAT3, NF- κ B	Hefetz-Sela <i>et al</i> ^[22] , 2013
Visfatin	Pro-inflammatory, angiogenic, promotion of cell survival and migration	ERK/MAPK, PI3K/AKT, NF- κ B, β 1-integrin	Adya <i>et al</i> ^[25] , 2008 Huang <i>et al</i> ^[26] , 2013
Lipid peroxidation products	Promotion of cell proliferation, differentiation, survival, migration, angiogenesis	PI3K/AKT/mTOR NF- κ B, PPAR, MAPK	Ayala <i>et al</i> ^[27] , 2014

PI3K: Phosphoinositide 3-kinase; NF- κ B: Nuclear factor-kappa B; JAK/STAT3: Janus kinase/signal transducers and activators of transcription 3; MAPK: Mitogen-activated protein kinases; AMPK: AMP-activated protein kinase; mTOR: Mammalian target of rapamycin; HIF-1 α : Hypoxia-inducible factor 1-alpha; TNF- α : Tumor necrosis factor alpha; IL-6: Interleukin-6; AKT: Protein Kinase B; ERK: Extracellular signal-regulated kinase; PPAR: Peroxisome proliferator-activated receptors.

an increase in size (hypertrophy) and/or increase in number (hyperplasia) of adipocytes. Healthy adipose tissue expansion consists in hypertrophic and hyperplastic white adipocytes, with appropriate angiogenic response, extracellular matrix remodelling and minimal inflammation. In contrast, pathological expansion of adipose tissue consists of adipocytes hypertrophy resulting in hypoxia, reduced angiogenesis, infiltration of macrophages and immune cells, low-grade inflammation, excessive production of reactive oxygen radicals, endoplasmic reticulum stress, mitochondrial dysfunction and remodelling of extracellular matrix^[13].

Inflammation is a recognised hallmark of cancer and pre-existing pro-inflammatory microenvironments are associated with increased cancer risk^[14]. Increasing evidence, in breast, prostatic, ovarian and colon cancer, suggests that dysfunctional adipocytes are involved in cancer cell proliferation and migration through dysregulation of local and systemic inflammatory-immune-angiogenic response system^[15]. Inflammation is initiated by adipose tissue hypertrophy leading to localized hypoxia which activates hypoxia-inducible factor 1-alpha (HIF-1 α). HIF-1 α up-regulates secretion of chemokines and proangiogenic factors including TNF- α , IL-6, IL-1, monocyte chemoattractant protein (MCP-1), plasminogen activator inhibitor-1 and VEGF, which are involved in the recruitment of macrophages and initiation of angiogenesis. Recruited macrophages contribute further to up regulation of inflammatory/immune cytokines favouring the acquisition of a systemic and local inflammatory phenotype^[16].

ROLE OF AT SECRETED FACTORS AND LIPID METABOLITES IN COLON CANCER

The adipose tissue secreted factors, lipid metabolites and signalling pathways have been summarized in Table 1.

TNF- α

TNF- α , secreted by dysfunctional adipose tissue, has been shown to support cancer cell proliferation, angiogenesis and metastasis through activation of key transcription factors, including PI3K/AKT/mTOR and nuclear transcription factor NF- κ B^[17-19]. TNF- α and hypoxic conditions also induce secretion of the proinflammatory cytokine IL-6, activator of Janus Kinase and signal transducers and activators of transcription 3 (Jak/STAT3) pathways, key regulators of cell proliferation and apoptosis^[20].

Adipocyte secreted hormones, including leptin, adiponectin and visfatin, have also been implicated in colon cancer progression (Figure 1).

Leptin

Leptin is a potent inflammatory agent involved in up regulation of pro-inflammatory cytokines such as TNF- α , MCP-1, and reactive oxygen species from endothelial cells and peripheral blood mononuclear cells^[21]. *In vitro* studies, in colon cancer cell lines, have demonstrated that leptin exerts pro-inflammatory, mitogenic, anti-apoptotic and angiogenic properties^[22,23].

Adiponectin

Adiponectin has a potent anti-inflammatory, anti-proliferative and pro-apoptotic activity. However, proliferative and pro-inflammatory properties of adiponectin on colonic epithelial cancer cells have also been reported. Several studies suggest local-paracrine pro tumorigenic effects of adiponectin according to tissue-specific expression of its receptor subtypes (ADIPOR1 and ADIPOR2). Increased AdipoR1 and AdipoR2 expression has been associated with cancer progression linked with the pro-angiogenic activity of adiponectin^[22,24].

Visfatin

Visfatin has been shown to exhibit pro-inflammatory

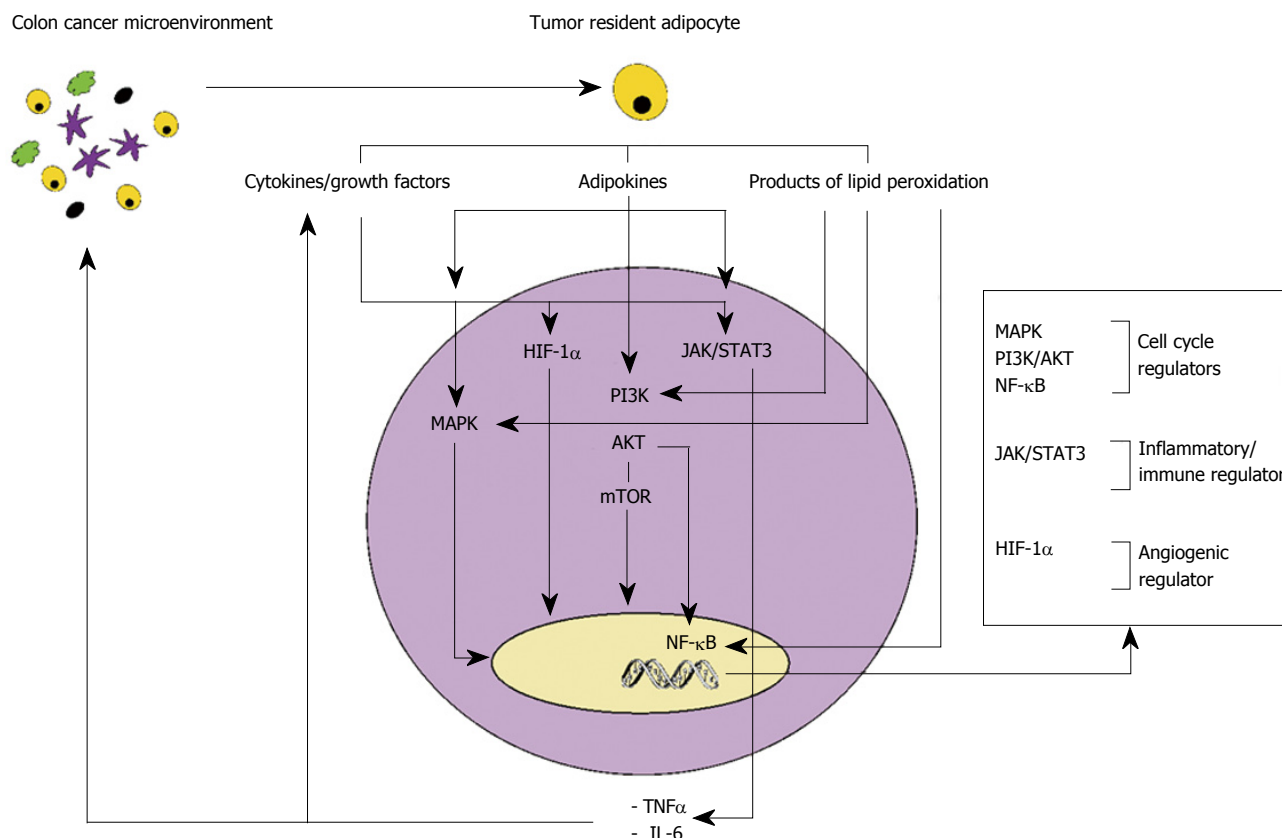


Figure 1 Signalling pathways activated by tumour resident adipocytes secreted factors. Tumour resident adipocytes secreted factors activate cell cycle regulators and inflammatory/immune/angiogenic regulators. Cancer cell secreted inflammatory cytokines activate host cells of TME constituting a paracrine/autocrine loop. MAPK: Mitogen-activated protein kinases; PI3K: Phosphoinositide 3-kinase; AKT: Protein Kinase B; mTOR: Mammalian target of rapamycin; NF-κB: Nuclear factor-κB; JAK/STAT3: Janus kinase/signal transducers and activators of transcription 3; HIF-1α: Hypoxia-inducible factor 1-alpha; TNF-α: Tumor necrosis factor alpha; IL-6: Interleukin-6.

and pro-angiogenic effects in endothelial cells^[25]. Studies have demonstrated a role of visfatin in CRC. CRC cells express chemokine receptors (CXCR4 and CXCR7), activated by visfatin, which bind stromal cell-derived factor-1, promoter of survival and migration of cancerous cells^[26].

Lipid peroxidation products

The chronic low-grade inflammatory state of dysfunctional adipocytes leads to activation of lipid peroxidation with the production and secretion of 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde. The secreted 4-HNE is responsible of deregulation of multiple pathways involved in tumour cell proliferation, differentiation, cell survival, migration, apoptosis and angiogenesis including MAPK, PI3K-AKT-mTOR, NF-κB. This also results in upregulation of prostaglandin E2 (PGE2) and cyclooxygenase-2, implicated in CRC^[27].

ACTIVATED PHENOTYPE OF TUMOUR RESIDENT ADIPOCYTES AND LOCAL PARACRINE REGULATION OF COLON CANCER

Recently great interest has emerged in reciprocal

signalling between tumour resident adipocytes and cancer cells. CRC progresses through sequential stages involving multiple layers of the colonic wall. TNM staging system is currently used for classifying CRC in 4 stages according to local invasion depth (T stage), lymph node involvement (N stage) and presence or absence of distant metastasis (M stage), providing indication for prognosis and therapeutic strategies. With cancer progression activation of complex signalling networks modify both cancer cells and stromal cells^[28]. Cancer cells and activated stromal cells communicate by autocrine/paracrine pathways contributing to dynamic modulation of TME through persistent recruitment of inflammatory and stromal cells in the TME. As a result, TME becomes increasingly populated with infiltrating innate immune cells (macrophages, neutrophils), adaptive immune cells (T and B lymphocytes) pericytes and stem cells contributing to cancer cell proliferation and invasion (Figure 2).

Adipocytes located in close proximity to tumour invasive front are reprogrammed by cancer cells into activated fibroblast-like cells. Tumour resident adipocytes exhibit morphological and functional modifications, known as adipocyte dedifferentiation, consisting in size reduction, due to enhanced lipolytic

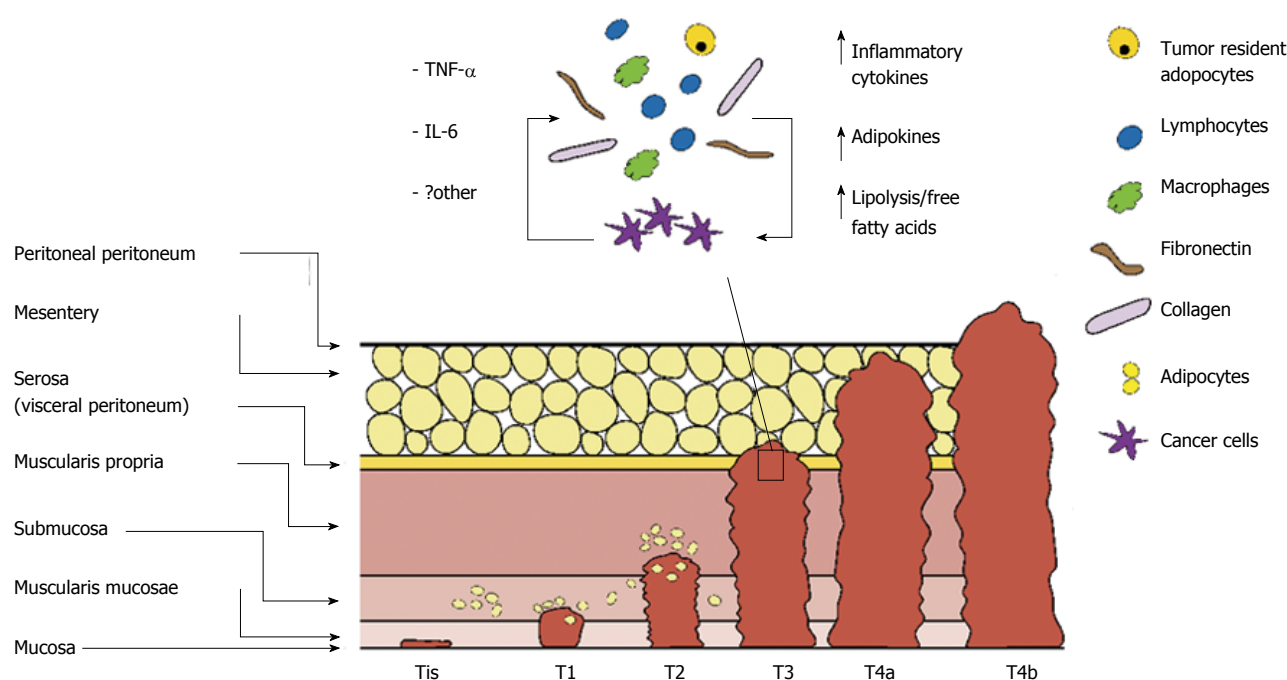


Figure 2 Colon cancer progression and tumour microenvironment. Bi-directional cross-talk between host cells of tumour microenvironment (TME) contributes to colon cancer progression. Activated tumour resident adipocytes increase secretion of cytokines, growth factors, adipokines and release lipid metabolites promoting colon cancer cell proliferation and migration. Cancer cells secrete inflammatory factors which repopulate TME, further enhancing cancer cell proliferation. Tis: carcinoma *in situ*; T1: Tumour invades submucosa; T2: Tumour invades muscularis propria; T3: Tumour invades through muscularis propria into pericorectal tissues; T4a: Tumour penetrates to the surface of the visceral peritoneum; T4b: Tumour directly invades or is adherent to other organs or structures.

activity, decreased adipocyte-differentiation markers (adiponectin, resistin, fatty acid binding protein-4, adipocyte protein 2), and increased secretion of inflammatory factors (IL-6, IL-8, IL-1 β , TNF- α), growth factors (insulin-like growth factor 1, IGF1 binding proteins), angiogenic factors (VEGF) and MCP-1 (CCL2)^[15]. *In vitro* studies, in breast and prostate cancer, have demonstrated that tumour resident adipocyte secreted factors activate signalling pathways involved in cancer cell survival, proliferation, invasion, epithelial to mesenchymal transition, angiogenesis and extracellular matrix remodelling, promoting cancer initiation and metastasis^[15,22]. Active recruitment of adipocytes to TME has not been reported, although it has been reported that bone marrow derived mesenchymal stem cells (progenitors of adipocytes) may be recruited to specific sites of neoplasia inducing metastatic properties^[29]. An open question is whether adipose tissue promotes cancer progression in subsets of molecular phenotypes or whether local tissue adipocytes are involved in inactivation of tumour suppressor genes and/or activation of oncogenes (Figure 3).

Adipocytes serve as a fuel rich source for increasing energy demand of rapidly proliferating tumour cells. Advanced stages of gastrointestinal malignancies often present with cancer-associated-cachexia as a result of lipolysis induced by cancer cells. Studies have described increased lipid droplets in colon adenocarcinoma and it has been implicated in PGE2 synthesis. Inhibition of lipid droplet formation by fatty

acid synthase inhibitors reduces cancer cell proliferation *in vitro*, suggesting a role of lipid droplets in colon adenocarcinoma^[30].

Metabolic and transcriptomic expression profile and direct paracrine effects of tumour resident adipocytes in colon cancer have not been evaluated. We have preliminary data (unpublished) indicating increased expression of pro-inflammatory/immune/angiogenic factors in colon cancer resident adipocytes, isolated from paraffin embedded sections using laser micro dissection system, compared to adipocytes isolated from the distal non neoplastic mucosa.

TME signalling pathways have recently been implicated in inducing chemoresistance in breast and prostate cancer^[31]. There is also evidence, in colon cancer cell lines, that leptin inhibits cytotoxic effects of 5-fluorouracil^[32].

Novel strategies in studying TME

Two dimensional (2D) cell culture models, widely used in basic science research, do not reproduce the complex interactions between host cells of TME. Recently, three-dimensional (3D) organoid models, derived from mouse and human intestinal tissue *ex vivo*, have been described. These *in vitro* organ-like cultures reproduce intestinal tissue micro-environment. Furthermore, they can be co-cultured with stromal components^[33]. Reproduction of colonic microenvironment *in vitro* will allow to decipher *ex vivo* bidirectional cross-talk mechanisms between adipocytes and colonic epithelial cells.

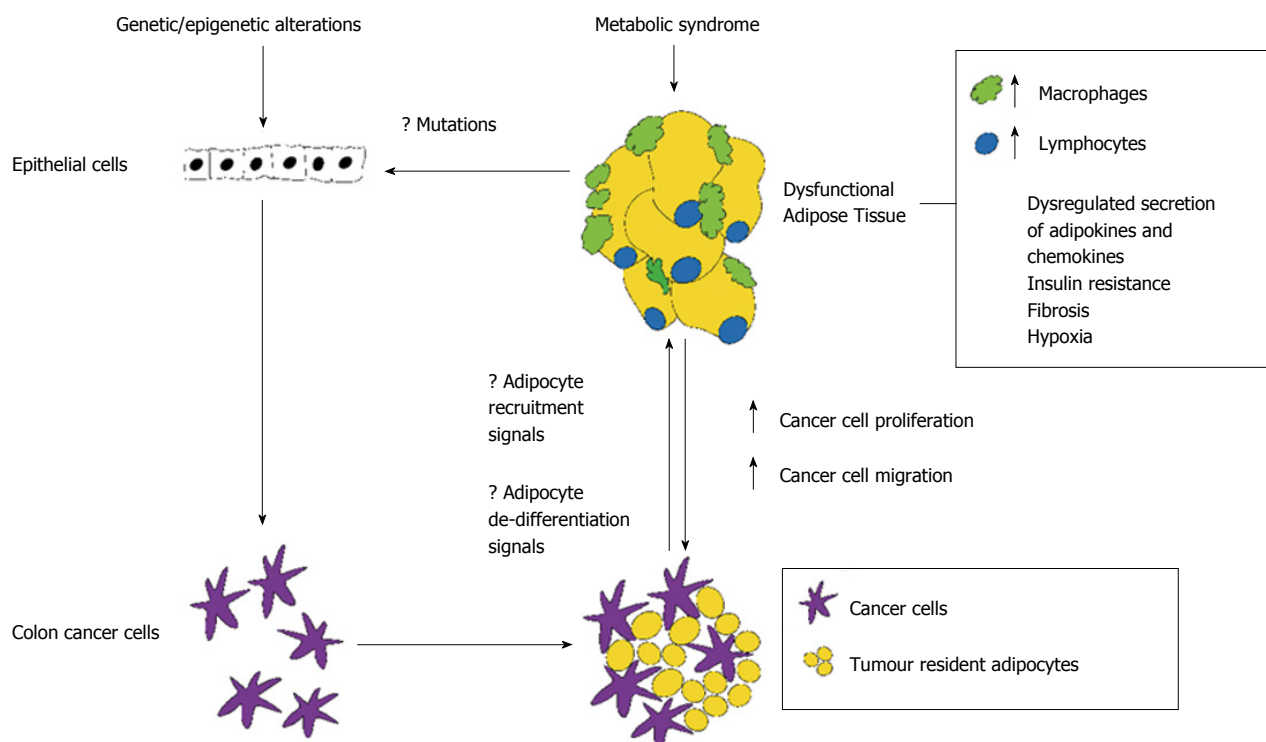


Figure 3 Paracrine interactions between dysfunctional adipose tissue, colon cancer cells and normal epithelial cells. Dysfunctional adipose tissue induces recruitment of macrophages and lymphocytes creating a favourable microenvironment for tumour initiation and progression. We propose that dysfunctional adipocytes may induce epigenetic mutations in neighbouring epithelial cells. Adipocyte activation and recruitment signals from colon cancer cells are not known.

CONCLUSION

The bidirectional cross talk between tumour resident adipocytes and colon cancer cells contributes to the progressive evolution of tumour microenvironment and cancer progression. It is therefore important to decipher the metabolic and transcriptomic expression profiles of colon cancer resident adipocytes in different stages of tumour progression. Colon organoid cultures combined with adipocytes and/or tumour resident adipocyte secreted factors will allow to identify critical signalling pathways of both early colonic carcinogenesis and cancer progression providing diagnostic biomarkers and novel therapeutic targets for colon cancer.

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Ophthalmic manifestations in patients with inflammatory bowel disease: A review

Leandro Lopes Troncoso, Ana Luiza Biancardi, Haroldo Vieira de Moraes Jr, Cyrla Zaltman

Leandro Lopes Troncoso, Ana Luiza Biancardi, Haroldo Vieira de Moraes Jr, Department of Ophthalmology, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941-913, Brazil

Cyrla Zaltman, Department of Internal Medicine, Gastroenterology Division, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941-913, Brazil

ORCID Number: Leandro Lopes Troncoso (0000-0002-2269-095X); Ana Luiza Biancardi (0000-0002-0169-7001); Haroldo Vieira de Moraes Junior (0000-0003-2562-6942); Cyrla Zaltman (00000-0002-5236-6501).

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Correspondence to: Leandro Lopes Troncoso, MD, Department of Ophthalmology, Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941-913, Brazil. leandrotroncoso@ufjf.br
Telephone: +55-21-988878988
Fax: +55-21-33115645

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Abstract

Clinical manifestations of inflammatory bowel disease (IBD) are not locally restricted to the gastrointestinal tract, and a significant portion of patients have involvement of other organs and systems. The visual system is one of the most frequently affected, mainly by inflammatory disorders such as episcleritis, uveitis and scleritis. A critical review of available literature concerning ocular involvement in IBD, as it appears in PubMed, was performed. Episcleritis, the most common ocular extraintestinal manifestation (EIM), seems to be more associated with IBD activity when compared with other ocular EIMs. In IBD patients, anterior uveitis has an insidious onset, it is longstanding and bilateral, and not related to the intestinal disease activity. Systemic steroids or immunosuppressants may be necessary in severe ocular inflammation cases, and control of the underlying bowel disease is important to prevent recurrence. Our review revealed that ocular involvement is more prevalent in Crohn's disease than ulcerative colitis, in active IBD, mainly in the presence of other EIMs. The ophthalmic symptoms in IBD are mainly non-specific and their relevance may not be recognized by the clinician; most ophthalmic manifestations are treatable, and resolve without sequel upon prompt treatment. A collaborative clinical care team for management of IBD that includes ophthalmologists is central for improvement of quality care for these patients, and it is also cost-effective.

Key words: Inflammatory bowel disease; Crohn's

disease; Ulcerative colitis; Ocular complications; Eye manifestations

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Core tip: Among all inflammatory bowel disease (IBD) patients, ophthalmic inflammatory disorders occur in 0.3% to 13.0% of cases, 1.6%-5.4% among the ulcerative colitis and 3.5%-6.8% among the Crohn's disease patients. Since asymptomatic inflammation of ocular tissues may occur, a routine ophthalmic follow-up is recommended in all IBD patients, mainly before changes in IBD therapy because some drugs may cause ocular adverse effects. Patients with chronic or recurrent use of systemic corticosteroids should be warned of the risk of cataracts and glaucoma. Patient awareness of possible eye involvement is important in improving understanding of their disease and health outcomes, supporting early diagnosis, which will contribute to success of the treatment.

Troncoso LL, Biancardi AL, de Moraes Jr HV, Zaltman C. Ophthalmic manifestations in patients with inflammatory bowel disease: A review. *World J Gastroenterol* 2017; 23(32): 5836-5848 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i32/5836.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i32.5836>

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic, immune-mediated inflammatory gastrointestinal disease of unknown etiology^[1-4]. Ulcerative colitis (UC) and Crohn's disease (CD) are the main types of IBD, with different pathophysiology and clinical features but both characterized by episodes of recurrent acute attacks^[1,4-9].

The incidence and prevalence of IBD are highly variable, depending on the population studied^[8,10-12], with an estimated global prevalence of 146.9 cases/100000 people. IBD has shown progressive increases in newly industrialized countries in Asia, South America and the Middle East, and has evolved into a global disease with a rising prevalence on every continent^[13]. The highest worldwide prevalence of IBD is found in Europe, with 322 and 505 cases per 100000 people for CD and UC, respectively^[3,8].

IBD is a chronic disease and requires chronic treatment. In addition, the disease has a great impact on the patient's quality of life, commonly requiring a lifetime of care. Ocular complaints can occur as an extraintestinal manifestation (EIM) of the disease or may be drug-related. These disorders can be non-specific, with no clinical relevance to the patient and/or the physician, but the risk of complications is a reality. Thus, early detection and treatment are necessary to

prevent poor outcomes, as discussed in this review.

EIMs

Considering the underlying disease, the prevalence of EIM is variable and ranges between 12% to 35% in UC and 25% to 70% in CD^[2,14-16]. Indeed, 6%-40% of patients with IBD have one or more EIMs that can be more debilitating than the underlying IBD itself^[17]. Although the EIMs have a multifactorial pathogenesis, it is not well understood^[16]. It seems to be related to an immune response against toxins and antigens that reach the bloodstream from the gastrointestinal tract, leading to an antigen-antibody complex deposition in different extraintestinal tissues^[2,18-21].

Other complex mechanisms are speculated in some cases. It has been suggested that a human epithelial colonic autoantigen that is also present in the skin, bile ducts, eyes and joints triggers an antibody-mediated immune response^[5,22,23] and that this is related to intestinal and extraintestinal symptoms in UC patients, but not in CD^[5]. A higher incidence of HLA-B27 and HLA-DRB1*0103 positivity was demonstrated in IBD patients, and an association with extensive disease and the development of EIMs, mainly ocular and articular, has been reported^[9,22,24]. However, Lanna *et al*^[25] analyzed 96 IBD patients and found no association between HLA positivity and ocular or joint manifestations, suggesting that this finding could be justified by the great genetic heterogeneity in different global populations.

The frequency of EIMs in each type of IBD patient is controversial. Most studies show a higher frequency of EIMs in CD patients^[2,20,26-28], while some report similar frequencies in both diseases^[29]. EIMs may occur before an IBD diagnosis and even before recurrent intestinal episodes^[3,30]. The diagnosis of IBD in those under 40 years old and of female sex are considered risk factors for the development of EIMs^[2,31].

Among EIMs, musculoskeletal conditions are most common, followed by mucocutaneous and ophthalmic diseases^[15,32]. However, nearly any organ, including those of dermatologic, hepatopancreatobiliary, renal, pulmonary and endocrinological systems, can be involved, leading to a significant challenge to physicians who manage IBD patients^[2,15,19]. Most IBD patients with EIMs have active colonic inflammation; although, they can occur prior to, or after, the onset of colonic symptoms^[20,26,33-35]. Early recognition of EIMs is important as they may characterize subclinical inflammation in IBD patients, with a possible increased risk of morbidity and mortality^[19,35,36].

The aim of this review was to evaluate the current literature about ocular EIMs, emphasizing inflammatory alterations that need prompt recognition to avoid irreversible visual impairment. Reference lists from the articles selected by electronic searching were manually reviewed to identify further relevant studies. Articles in each of these multiple searches were

reviewed, and those meeting the inclusion criteria, that is, publications providing data on the incidence, prevalence, clinical features and management of ophthalmologic manifestations in IBD, were recorded.

OCULAR INVOLVEMENT

The ocular system can be affected by several immune systemic diseases, including IBD^[29,37-42]. Crohn^[42] published the first report of ocular involvement in IBD in 1925, in which it was suggested that two patients he treated probably suffered from keratomalacia and xerophthalmia.

Since the first report, the main ocular findings have been related to inflammatory manifestations^[20,25,27,29,43-45] that occur in the early years after an IBD diagnosis^[2,15]. Although these findings can indicate disease activity^[15,18], the association with the gastrointestinal tract-affected area is not well established^[2,15]. It has been demonstrated that there is a greater tendency of ocular inflammation in CD patients, mainly with colitis or ileocolitis, and UC patients with pancolitis^[2,34,46]. Zippi *et al.*^[28] corroborate the literature data in their retrospective study in Italian IBD patients, demonstrating a significant association between ocular EIM and CD.

The prevalence of ophthalmic inflammatory disorders is variable, according to the population studied, ranging from 0.3% to 13.0% among all IBD patients^[14,20,25,27,29,34,45,47], 1.6%-5.4% among those with UC and 3.5%-6.8% among those with CD^[25,29]. Although a greater frequency of ocular involvement has been demonstrated in CD vs UC patients^[23,48], the results are controversial^[25,27].

Considering the risk factors for developing ocular manifestation in IBD, an association has been reported with female sex^[17,23,31], and the presence of arthritis or arthralgia in CD patients^[23,46]. A paradoxical positive association has been demonstrated between smoking and ocular manifestation in UC patients^[49], because it is well known that smoking exerts a protective effect against both the development and progression of UC^[50,51].

The physiopathology of ocular EIMs remains unclear^[2,25,29,52-54]. It has been suggested that local action of antigen-antibody complexes produced against the bowel wall vessels and transported *via* the bloodstream could be responsible for eye involvement^[18,19]. However, Santeford *et al.*^[52] suggested a disturbance in physiological macrophage-mediated autophagy as a potential molecular link between systemic disease and uveitis. Lin *et al.*^[55], in a large retrospective analysis, suggested that a family history of IBD itself may confer an independent, increased susceptibility to the development of ocular inflammation, despite the absence of bowel disease or of known genetic susceptibility (HLA-B27).

The most common ocular manifestations related

to IBD are episcleritis (2%-5%) and uveitis (0.5%-3.5%)^[15,17,29,32], as listed in Table 1.

Karmiris *et al.*^[56] performed an important study due to the large number of subjects evaluated. They retrospectively analyzed 1860 (1001 with CD and 859 with UC) Greek IBD patients' medical reports. Arthritic, mucocutaneous and ocular (3% of IBD patients; 8.9% of all EIM occurrences) were the most common types of manifestations. Ocular EIMs were more frequent in women (54.55%) and CD patients (81.82%), with the exception of posterior uveitis, which had a predominance in UC patients. The authors mentioned episcleritis as the most frequent manifestation, although 31 cases of anterior uveitis and 16 cases of episcleritis were found. Disease activity was evaluated clinically in 346 participants, according to the treating physician's assessment (presence of symptoms associated with elevated inflammatory markers, mainly C-reactive protein and erythrocyte sedimentation rate, despite appropriate treatment at the time of the EIM diagnosis). They found 225 (65%) active IBD and 121 (35%) quiescent cases. The relationships between ocular EIM and IBD activity and extent, or behavioral and smoking habits were not clearly mentioned in the study.

Similarly, Bandyopadhyay *et al.*^[27] reported in their study of 120 Indian IBD patients an association between general EIMs and female sex, Hindu religion, severe gastrointestinal disease and steroid usage, but did not mention specific associations with ocular EIMs. The frequency of ocular EIM reported was similar to that among American and European populations. Manser *et al.*^[57] detected uveitis in 15.7% of patients with extraintestinal complications and 12.3% of all 179 UC patients evaluated. They suggested that the introduction of early mesalazine therapy, up to 2 mo after UC diagnosis, could be a protective factor against the development of EIMs^[58].

Cloché *et al.*^[59] evaluated 74 of 305 IBD patients with ophthalmological symptoms. Only one patient presented with scleritis and they concluded that ocular symptoms were neither specific nor associated with ocular inflammation. A limitation of the study was that only symptomatic patients underwent examinations. No subclinical occurrence was investigated; thus, it is not possible to determine the actual occurrence of ocular manifestations in the total sample. Even evaluating only symptomatic patients, a frequency of ocular manifestation of 1.4% was found, lower than that found in the literature. A possible explanation is based on the large number of patients receiving biological agents, about 50%, which may have treated the IBD and prevented ocular inflammation^[60].

In an important prospective study, Felekis *et al.*^[61] performed complete eye examinations in 60 IBD patients, finding a high frequency of 43% of ocular EIMs. However, in some cases these findings could

Table 1 Studies evaluating ocular manifestations in inflammatory bowel disease patients

Ref.	Country	Study design	Ocular exam sample	Ocular manifestation frequency	Comment
Karmiris <i>et al</i> ^[56] (2016)	Greece	Prospective cohort	1860 (1001 CD; 859 UC)	55 (3%) (45 CD; 10 UC): 31 Anterior uveitis (25 CD; 6 UC); 16 Episcleritis (16 CD); 7 Posterior uveitis (3 DC; 4 UC); 1 Central serous retinopathy (CD)	Ocular EIMs represented the third most frequent group of EIM in the study All patients with episcleritis suffered from CD. There were patients with anterior and posterior uveitis
Manser <i>et al</i> ^[57] (2016)	Switzerland		140 UC patients with EIM or complications	22 (15.7%) Uveitis	Investigated prevalence of uveitis in patients with UC
Bandyopadhyay <i>et al</i> ^[27] (2015)	India		120 (62 CD; 58 UC)	16 (13%) (8 CD; 8 UC): 7 Uveitis (7 CD); 9 Episcleritis (1 CD; 8 UC)	Authors describe two cases of scleritis (2 CD) and one of endophthalmitis (CD) that were not accounted as ocular manifestations. Authors consider a selection bias, as most participants had severe intestinal disease
Isene <i>et al</i> ^[58] (2015)	Europe (Norway, Denmark, Netherlands, Spain, Italy, Greece, and Israel)	Prospective cohort	1145 (364 CD; 781 UC)	12 (1.0%) 10 (0.9%) Anterior uveitis; 2 (0.2%) Episcleritis	Authors concluded that familial IBD does not predict increased risk of immune-mediated EIM, as smoking does not seem to influence the risk
Zippi <i>et al</i> ^[28] (2014)	Italy	Retrospective	811 (216 CD; 595 UC)	26 Uveitis (3.2%) (16 CD; 10 UC)	It is not informed if other ocular manifestations have been investigated in addition to uveitis.
Cloch�� <i>et al</i> ^[59] (2013)	France		74 IBD (no underlying disease specification)	1 (1.4%): Scleritis	A large number of patients were receiving biological agents, approximately 50%, that may treat IBD and prevent ocular inflammation. Authors do not define the underlying IBD of the scleritis patient
Vavricka <i>et al</i> ^[22] (2011)	Switzerland	Prospective Cohort	950 (580 CD; 370 UC)	50 (5.3%) (36 CD; 14 UC): 50 Uveitis	Only uveitis was considered ocular EIM, and it was associated to active CD, but no relation was found to UC activity
Cury <i>et al</i> ^[60] (2010)	Brazil		88 (48 CD; 40 UC)	7 (6.25%) (no underlying disease specification): 1 Conjunctivitis; 3 Blepharitis; 1 Episcleritis; 2 Uveitis; 2 Cataracts	The study used a control group of 24. Considered also unspecific ocular abnormalities, as cataract and blepharitis
Felekis <i>et al</i> ^[61] (2009)	Greece	Prospective cohort	60 (23 CD; 37 UC)	26 (43%) (12 CD; 14 UC): 13 Dry eye; 8 Glucocorticoid-induced cataract; 3 Iridocyclitis; 3 Retinal pigment epithelium disturbances; 2 Episcleritis; 2 Serous retinal detachment; 1 Conjunctivitis; 1 Choroiditis; 1 Vasculitis; 1 Optic neuritis	The study used a control group of 276. Authors conclude that ocular manifestations occur in UC patients as frequently as in CD patients; however, the results of the statistical analysis are not mentioned for any of the study variables
Lanna <i>et al</i> ^[25] (2008)	Brazil		96 (59 CD; 37 UC)	6 (6.2%) (4 CD; 2 UC): 4 Uveitis (2 bilateral; 2 CD; 2 UC); 1 Scleritis (CD); 1 Episcleritis (CD)	It was not possible to analyze the association between the HLA-B27 and ocular abnormalities because only 3 of the 6 patients had been tested for HLA-B27; all of them were negative for this antigen
Yilmaz <i>et al</i> ^[35] (2007)	Turkey	Prospective cohort	116 (20 CD; 96 UC)	28 (24.13%) (12 CD; 22 UC): 10 Conjunctivitis; 8 Blepharitis; 6 Uveitis; 6 Cataracts; 4 Episcleritis	Study considered unspecific ocular abnormalities, as cataract and blepharitis, which are very frequent in the general population

Mendoza <i>et al</i> ^[29] (2005)	Spain	Prospective cohort	566 (295 CD; 271 UC)	13 (2.3%) (6 CD; 7 UC); 8 Uveitis (2 CD; 6 UC); 5 Episcleritis (4 CD; 1 UC)	In 2 patients the ophthalmologic clinical presentation preceded the diagnosis of IBD, but its frequency is probably undervalued considering the high prevalence of asymptomatic uveitis
Ricart <i>et al</i> ^[47] (2004)	United States		243 IBD [47 familial IBD (25 CD; 22 UC); 196 sporadic IBD (114 CD; 82 UC)]	Familial IBD: 3 (2 CD; 1 UC) Sporadic IBD: 10 (7 CD; 3 UC) Authors don't specify which ocular EIM was found	Significant association between EIM and disease status (familial <i>vs</i> sporadic) was not detected. This suggests that susceptibility genes for the development of IBD and the susceptibility genes for the development of EIM are different
Lakatos <i>et al</i> ^[2] (2003)	Hungary	Prospective cohort	873 (254 CD; 619 UC)	28 (3.2%) (8 CD; 20 UC); 13 Conjunctivitis (4 CD; 9 UC), 10 Anterior uveitis (4 CD; 6 UC); 5 Scleritis (1 CD; 4 UC); 1 Orbital pseudotumor (female UC patient)	The prevalence was more frequent in women in both UC and CD. In UC more than half of the patients with ocular complication had pancolitis
Christodoulou <i>et al</i> ^[62] (2002)	Greece	Retrospective	248 (37 CD; 215 UC)	4 (1.61%) (1 CD; 3 UC); 4 Iridocyclitis	Evaluated only iridocyclitis as ocular EIM

CD: Crohn's disease; EIM: Extraintestinal manifestation; IBD: Inflammatory bowel disease; UC: Ulcerative colitis.

have been coincidental and not related to IBD, such as dry eye and blepharitis. According to the methodology, individuals with ocular symptoms were excluded from the control group, and half of the sample of IBD patients was selected during hospitalization (severe disease activity), suggesting selection bias. Even so, the article presents some ocular findings that are infrequent in the literature, because the subjects underwent complementary fundus examinations with fluorescein angiography, increasing the importance of the study.

Lanna *et al*^[25] performed eye examinations in 96 of 130 IBD patients. Six patients (four with CD, two with UC) presented ocular manifestations. Uveitis was diagnosed in four patients, anterior nodular scleritis in a woman with CD, and episcleritis in a man with CD with recurrent peripheral arthritis and psoriasis. One of the two men with anterior uveitis also had ankylosing spondylitis.

Yilmaz *et al*^[35] and Cury *et al*^[60] considered all ocular findings as EIMs, including conjunctivitis, blepharitis, and cataracts. Some entities have a high prevalence in the general population, regardless of the presence of intestinal disease, what can be considered a confounding factor in the analysis. It is difficult to establish any relationship between these occurrences and IBD because they may be related to other factors, such as age and other underlying factors.

Cury *et al*^[60] described a correlation between dry eye and the use of 5-aminosalicylates in IBD patients. Apparently, dry eye disease may be associated with IBD and also may be related to its treatment. Blepharitis was less common in IBD patients than controls (3% *vs* 33%)^[60], suggesting a protective action of the drug used in IBD treatment.

Episcleritis

Episcleritis is a benign inflammation of the episclera, the thin blood-rich layer of tissue that covers the sclera. It is the most common ocular manifestation and causes moderate discomfort, acute redness in one or both eyes^[34], and diffuse or localized episcleral edema, particularly surrounding the episcleral vessels^[40]. Its classification as nodular or diffuse does not affect the prognosis^[62-64].

Episcleritis seems to be more associated with IBD activity when compared with other ocular EIMs. It appears during flares of IBD, and its resolution occurs with effective treatment of the intestinal disease^[20,32,41,46,54]. It is usually recurrent and can spread to the sclera, causing scleritis^[40,43]. To an untrained observer and without a slit lamp examination, it is not easy to distinguish the two entities^[65]. Differentiation from conjunctivitis, which is a frequent condition in the general population and may occur coincidentally in patients with IBD, may also be difficult^[43,66,67].

The differential diagnosis between episcleritis, uveitis, and scleritis is based on the absence of moderate-to-severe eye pain, photophobia, blurring, and low vision in the former^[33]. Episcleral injection blanches with topical application of phenylephrine and softens with palpation^[66]. As a benign condition, specific treatment is not always necessary; however, cool compresses, lubricant eye drops, topical non-steroidal anti-inflammatory drugs, and topical corticosteroids are occasionally required^[20,33,34,54,66]. Figure 1 illustrates diffuse episcleritis.

Scleritis

Scleritis is an inflammation of the sclera, the opaque and protective outer layer of the eye, that causes

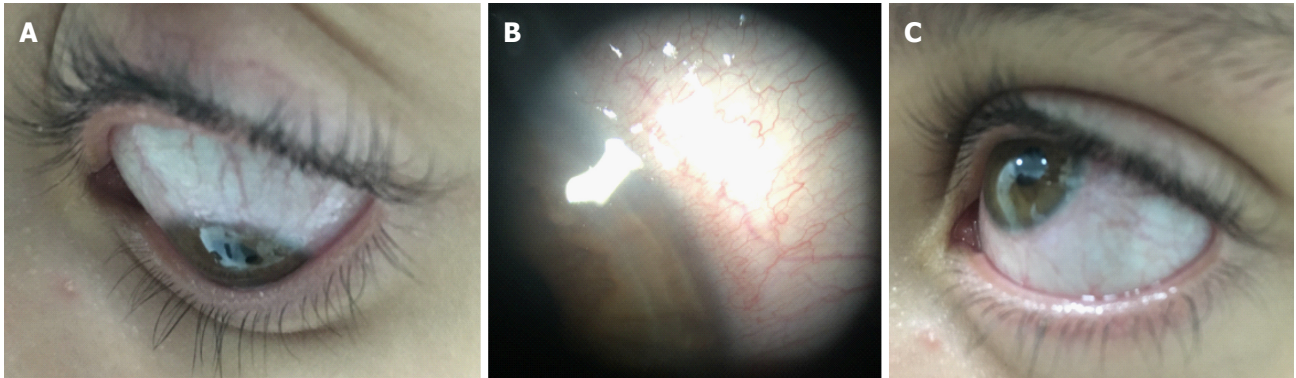


Figure 1 Diffuse episcleritis. A: Superior view; B: Episcleral injection at slit lamp exam; C: Inferior view. Personal archive.

Table 2 Uveitis classification

SUN classification	Primary site of inflammation	Manifestation
Anterior uveitis	Anterior chamber	Iritis, iridocyclitis, anterior cyclitis
Intermediate uveitis	Vitreous	Pars planitis, posterior cyclitis, hyalitis
Posterior uveitis	Retina or choroid	Focal, multifocal or diffuse choroiditis, chorioretinitis, retinochoroiditis, retinitis, neuroretinitis
Panuveitis	Anterior chamber, vitreous, and retina or choroid	

Adapted from Standardization of Uveitis Nomenclature Working Group^[70]. SUN: Standardization of Uveitis Nomenclature Working Group.

ocular pain, which radiates to the face and scalp. Characteristically, it worsens at night, and is associated with ocular hyperemia and visual loss^[34,40]. It can present with a deep scleral injection that does not blanch with phenylephrine^[66].

Scleritis and intermediate or posterior uveitis are much rarer than episcleritis and anterior uveitis in IBD, occurring in less than 1% of cases, but should be evaluated with caution because, if left untreated, it may progress to permanent visual loss^[33]. Scleritis classification is important because it is related to severity and prognosis. Watson and Hayreh^[63] classified scleritis as anterior (diffuse, nodular, or necrotizing, with or without inflammation) and posterior. Involvement of the anterior part of the sclera is more common and posterior scleritis is not associated with ocular hyperemia. A modified classification of scleritis was proposed by Watson *et al.*^[64] (Figure 2) in accordance with location (anterior or posterior), and clinical presentation (diffuse, nodular, or necrotizing). The necrotizing anterior scleritis was classified according to its etiology, as vaso-occlusive, granulomatous, surgically induced, and scleromalacia perforans. Figure 3 illustrates the different types of scleritis.

Systemic treatment is necessary in all cases, usually with oral non-steroidal anti-inflammatory drugs

but they should be used with great caution in active IBD^[68]. Systemic steroids or immunosuppressants may be necessary in severe cases, and control of the underlying bowel disease is important to prevent recurrence^[34,68]. To avoid side effects of long-standing corticosteroids use, immunosuppressive therapy is required^[40], which will be discussed regarding uveitis treatment.

Uveitis

Uveitis is the third leading cause of irreversible blindness in developed countries^[37,52,69]. It is defined as inflammation of the uveal tract, the middle layer of the eye, which includes the iris, ciliary body, and choroid^[70]. It is classified according to the primary site of inflammation as anterior, intermediate, posterior, or panuveitis^[70] (Table 2).

Uveitis is characterized by vascular dilation, leading to conjunctival injection, aqueous flare related to increased vascular permeability, and aqueous and vitreous inflammatory cells^[69]. Uveitis can be idiopathic^[38,71], drug-related^[72,73], or systemic disease-related^[37,38,74]; in approximately 50% of cases, an underlying disease can be identified^[38]. Anterior uveitis is the most common pattern, related to seronegative spondyloarthropathies^[40,55,74]. Figure 4 shows the clinical signs of anterior uveitis.

In IBD patients, anterior uveitis has an insidious onset, it is longstanding and bilateral^[25,33,46,75], and not related to the intestinal disease activity^[32,54,68,76]. In contrast, Vavricka *et al.*^[22], after prospectively evaluating a large sample of IBD patients, demonstrated an association between uveitis and CD activity, but not with UC.

A clinical overlap of anterior uveitis, dermatologic manifestations (erythema nodosum)^[43], and musculoskeletal symptoms (arthritis and sacroileitis)^[18,75,77] in CD was reported. It was proposed that a common antigen (an isoform of tropomyosin) in the non-pigmented ciliary epithelium of the eye, the keratinocytes, chondrocytes and the gut triggered an autoimmune reaction^[22,23]. Thus, in IBD patients with eye complaints and others EIMs, the presence of uveitis

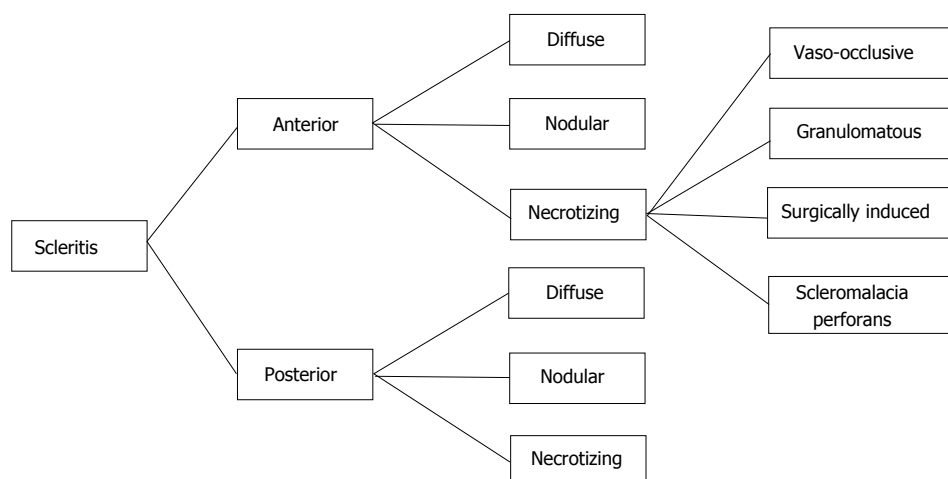


Figure 2 Classification of scleritis^[64].

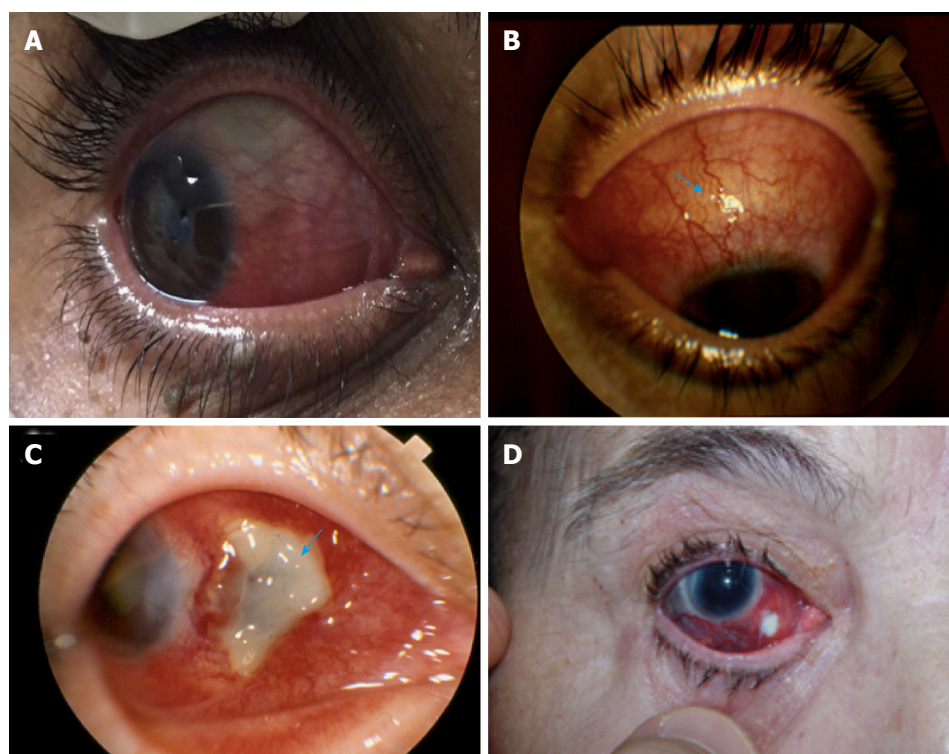


Figure 3 Clinical presentation of scleritis. A: Anterior diffuse scleritis (personal archive); B: Anterior nodular scleritis (personal archive). The differential diagnosis is based on the presence of a sclera nodule (arrow); C: Anterior necrotizing scleritis, showing the avascular area of necrosis (arrow) (personal archive); D: Anterior necrotizing surgically-induced scleritis, induced by scleral biopsy (courtesy of Prof. Andre Curi).

must be considered^[34,68]. Because uveitis has a variable chronicity and severity, it may be complicated, according to its primary site of inflammation, by cataracts, glaucoma, band keratopathy, hyphema, vitreous hemorrhage, cystoid macular edema, retinal detachment, retinal ischemia, optic atrophy, chronic eye pain and blindness^[69].

Treatment

Prompt treatment can avoid complications and visual impairment^[38,40,76]. Treatment of anterior uveitis is based on topical steroids, to reduce inflammation,

and topical cycloplegics, to prevent ciliary body and pupillary spasms related to ocular pain. Also, cycloplegics prevent posterior synechiae because they dilate the pupil and stabilize the blood-aqueous barrier, avoiding protein leakage (flare)^[33,34,68]. According to the gravity of uveitis, periocular corticosteroid injections or systemic corticosteroids may also be necessary^[37,53]. Uveitis with a chronic course requires immunosuppressive therapy to spare the prolonged use of corticosteroids and their side effects^[38,53]. However, the choice of immunosuppressive therapy requires a multidisciplinary decision, especially if there

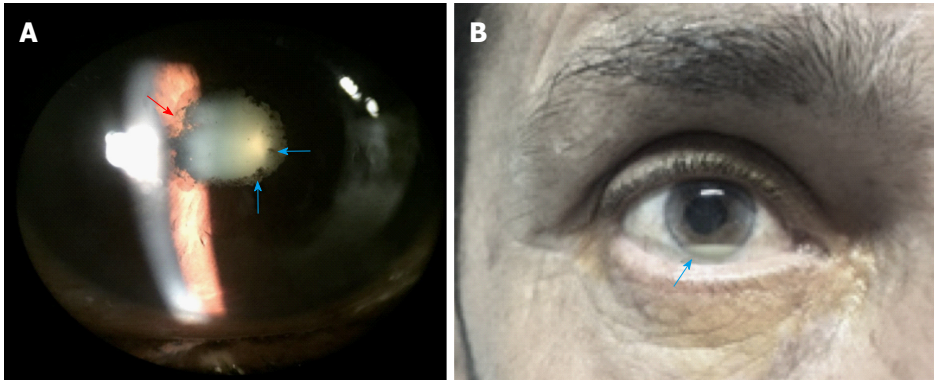


Figure 4 Anterior uveitis. A: Slit lamp exam revealed posterior synechiae (red arrow) and pigment deposits on the anterior lens capsule (blue arrow) (personal archive); B: Inflammatory cells in the anterior chamber of the eye causing hypopyon (arrow) (personal archive).

is another associated EIM^[20].

Cyclosporine, a T-cell inhibitor^[24,32,41], thiopurines (antimetabolites)^[33,41,66,78], methotrexate^[33,41,79], sulfasalazine (5-ASA derivate)^[68,80], and biological anti-tumor necrosis factor (TNF) agents (mainly infliximab and adalimumab)^[33,81] are effective in treating both the IBD and the inflammatory IBD-related ocular impairment^[6,34,44,53,82-85]. Although vedolizumab and certolizumab pegol have been introduced more recently in the therapy of CD^[8,82,83], their efficacy in ocular inflammation is unknown. Despite the fact that the anti-metabolite mycophenolate can be used to treat uveitis^[68,78,86,87], it is not indicated as an IBD treatment^[81].

Patient awareness of EIMs is important in improving patient understanding of their disease and health outcomes^[88]. It also increases the likelihood of early diagnosis, contributing to success of the applied treatment.

Other ophthalmic manifestations

Other ophthalmic manifestations have been described in relation to IBD. Table 3 presents observational case reports, interventional case reports, and case series describing other ocular disorders in IBD patients. Some of these manifestations can be debilitating if not recognized and treated at an early stage.

Ocular complications

Ocular impairment may be related to IBD, drug therapy or to other factors, such as age, genetics and other concomitant diseases^[34,38]. Cataracts and open-angle glaucoma are complications of long-standing ocular inflammation or the prolonged use of corticosteroids^[34,37,41,43]. Some ocular manifestations have been related to drugs used in the treatment of IBD, such as corneal immune infiltrates^[121] and diffuse retinopathy^[122] related to adalimumab, anterior optic neuropathy^[123] and retinal vein thrombosis^[124,125] developing after infliximab, and cyclosporine, used in CD, causing rare optic neuropathy^[66]. Levels of methotrexate in tears approximate serum levels after

short-term use, which may lead to irritation of the conjunctiva, cornea, and eyelids^[67].

Uveitis has been associated with the use of biological anti-TNF drugs. It has been described in association with etanercept, infliximab, adalimumab, and rifabutin^[72,73]. Inflammation declines with drug withdrawal, which is recommended, and the use of topical corticosteroids may be necessary to complete the remission of the inflammatory condition^[38,72]. Furthermore, neurological side effects from drug therapy can cause visual impairment without directly affecting the eyes^[126].

Katsanos *et al*^[48] performed a review of orbital and optic nerve involvement in IBD. It was found that optic nerve impairment can occur as a result of damage of the optic nerve tissue *per se*, as a result of inflammation and/or ischemia, due to intracranial hypertension, and secondary to anti-TNF agents. In some cases, it was difficult to determine the exact cause of ocular involvement in IBD.

After bowel resection in the IBD context, short bowel and malabsorption syndromes can lead to vitamin A deficiency, which may result in night blindness (nyctalopia) and keratoconjunctivitis sicca^[127,128]. Vomiting and unilateral painful red eye lead to a suspicion of acute angle closure glaucoma^[67], a threatening ophthalmological urgency that has not been described in IBD but which may confound the clinician.

Finally, an association between the use of latanoprost eye drops for glaucoma treatment and IBD relapse has been reported^[129]. It was concluded that the systemic absorption of the prostaglandin analog could have caused an increase in intestinal inflammation in IBD patients.

CONCLUSION

Physicians must remember that ocular involvement is more prevalent in CD and in active IBD, primarily in the presence of others EIMs. The ophthalmic symptoms in IBD are mainly non-specific and their relevance may not be recognized by the clinician. Moreover,

Table 3 Case reports and case series of other ocular manifestations associated with inflammatory bowel disease

Ref.	Country	Ocular impairment	IBD
Hwang <i>et al</i> ^[89] (2001)	Canada	Dacryoadenitis	CD
Mochizuki <i>et al</i> ^[90] (2010)	Japan		UC
Boukouvala <i>et al</i> ^[91] (2012)	United Kingdom		CD
Jakobiec <i>et al</i> ^[92] (2014)	United States		2 CD
Ruiz Serrato <i>et al</i> ^[15] (2013)	Spain	Palpebral ptosis	CD
Diaz-Valle <i>et al</i> ^[93] (2004)	Spain	Lid margin ulcers	CD
Leibovitch <i>et al</i> ^[94] (2005)	Australia	Pyodermatitis-pyostomatitis vegetans of eyelids	UC
Garrity <i>et al</i> ^[95] (2004)	United States	Orbital myositis	2 CD
Verma <i>et al</i> ^[96] (2013)	Canada		CD
Foroozan <i>et al</i> ^[97] (2003)	United States	Ocular miasthenia graves	UC
Pham <i>et al</i> ^[31] (2011)	United States	Peripheral ulcerative keratitis	3 CD
Roszkowska <i>et al</i> ^[98] (2013)	Italy	Salzmann nodular corneal degeneration	CD
Zullow <i>et al</i> ^[99] (2017)	United States	Central serous	UC
Geyshis <i>et al</i> ^[100] (2013)	Israel	chorioretinopathy	UC
Assadsangabi <i>et al</i> ^[101] (2010)	United Kingdom		CD
Ugarte <i>et al</i> ^[102] (2002)	United Kingdom	Serpiginous chorioretinopathy	CD
Casalino <i>et al</i> ^[103] (2014)	Italy	Choroidal	CD
Thomas <i>et al</i> ^[104] (2014)	United States	neovascularization	CD
Unal <i>et al</i> ^[105] (2008)	Turkey		CD
Saatci <i>et al</i> ^[106] (2002)	Turkey	Retinal vasculitis	CD
Larsson <i>et al</i> ^[107] (2000)	Sweden	Retinal vein occlusion	1 CD, 1 UC
Buchman <i>et al</i> ^[108] (2006)	United States		UC
Unal <i>et al</i> ^[105] (2008)	Turkey		CD
Yamane <i>et al</i> ^[109] (2007)	Brazil		CD
Vayalambone <i>et al</i> ^[110] (2011)	United Kingdom		UC
Falavarjani <i>et al</i> ^[111] (2012)	Iran	Retinal artery	CD
Abdul-Rahman <i>et al</i> ^[112] (2010)	New Zealand	occlusion	CD
Saatci <i>et al</i> ^[106] (2002)	Turkey		CD
Siqueira <i>et al</i> ^[113] (2016)	Brazil		CD
Saatci <i>et al</i> ^[106] (2002)	Turkey	Retinal neovascularization	CD
Fuentes-Páez <i>et al</i> ^[114] (2007)	Spain	Subretinal fibrosis and uveitis syndrome	UC
Munk <i>et al</i> ^[115] (2016)	United States	Acute macular neuroretinopathy	UC
McClelland <i>et al</i> ^[116] (2012)	United States	Optic perineuritis	CD
Felekis <i>et al</i> ^[117] (2010)	Greece	Anterior ischemic optic neuropathy	CD
Mason <i>et al</i> ^[118] (2002)	United States	Macular edema	CD
De Franceschi <i>et al</i> ^[119] (2000)	Italy	Dystrophy of the retinal pigment epithelium	CD
Villain <i>et al</i> ^[120] (2002)	France	Pseudotumor cerebri	CD

CD: Crohn's disease; IBD: Inflammatory bowel disease; UC: Ulcerative colitis.

asymptomatic inflammation of ocular tissues may occur, so a routine ophthalmic follow-up is recommended in all IBD patients (with or without ocular symptoms), mainly before changes in IBD therapy, because some

drugs may cause ocular adverse effects. It is important to remember that most ophthalmic manifestations are treatable without sequel if recognized promptly.

Ophthalmologists must consider that ophthalmic manifestations of IBD may precede the systemic disease, and systematic anamnesis must be done in chronic uveitis of unknown etiology. Patients with chronic or recurrent use of systemic corticosteroids should be warned of the risk of cataracts and glaucoma. A collaborative clinical care team for management of IBD that includes ophthalmologists is central for improvement of the quality care for these patients, and is also cost-effective.

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Laparoscopic appendectomy for acute appendicitis: How to discourage surgeons using inadequate therapy

Tomohide Hori, Takafumi Machimoto, Yoshio Kadokawa, Toshiyuki Hata, Tatsuo Ito, Shigeru Kato, Daiki Yasukawa, Yuki Aisu, Yusuke Kimura, Maho Sasaki, Yuichi Takamatsu, Taku Kitano, Shigeo Hisamori, Tsunehiro Yoshimura

Tomohide Hori, Takafumi Machimoto, Yoshio Kadokawa, Toshiyuki Hata, Tatsuo Ito, Shigeru Kato, Daiki Yasukawa, Yuki Aisu, Yusuke Kimura, Maho Sasaki, Yuichi Takamatsu, Taku Kitano, Shigeo Hisamori, Tsunehiro Yoshimura, Department of Gastrointestinal Surgery, Tenriyodousoudanjyo Hospital, Tenri, Nara 632-8552, Japan

Author contributions: Hori T drew all of the schemas, collected the data, and wrote this review; Machimoto T, Kadokawa Y, Hata T, Ito T, Kato S, Yasukawa D, Aisu Y, Kimura Y, Sasaki M, Takamatsu Y, Kitano T and Hisamori S provided academic opinions on the review and helped to assess important papers; Yoshimura T supervised this review.

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Correspondence to: Tomohide Hori, PhD, MD, FACS, Department of Gastrointestinal Surgery, Tenriyodousoudanjyo Hospital, 200 Mishima-cho, Tenri, Nara 632-8552, Japan. horitomo@tenriyoro.jp
Telephone: +81-743-635611
Fax: +81-743-631530

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Abstract

Acute appendicitis (AA) develops in a progressive and irreversible manner, even if the clinical course of AA can be temporarily modified by intentional medications. Reliable and real-time diagnosis of AA can be made based on findings of the white blood cell count and enhanced computed tomography. Emergent laparoscopic appendectomy (LA) is considered as the first therapeutic choice for AA. Interval/delayed appendectomy at 6-12 wk after disease onset is considered as unsafe with a high recurrent rate during the waiting time. However, this technique may have some advantages for avoiding unnecessary extended resection in patients with an appendiceal mass. Non-operative management of AA may be tolerated only in children. Postoperative complications increase according to the patient's factors, and temporal avoidance of emergent general anesthesia may be beneficial for high-risk patients. The surgeon's skill and cooperation of the hospital are important for successful LA. Delaying appendectomy for less than 24 h from diagnosis is safe. Additionally, a semi-elective manner (*i.e.*, LA within 24 h after onset of symptoms) may be paradoxically acceptable, according to the factors of the patient, physician, and institution. Prompt LA is mandatory for AA. Fortunately, the Japanese government uses a universal health insurance system, which covers LA.

Key words: Laparoscopic appendectomy; Acute appendicitis; Interval appendectomy; Surgery; Delayed appendectomy

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Core tip: Acute appendicitis develops in a progressive and irreversible manner, and emergent laparoscopic appendectomy (LA) is mandatory. The Japanese government uses a universal health insurance system. Any physician and institution can routinely perform expensive emergent LA in Japan, in accordance with medical ethics. Unsafe, but cost-effective, treatments such as interval/delayed appendectomy and conservative management only are unsuitable in Japan. Time-honored practices, (*i.e.*, emergent LA) should be respected in Japan.

Hori T, Machimoto T, Kadokawa Y, Hata T, Ito T, Kato S, Yasukawa D, Aisu Y, Kimura Y, Sasaki M, Takamatsu Y, Kitano T, Hisamori S, Yoshimura T. Laparoscopic appendectomy for acute appendicitis: How to discourage surgeons using inadequate therapy. *World J Gastroenterol* 2017; 23(32): 5849-5859 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i32/5849.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i32.5849>

INTRODUCTION

Acute appendicitis (AA) is a clinical diagnosis^[1,2]. The first appendectomy was performed in New York in 1886^[1], and thereafter, appendectomy was considered the most common emergency surgery^[2-4]. Prompt appendectomy has long been the standard of care for AA because of the risk of progression to advanced pathophysiology^[3].

Currently, laparoscopic appendectomy (LA) is available as the first therapeutic choice for AA^[1,3]. However, surgeons on call when a patient is admitted is an important factor for determining whether a patient can receive an advantageous LA or conventional open surgery^[5]. Additionally, a poor clinical course in a hospital with an unfavorable combination for emergent laparoscopic surgery is a critical matter^[3,6]. In brief, factors of hospitals and physicians affect the severity of AA^[3,5-7].

A 24-h surgical shift in a quadratic/tertiary care hospital is stressful for surgeons^[8]. Emergent surgeries during overtime service and off-days result in higher rates of morbidity and mortality^[9,10]. However, AA indicates a surgical emergency.

AA may be managed in an elective manner once antibiotic therapy is initiated^[2,11-14]. Initial non-operative management followed by interval and/or delayed appendectomy for AA has been challenged, especially in pediatric patients^[3]. However, the necessity and validity of an interval/delayed appendectomy is still controversial in adult patients^[2,3,11-18], though some researchers believed that interval/delayed

appendectomy has some advantages only for treatment of appendiceal mass^[12,14].

We review major controversy in management of AA based on previous studies, and discuss what practice is the best option for patients with AA.

PATHOPHYSIOLOGY

The exact mechanism of AA is still unclear, but is believed to be multifactorial. Inadequate dietary fiber, familial factors, and luminal obstruction from fecalith impaction or lymphoid hyperplasia, and other processes, such as parasitic infestation, may be involved^[19-22]. Luminal obstruction by external (*i.e.*, lymphoid hyperplasia) or internal (*i.e.*, inspissated fecal material and appendicoliths) compression plays a major pathophysiological role^[3]. This subsequently leads to increased mucus production, bacterial overgrowth, viral infection, and stasis, which increase appendiceal wall tension^[3,19-24]. Consequently, blood and lymph flow is diminished, and necrosis and perforation follow^[3]. Because these events occur over time, only an early surgical approach might prevent progression of disease^[3,4]. The immunological orchestra around the ileocecal portion is well developed and complicated, and the appendix has its own immunological features^[25-28]. Many major immunological and cellular function-associated gene sets involved in the protective effect of AA followed by appendectomy in experimental colitis have been identified^[25].

Once disease is triggered, AA develops in a progressive and irreversible manner^[3,4,29]. Even a histologically normal appendix clearly shows evidence of inflammatory responses against AA, as shown by cytokine production/expression^[29].

An appendiceal mass (tumor formation after perforating AA) is the end result of a walled-off appendiceal perforation^[2,16]. Pathologically, this mass may range from phlegmon to abscess^[16]. A pus-containing mass is an inflammatory tumor consisting of an inflamed appendix, its adjacent viscera, and the greater omentum^[16].

In AA, tumor necrosis factor- α is at the top of the pathway^[29,30], and interferon- γ and interleukin-6 play an important role^[30,31]. Fas-mediated induction of apoptosis is a major factor in selection of lymphocytes and downregulation of immunological processes, and endothelial Fas-ligand expression is elevated in AA^[26]. AA develops in a progressive and irreversible pathway^[2,16], even if the clinical course of AA can be temporarily modified by intentional medications^[32].

DIAGNOSTIC VALUE

Physical findings of AA are well established^[33]. Many researchers, such as Charles McBurney, Niels Thorkild Rovsing, Jacob Moritz Blumberg, Otto Lanz, Frederic

Treves, and others were involved in the initial study of AA^[1,34]. Most patients present early in the disease process^[2], although, in 2%-6% of patients, diagnosis is made when an appendiceal mass is discovered on preoperative imaging^[16,35]. Young female patients have the highest risk of being falsely diagnosed with AA and thus have unnecessary surgery^[36].

Computed tomography (CT) is more reliable for diagnosis than an ultrasound examination^[37,38], and enhanced CT should be routinely performed for suspected appendicitis^[39]. Enhanced CT scans have become the main diagnostic tool for patients with AA and have a high sensitivity and specificity^[39]. Briefly, enhanced CT is a powerful tool for a strict diagnosis and assessment of the degree of inflammation^[15,36,37,39-42], and enhanced CT is superior to a physician's clinical examination^[36,37,39,40]. A helical CT image study should be performed with contrast enhancement, even with lower doses^[37,43]. Routine CT for suspected appendicitis improves patients' care, shortens the duration to surgery, and reduces the use of hospital resources and overall admission costs^[39].

Laboratory data show that serum levels of the white blood cell (WBC), C-reactive protein (CRP), and interleukin-6 are related to AA^[44-46]. The most reliable marker is neither the neutrophil count nor CRP, but the WBC count^[1,44].

The WBC count and CT findings equally provide surgeons with complementary information in discerning the necessity for an urgent operation^[36,44,47]. With development of WBC measurement and enhanced CT imaging, the rate of negative appendectomy has decreased to as low as < 5%^[1,36,40,48].

HISTORY OF LA

LA was reported in 1983^[49]. Thereafter, some advantages of LA, such as less pain, fine cosmetics, shorter hospital stay, faster recovery, less wound infection, and lower cost, compared with conventional open surgery were shown from the 1990s^[50-63]. Postoperative complications are also lower in LA than in conventional open surgery^[56,64-66]. Therefore, LA has spread to become the standard surgery worldwide^[1,15,50,62,65-67]. Although the operative time, including buried sutures, may be longer in LA^[50-52,55], there are no significant differences in the rate of severe morbidity/mortality between open and laparoscopic appendectomies^[68]. Currently, natural orifice trans-luminal endoscopic surgery is also considered as safe and feasible^[69,70].

DURATION TO SURGERY

Many physicians have an interest in the duration from onset of symptoms to surgery. In adult patients with AA, the risk of developing advanced pathophysiology and postoperative complications increases with

time^[3,4]. However, there is the minority opinion that the duration from surgical admission to induction of anesthesia is not predictive in regression models for overall morbidity or serious morbidity/mortality^[71].

Interval/delayed appendectomy is considered as unsafe^[3], although the term of "interval appendectomy" can be used only in case of appendiceal mass and performed after 6-12 wk of the beginning of disease^[1,12,14]. Any delays in seeking medical help results in difficulty in controlling AA, and prompt appendectomy is mandatory^[3,4]. Interval/delayed appendectomy may not increase the risk of perforation and moderate/severe complications^[1,71,72], but is significantly associated with an increased risk of surgical site infection (SSI) in patients with nonperforated appendicitis and prolongation of the hospital stay^[71,73]. Prompt surgical intervention is warranted to avoid additional morbidity in this population^[73].

Transferred patients are less likely to be ruptured, primarily because they present earlier^[72]. Morbidity is not increased in patients who have appendectomy that is delayed for up to 24 h^[1]. Delaying an appendectomy for longer than 6 hours, but less than 24 h, from diagnosis is safe and does not lead to worse outcomes^[74]. This can help limit disruption to the schedules of the surgeon and the operating room^[74]. Paradoxically, AA that is approached in a semi-elective manner (*i.e.*, LA within 24 h after symptom onset) may be acceptable, according to the factors of physicians and hospitals^[1,3,5-7,74].

A physician's delay in avoiding negative appendectomy does not affect the stage of disease^[7]. A surgeon's decision to observe patients in hospital to clarify the diagnosis is justified^[7], as it does not adversely affect the outcome^[7].

SURGICAL PROCEDURES

Actual procedures of LA are shown in detail in Figures 1-3. Gastric and bladder catheters are placed only during surgeries for decompression to avoid unexpected injuries^[1].

Stump appendicitis is a critical result of an incomplete surgery^[75], and management of the base of the appendix during LA is important. Surgeons need to decide the best management of the base of the appendix; choices include a clip, ligate, or staple. A flexible endostaple has some advantages in application of LA for day surgery and extended resection to the cecum^[76-79]. However, an endostaple (Tri-staple camel 45 mm and iDrive; Medtronic, Minneapolis, MN, United States) may be excessive in quality and have a higher cost than a ligation tool (Endoloop; Ethicon, Cincinnati, OH, United States) or clip closure^[80]. If there is any concern about the stump, a couple of interrupted seromuscular sutures can be added, although the suture technique is technically demanding^[80].

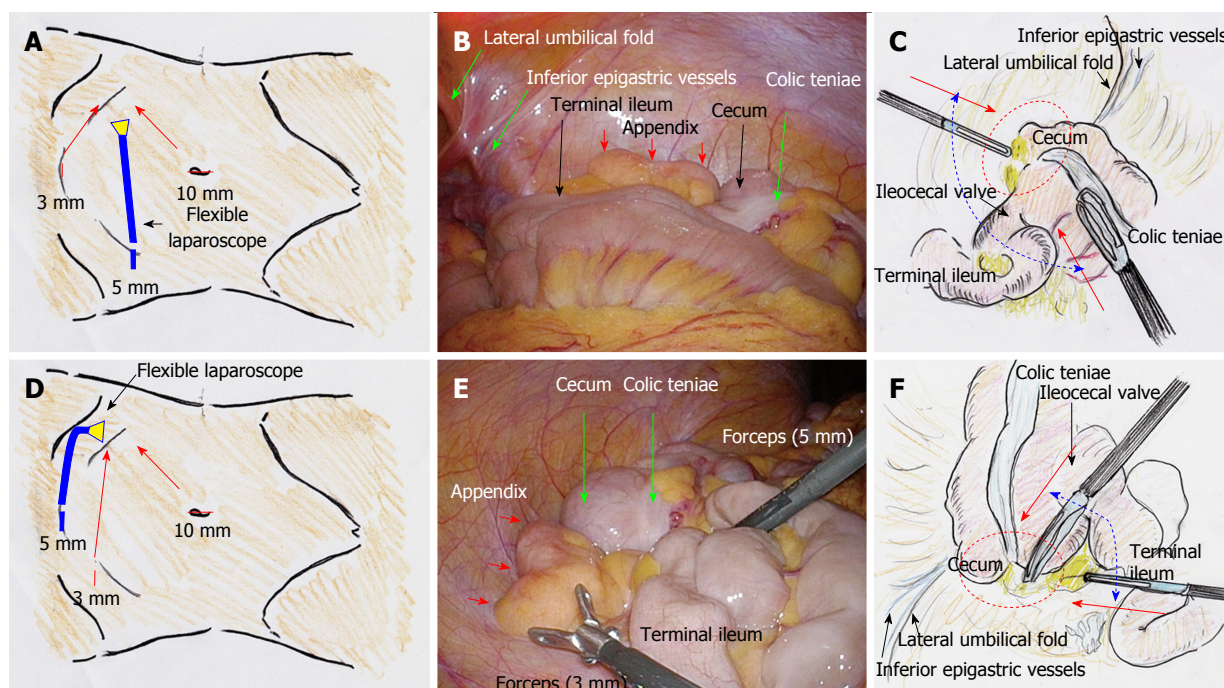


Figure 1 Port placement and laparoscopic view. A-C: If the left lateral port is set for laparoscope, a wider angle of working forceps can be made. However, a stab scar of 5 mm remains visible; D-F: Port placements for LA using an endostaple with the best cosmesis are shown. LA: Laparoscopic appendectomy.

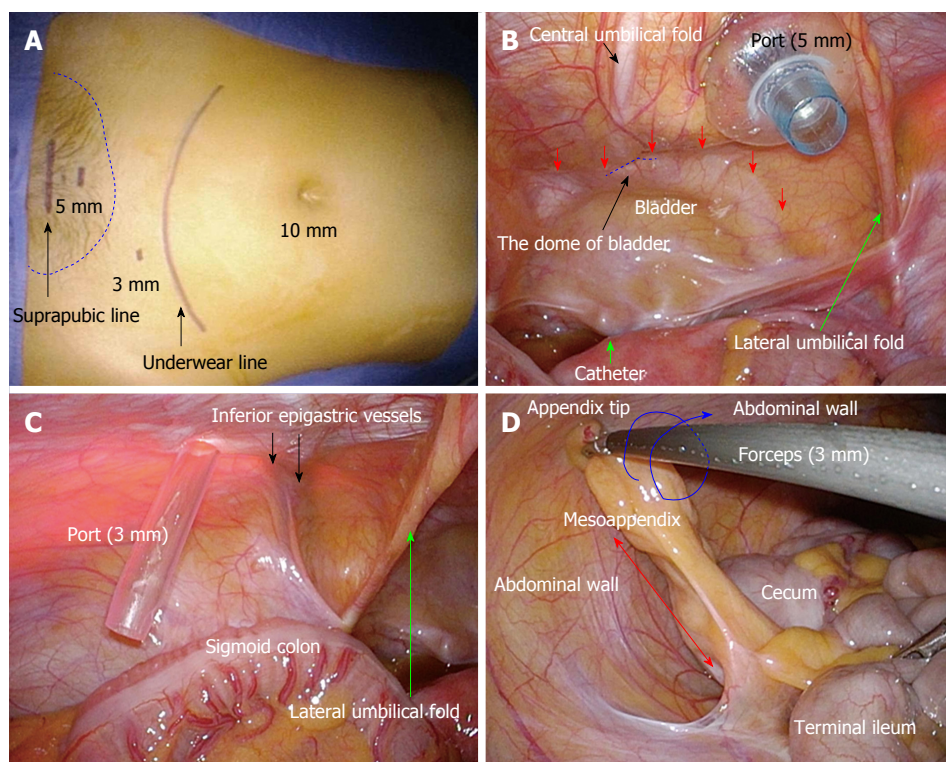


Figure 2 Major techniques during laparoscopic appendectomy. A: A suprapubic port (5 mm) for a flexible laparoscope is placed within the area of pubic hair (dotted blue line) to hide the postoperative stab scar. A left lateral port (3 mm) is placed as low as possible, to enable an adequate angle for the working forceps and to hide the postoperative stab scar by underwear; B: The bladder wall (red arrows), the dome of the bladder (dotted blue line), and the central umbilical fold should be recognized. Although the suprapubic peritoneum easily extends during port insertion, a suprapubic port should be placed without bladder injury; C: Any injury of the left inferior epigastric vessels should be avoided; D: Countertraction of the mesoappendix (red arrow) should be made without obstruction of the abdominal wall. Gripping and rotating forces of 3-mm forceps are sufficient. The appendix can be shortened in a rolled-in fashion (blue arrow) to avoid any disturbance by the abdominal wall.

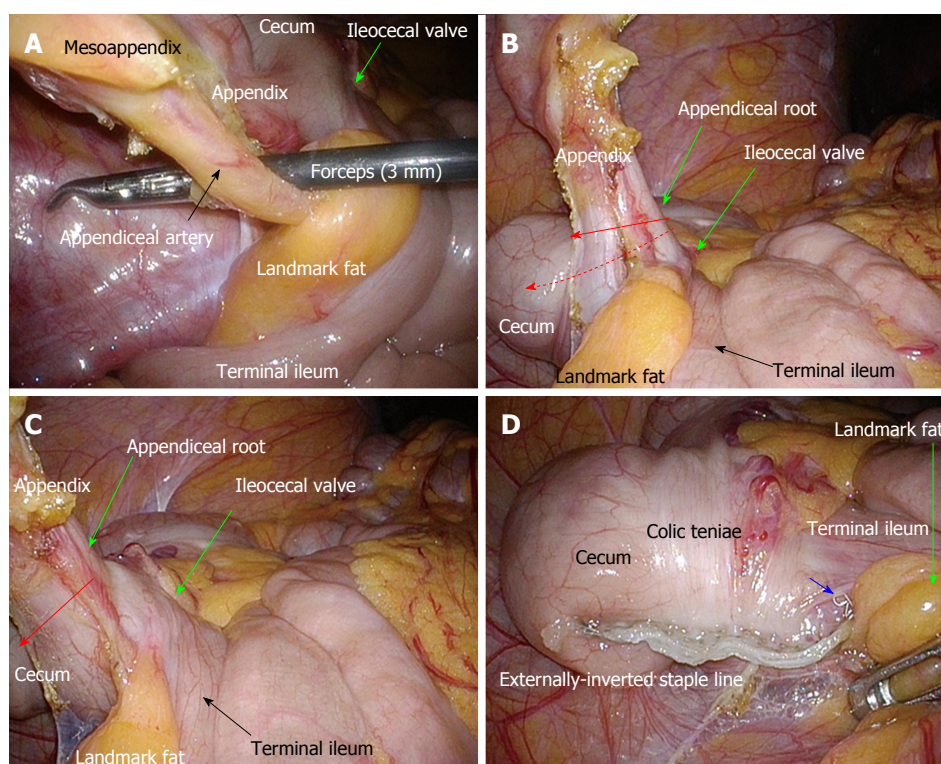


Figure 3 Key techniques during laparoscopic appendectomy. A: Appendiceal vessels should be clearly dissected and be sealed without a clip for subsequent use of an endostaple; B: Total resection of the appendiceal root should be made (red solid arrow). A flexible endostaple has an advantage in extended resection to the cecum (red dotted arrow); C: The ileocecal valve should be recognized before an endostaple is placed (red arrow). Any involvement of this valve should be avoided; D: The externally-inverted staple line should be carefully checked. If there is any concern about a stump, a couple of interrupted seromuscular sutures can be added. Unrelated and spilled staples (blue arrow) should be removed.

PERITONEAL LAVAGE IN PERFORATING AA

Surgeons should be aware of a potentially higher incidence of intraabdominal abscess formation following LA^[68,81,82]. Use of endobags (Rüsch MemoBag; Teleflex, Wayne, PA, United States), inversion of the appendiceal stump, and carefully conducted local irrigation of the abdomen in the supine position may reduce the incidence of abscess formation^[81]. Peritoneal lavage during surgery is an effective, safe, and simple treatment for generalized peritonitis^[83]. Irrigation of the abdominal cavity with more than 10 L of saline should be performed^[84,85], and a cut-off level of saline volume to prevent intraabdominal abscess formation after surgery is 12 L^[83]. Do not hesitate to place a drain. A drain pathway through the abdominal wall is adequately made at the right abdomen, to prevent a drain dislocation.

COSMESIS

Primary closure is currently accepted, even in complicated appendicitis with a dirty abdominal wound^[86-88]. However, delayed closure, which can occur several days after surgery, can lead to a decrease in SSI, a shorter hospital stay, and lower cost^[89,90]. LA has an

advantage of a lower rate of SSI, even in complicated appendicitis^[82,91-93].

STABILITY OF RESIDENTS ON LA

Video game playing, such as Nintendo Wii (Nintendo Co., Ltd., Kyoto, Japan) and Playstation 2 (Sony Interactive Entertainment Inc., Tokyo, Japan)^[94-101], and laparoscopic performance skill are well associated. Therefore, the younger generation may be suitable for performing laparoscopic surgery^[94-101]. LA performed by residents under the guidance of a staff surgeon is safe and feasible^[102,103]. Operative time and postoperative complications can be reduced with increasing experience of a resident^[104]. Incidental appendectomy during conventional open surgery is also important to educate young surgeons^[105].

INTERVAL/DELAYED APPENDECTOMY AND RECURRENCE DURING THE WAITING TIME

Some physicians consider that management of AA remains controversial^[2,42]. An appendiceal mass is a misery form of perforated AA^[2,16]. Initial conservative management of an appendiceal mass was first

advocated in 1901 as a solution^[106]. Interval/delayed appendectomy is performed electively after initial non-operative management^[2,11-14], but has been questioned by a growing amount of evidence^[2,11-18,107,108].

The recurrence rate of AA during the waiting time for interval/delayed appendectomy is 6%-37%^[13-15,109-114], and the complication rate of surgery for recurrent AA is also not low (3%-23%)^[11,107,109,115-117]. Advocates of interval/delayed appendectomy believe that the recurrence of AA is low, even though the actual rate is high, during the waiting period^[3,13-15,109-114]. Interval/delayed appendectomy is routinely performed at 6-12 wk, mainly because of fear of recurrent appendicitis or because of concerns about the presence of malignancy^[12,14,118,119].

Especially in a phlegmon or appendiceal mass, interval/delayed appendectomy may have some advantages. These advantages include providing a definite diagnosis, to rule out any underlying masquerading malignancy and to avoid an unnecessary extended resection^[12,14,108,118-121].

POSTOPERATIVE COMPLICATIONS

This review shows that the severity of pathophysiology and the complication rate in adult patients with AA are time-dependent^[3], and thus suggests that delaying appendectomy is unsafe^[3,4].

Mortality due to AA is difficult to observe^[3,76], and the mortality rate after appendectomy is nearly zero^[76]. However, the rates of morbidity and mortality are clearly increased in older patients, male patients, and patients with steroid use, baseline disease, active pneumonitis, and a bleeding tendency^[3,56,122]. Perioperative injection of antibiotics should be considered to reduce complications, including SSI^[1,123,124]. Postoperative complications are also lower in LA than in conventional open surgery^[56,64,65].

CONSERVATIVE TREATMENT ALONE

Notably, non-operative management has a cost advantage over routine interval/delayed appendectomy after initial successful conservative management^[2]. Patients who recover from conservative treatment of an appendiceal mass should undergo colonoscopy or barium enema to detect any underlying diseases and to rule out coexistent colorectal cancer^[12,14,108,118-121].

Laparoscopic surgery by experienced surgeons is a safe and feasible first-line treatment for appendiceal abscess^[32,67]. Additionally, laparoscopic surgery is associated with fewer readmissions and fewer additional interventions than conservative treatment with a comparable hospital stay^[67]. However, non-operative management is well tolerated and efficacious in select populations, especially in children^[125-127]. Some patients who initially receive conservative treatments do not require surgical intervention^[13,17,107,120,128], and AA should no longer be regarded as an indication for

interval/delayed appendectomy^[13,17,107,120,128,129]. Routine interval/delayed appendectomy benefits less than 20% of patients^[14]. The majority of recurrence occurs in the first 6 mo^[14,109-112], but the rate decreases to approximately 2% at 1 year^[107,112,118]. Importantly, AA develops in a progressive and irreversible pathway^[2,16], even if the clinical course of AA can be temporarily modified by intentional antibiotics^[32]. The length of hospital stay and postoperative complications increase with advanced pathology during antibiotic treatment^[3].

A gradual, adapted antibiotherapy in non-operative management of an appendiceal abscess and mass is effective^[130]. There is no relevant predictive factor of failure of first-line antibiotics^[130]. Monotherapy with a second-generation, broad-spectrum cephalosporin, such as cefotetan, administered twice a day, is an economical and effective adjunctive regimen^[38]. A third-generation cephalosporin can be used^[15], but is not recommended yet^[131].

SPECIFIC SITUATIONS

Some situations of patients are especially listed in surgical indications, such as older people, pregnancy, and negative appendectomy^[1,48,129]. Although LA in pregnant women has been already reported, fetal loss and negative appendectomy should be avoided in this population^[132,133]. The available low-grade evidence suggests that LA in pregnant women is associated with a greater risk of fetal loss^[132,134]. Appendectomy and early appendicitis are associated with increased pregnancy rates^[135]. Young women with early appendicitis have better pregnancy rates than those with advanced appendicitis. Early referral for laparoscopy and appendectomy is advocated^[135]. Appendiceal tumors may be incidentally detected^[136,137].

REASONABLE COST

The cost effectiveness of LA has been reported^[57]. Non-operative management without LA is the least costly^[138]. Non-operative management has a cost advantage over routine interval appendectomy after initial successful conservative management^[2].

Despite liberal use of disposable equipment, LA can still be performed within the confines of the national tariffs^[139]. There is considerable variation in the cost of this procedure, and it may be possible to reduce costs by more stringent use of disposable equipment and standardizing recovery protocols^[139].

DISCUSSION

Clinically, many surgeons believe that LA is an appropriate treatment^[76]. However, LA requires general anesthesia, although LA under combined spinal-epidural or local anesthesia is currently being attempted^[140,141]. LA in a semi-elective manner (within 24 h after onset of symptoms) may be beneficial for avoiding

uncomfortable situations for anesthetic induction, such as a full stomach and dehydration^[142,143]. However, even in a high-volume center, unfavorable combinations, such as low activity of the operation room during night time, affects the clinical course of AA^[6]. Overall, LA in a semi-elective manner may be acceptable.

Each country has its own health insurance system. The Japanese government uses a universal health insurance system. Therefore, expensive imaging studies and emergent surgery can easily be performed in Japan. However, expensive studies and therapeutic options may be uncertain in the United States and Europe^[144,145]. Novel procedures in Japan are not authorized until they are included in the health insurance system's listing by the governmental council^[146]. Paradoxically, if a surgery is once listed in Japanese health insurance system, any physician and institution can routinely perform it, even an expensive emergent surgery, in accordance with medical ethics. We have to consider how to dismantle interval/delayed appendectomy and antibiotics alone, in Japan.

Diagnostic methodology and subsequent management pathway have been already established^[147]. The LA under the hands or directions of experienced surgeons is safe and has a lot of beneficial advantages^[148,149]. High-volume centers should routinely perform emergent laparoscopic surgery including LA^[148]. Physicians and surgeons have a large interesting frontier.

CONCLUSION

AA is pathophysiologically progressive. As time passes, AA is difficult to control. Prompt LA in a quadratic/tertiary care hospital is mandatory for AA, and this time-honored practice should be respected. Interval/delayed appendectomy and conservative management are unsuitable in Japan. The question can be asked: "Where should emergency physicians or general surgeons head in the next decade?". We consider that it is important to focus on stabilizing prompt LA for AA around the world.

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Long non-coding RNAs in hepatocellular carcinoma: Potential roles and clinical implications

Zhao-Shan Niu, Xiao-Jun Niu, Wen-Hong Wang

Zhao-Shan Niu, Laboratory of Micromorphology, School of Basic Medicine, Medical Department of Qingdao University, Qingdao 266071, Shandong Province, China

Xiao-Jun Niu, Oncology Specialty, Medical Department of Qingdao University, Qingdao 266071, Shandong Province, China

Wen-Hong Wang, Department of Pathology, School of Basic Medicine, Medical Department of Qingdao University, Qingdao 266071, Shandong Province, China

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Correspondence to: Zhao-Shan Niu, MD, Laboratory of Micromorphology, School of Basic Medicine, Medical Department of Qingdao University, Room 201, Building Boya, 308 Ningxia Road, Qingdao 266071, Shandong Province, China. z.s.niu@qdu.edu.cn
Telephone: +86-532-83780012
Fax: +86-532-83780012

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Abstract

Long non-coding RNAs (lncRNAs) are a subgroup of non-coding RNA transcripts greater than 200 nucleotides in length with little or no protein-coding potential. Emerging evidence indicates that lncRNAs may play important regulatory roles in the pathogenesis and progression of human cancers, including hepatocellular carcinoma (HCC). Certain lncRNAs may be used as diagnostic or prognostic markers for HCC, a serious malignancy with increasing morbidity and high mortality rates worldwide. Therefore, elucidating the functional roles of lncRNAs in tumors can contribute to a better understanding of the molecular mechanisms of HCC and may help in developing novel therapeutic targets. In this review, we summarize the recent progress regarding the functional roles of lncRNAs in HCC and explore their clinical implications as diagnostic or prognostic biomarkers and molecular therapeutic targets for HCC.

Key words: Hepatocellular carcinoma; Long non-coding RNAs; Function; Biomarker; Therapeutic target

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Core tip: Emerging evidence indicates that long non-coding RNAs (lncRNAs) may play important regulatory roles in the pathogenesis and progression of human cancers, including hepatocellular carcinoma (HCC). Therefore, elucidating the functional roles of lncRNAs in tumors can contribute to a better understanding of the molecular mechanisms of HCC and may help in developing novel therapeutic targets. In this review, we summarize the recent progress regarding the functional

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INTRODUCTION

Hepatocellular carcinoma (HCC), a major type of primary liver cancer, is the second leading cause of cancer death worldwide^[1]. Unfortunately, the incidence and mortality rates of HCC have continued to increase globally. The high mortality of HCC patients is mainly due to late diagnosis, leading to limited therapeutic options. Accordingly, there is an urgent need to elucidate the molecular mechanisms involved in the initiation and progression of HCC to identify reliable biomarkers for early diagnosis and therapeutic targets to improve the survival of these patients. Recent data have demonstrated that the complexity of human carcinogenesis cannot be accounted for by genetic alterations alone and that epigenetic changes may also be involved^[2]. In fact, it is becoming increasingly evident that dysregulated epigenetic regulatory processes play a central role in cancer onset and progression^[3]. In human HCC, for example, epigenetic changes in various cancer-related genes are more frequently observed than genetic changes^[4], suggesting the crucial impact of epigenetic alterations in hepatocarcinogenesis.

Epigenetic alterations include changes in DNA methylation, histone modifications, and non-coding RNA-mediated gene silencing^[5]. Recent studies have revealed that the vast majority of the human genome is actively transcribed into non-coding RNAs (ncRNAs), only 1%-2% of which encode proteins^[6,7]. As most cancer studies to date have principally focused on protein-coding genes, the function of ncRNAs in cancer remains largely unknown. Nonetheless, accumulating evidence is shedding light on the functional importance of ncRNAs in cancer biology, and these molecules are emerging as new regulators of diverse biological functions, with important roles in oncogenesis and tumor progression^[8]. ncRNAs can be roughly classified into the following two groups based on length: small ncRNAs (< 30 nucleotides) and long ncRNAs (lncRNAs; > 200 nucleotides)^[9]. Small ncRNAs, especially microRNAs (miRNAs), have been studied extensively. In contrast, lncRNAs are the least studied transcripts and their functions remain largely unknown, even though they constitute the majority of ncRNAs.

lncRNAs were initially regarded as "transcriptional

noise" of the transcriptome. However, the recent application of next-generation sequencing, particularly RNA-sequencing (RNA-Seq), has broadened and deepened our knowledge of lncRNAs related to various types of diseases, including cancer. It is clear that lncRNAs act as critical regulators of multiple cellular processes, especially gene expression. It has been well documented that many lncRNAs are frequently aberrantly expressed in human cancers in which they may serve as oncogenes or tumor suppressors^[10-12], suggesting that they may act as novel drivers of tumorigenesis. Compared with protein-coding genes, lncRNA alterations are highly tumor- and cell line-specific^[13], and this characteristic of specificity makes lncRNAs promising biomarkers for diagnosis. Importantly, lncRNAs play critical regulatory roles in the pathogenesis and progression of cancers, including cell proliferation, differentiation, apoptosis, tumorigenesis, and progression^[14-17]. All of these findings point to lncRNAs as promising diagnostic or prognostic biomarkers and potential therapeutic targets for cancer.

Given the critical roles of lncRNAs in the initiation and progression of cancer, it is not surprising that lncRNAs have aroused considerable interest in HCC research. To date, multiple HCC-related lncRNAs have been identified. *In vitro* and *in vivo* functional experiments have shown that in HCC cells, lncRNAs are involved in the regulation of diverse biological processes, such as proliferation, migration, apoptosis, the cell cycle, tumorigenesis, and metastasis. Moreover, increasing evidence indicates that lncRNAs may play irreplaceable roles in the initiation and progression of HCC. As lncRNAs may serve as diagnostic or prognostic biomarkers and therapeutic targets for HCC, elucidating the roles of lncRNAs in tumors can contribute to a better understanding of the molecular mechanisms of HCC and assist in the development of novel therapeutic targets. In this review, we summarize the recent progress regarding the functions of lncRNAs in HCC and explore their clinical implications as diagnostic or prognostic biomarkers and molecular therapeutic targets.

CLASSIFICATION OF LNCRNAs

As they can be categorized according to their various properties, such as transcript length, genomic location and context, sequence and structure conservation, effects on DNA sequences, functional mechanisms and targeting mechanisms, association with protein-coding genes or subcellular structures, many different classifications of lncRNAs have been proposed^[18,19]. For example, according to their genomic location relative to neighboring protein-coding genes, lncRNAs have generally been categorized into five classes: sense, antisense, intronic, intergenic, and bidirectional lncRNAs^[20]. lncRNAs may also be classified according to their targeting mechanisms: signal, decoy, guide,

and scaffold^[21].

However, there has been no systematic and unambiguous classification of lncRNAs to date, and many existing lncRNA classifications are conflicting and overlapping. Different criteria (databases, projects, and methodologies) used to classify lncRNAs may be primarily responsible for the classification overlap. In reality, lncRNAs are not a homogeneous class of molecules but rather a mixture of multiple functional classes with distinct biological mechanisms and/or roles^[22]. Many lncRNAs are not easily classified into any particular category, and it is likely that the same lncRNAs may be listed in different groups in all classifications^[23,24]. In addition, the vast majority of lncRNAs remain functionally uncharacterized, which hampers their functional classification.

Given their complexity, from biogenesis to function, these overlapping and conflicting classifications would inevitably add another layer of difficulty to our understanding of lncRNA biology. Interestingly, the authors of a recent review highlight the roles of large systems biology-based datasets as conceptual guidelines for lncRNA classification and functional annotation^[19]. Specifically, advances in high-throughput transcriptome sequencing technologies will contribute to uncovering previously unknown functions of lncRNAs, and as such, the arbitrary classifications will need to be redefined.

SUBCELLULAR LOCALIZATION PATTERNS OF LNCRNAs

lncRNAs have diverse subcellular localization patterns, ranging from bright sub-nuclear foci to almost exclusive cytoplasmic localization; some lncRNAs are found in both compartments^[25,26], with the majority preferentially localized to the nucleus and chromatin^[20,27-29]. Importantly, it is becoming increasingly clear that the function of lncRNAs depends on their subcellular localization^[30]. In general, nuclear lncRNAs are recognized as important transcriptional and epigenetic modulators of nuclear functions^[15,31,32], whereas cytoplasmic lncRNAs have been described as modulating mRNA stability and translation^[32,33]. Compared with the mostly highly abundant cellular RNAs, the vast majority of lncRNAs that are typically less abundant in a population of cells can be highly abundant in individual cells^[25,34]. To more precisely locate and confirm the sub-cellular localization of lncRNAs, two recent reports have suggested that rather than using conventional RNA fluorescence *in situ* hybridization (FISH) techniques that have a relatively low sensitivity, it may be more effective to study lncRNAs by applying single-molecule RNA FISH^[25,35].

MECHANISMS OF LNCRNA-MEDIATED GENE EXPRESSION

To date, the biological functions and molecular

mechanisms of most lncRNAs remain largely elusive, with only very few being partially characterized. Nevertheless, existing evidence demonstrates that these molecules play critical roles in the regulation of specific cellular processes, specifically in protein-coding gene expression at the epigenetic, transcriptional and post-transcriptional levels^[36-40].

Epigenetic regulation

Epigenetic regulatory mechanisms can act at genomic (DNA methylation or demethylation) or nucleosomal and chromatin (post-translational histone modifications and chromatin remodeling complexes) levels^[41]. As stated above, the majority of lncRNAs localize preferentially to the nucleus and chromatin, and increasing evidence indicates that some nuclear lncRNAs epigenetically regulate gene expression by altering chromatin structure^[42]. There are two underlying mechanisms by which lncRNAs mediate changes in chromatin and gene expression. First, they can directly interact with chromatin-modifying enzymes, functioning as guides in *cis* or *trans* by recruiting chromatin modifiers to specific genomic loci to mediate DNA methylation or histone modification, thereby modulating chromatin states and impacting gene expression^[32,43-47]. Second, lncRNAs function as adaptors that link specific chromatin loci with ATP-dependent chromatin-remodeling complexes^[48,49], serving as guides to target these complexes to regulate nucleosome remodeling and gene expression^[47,50,51].

In addition, lncRNAs have been identified as crucial regulators of epigenetic processes such as X-chromosome inactivation^[52,53], genomic imprinting^[53,54], cellular differentiation determination^[55,56], and cell identity maintenance^[57]. Thus, lncRNAs play crucial roles in the epigenetic regulation of gene expression. In particular, investigation of the interrelationships between lncRNAs and epigenetic modifications will provide new insight into cancer diagnosis and therapy.

Transcriptional regulation

At the level of transcriptional regulation, lncRNAs regulate gene expression by (1) recruiting and guiding transcription factors to the promoter region of target genes to regulate their transcription; (2) functioning as transcriptional activators or repressors to mediate gene transcription; (3) interacting with RNA polymerase II to regulate gene transcription; (4) interfering with transcription of adjacent genes in *cis*; (5) forming lncRNA-DNA hybrids to repress transcription of a target; and (6) affecting protein localization to regulate gene expression^[24,58-63].

Post-transcriptional regulation

lncRNAs regulate the expression of genes responsible for biological functions at the post-transcriptional level by modulating messenger RNA (mRNA) stability, translation, degradation, and pre-mRNA alternative

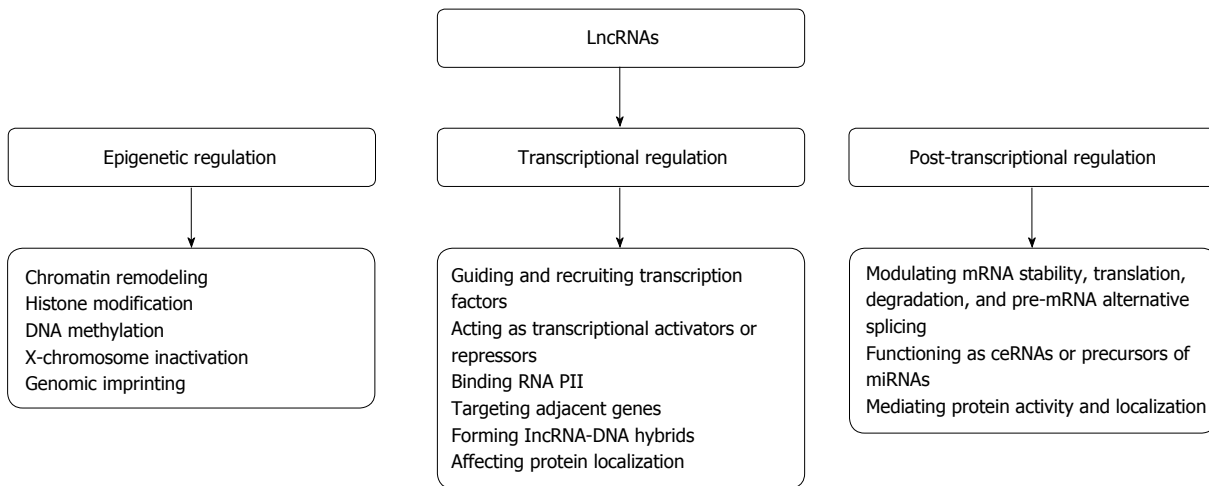


Figure 1 The regulatory mechanisms of long non-coding RNAs. LncRNAs: Long non-coding RNAs; RNA PII: RNA polymerase II; ceRNAs: Competing endogenous RNAs; mRNA: Messenger RNA; miRNAs: MicroRNAs.

splicing genes. These molecules also function as competing endogenous RNA (ceRNA) or endogenous microRNA (miRNA) sponges, act as precursors of miRNAs, and interact with proteins to mediate their activity or alter their localization^[58,64-71]. Through these mechanisms, lncRNAs play crucial roles in the post-transcriptional regulation of gene expression.

Taken together, these distinct molecular mechanisms allow dysregulated lncRNAs to up-regulate or down-regulate gene expression, thereby determining their regulatory functions in various biological processes. Nevertheless, the complicated mechanisms underlying such regulatory behaviors need further investigation. The biological functions and molecular mechanisms of action of lncRNAs are presented in Figure 1.

FUNCTIONAL ROLES OF LNCRNAs AND MECHANISMS UNDERLYING LNCRNAs DYSREGULATION IN CANCER

Numerous investigations have indicated that aberrantly expressed lncRNAs play critical roles in cancer initiation and progression. However, the biological functions and mechanisms of the majority of lncRNAs in cancer remain largely unknown. In general, lncRNAs regulate gene expression in cancer at the epigenetic, transcriptional, and post-transcriptional levels. Consequently, lncRNAs affect cell proliferation, survival, migration, or genomic stability^[72], thereby contributing to tumor development. Specifically, evidence to date demonstrates that lncRNAs are frequently aberrantly expressed in human cancers in which they may serve as oncogenes or tumor suppressors^[73,74]. These lncRNAs can mediate several cancer-associated processes, including epigenetic regulation, the DNA damage response, cell cycle control, and miRNA silencing^[75]. Furthermore, dysregulated lncRNAs

can disrupt multiple cellular oncogenic pathways by exerting oncogenic and/or tumor suppressive functions. LncRNAs also drive many important cancer phenotypes through interactions with other cellular macromolecules, including DNA, protein, and RNA^[76]. In brief, the role of lncRNAs in cancer initiation and progression is evident, yet the detailed mechanisms of their involvement in this process need to be clarified.

To date, researchers have elucidated genetic, epigenetic, and transcriptional regulatory mechanisms responsible for dysregulation of lncRNAs in cancer^[77]. For instance, genetic regulatory factors, such as genetic instability and single-nucleotide polymorphisms, can be found in lncRNAs and might contribute to their aberrant expression in cancer^[77]. Additionally, aberrant expression of lncRNAs with oncogenic properties can be caused by gene amplifications and point mutations^[78]. Epigenetic regulation, such as DNA methylation or histone acetylation in the promoter region of lncRNAs, can alter their expression in cancer^[79,80], and expression of some cancer-associated lncRNAs can also be initiated by some key transcription factors, such as Myc and p53^[81,82], or signaling cascades such as Notch^[83]. Taken together, the above-mentioned regulatory factors contribute to aberrant expression of lncRNAs in cancer, with the dysregulated lncRNAs consequently acting as important regulators of cancer initiation and progression.

DYSREGULATED EXPRESSION OF LNCRNAs IN HCC

It has been proven that aberrant lncRNA expression leads to dysregulation of downstream effectors and that lncRNAs may provide a cellular growth advantage resulting in HCC^[84], suggesting that lncRNAs may serve as promising diagnostic biomarkers and potential therapeutic targets for HCC. Thus far, multiple

Table 1 Hepatocellular carcinoma associated long non-coding RNAs in this review

LncRNA	Chromosomal location	Dysregulation	Biological roles	Ref.
<i>H19</i>	11p15.5	Up-regulated	Promotes HCC growth	Matouk <i>et al</i> ^[93]
		Down-regulated	Inhibits migration and invasion of HCC cells	Lv <i>et al</i> ^[98]
<i>HOTAIR</i>	12q13.13	Up-regulated	Promotes HCC growth	Geng <i>et al</i> ^[107]
<i>HOTTIP</i>	7p15.2	Up-regulated	Promotes proliferation of HCC cells	Quagliata <i>et al</i> ^[115]
<i>HULC</i>	6p24.3	Up-regulated	Promotes HCC growth	Zhang <i>et al</i> ^[127]
<i>MALAT1</i>	11q 13.1	Up-regulated	Promotes invasion	Lai <i>et al</i> ^[148]
<i>MVIH</i>	10q22-q23	Up-regulated	Promotes HCC growth, microvascular invasion, and intrahepatic metastasis	Shi <i>et al</i> ^[153]
<i>MEG3</i>	14q32.2	Down-regulated	Inhibits cell growth	Zhu <i>et al</i> ^[166]
<i>Lnc-FTX</i>	Xq13.2	Up-regulated	Promotes proliferation and cell cycle progression of HCC cells	Liu <i>et al</i> ^[175]
		Down-regulated	Inhibits proliferation and cell cycle progression of HCC cells	Liu <i>et al</i> ^[176]

HCC: Hepatocellular carcinoma; LncRNA: Long non-coding RNA; *H19*: *H19*, imprinted maternally expressed transcript; *HOTAIR*: HOX antisense intergenic RNA; *HOTTIP*: HOXA transcript at the distal tip; *HULC*: Highly up-regulated in liver cancer; *MALAT1*: Metastasis-associated lung adenocarcinoma transcript 1; *MEG3*: Maternally expressed gene 3; *MVIH*: Microvascular invasion in HCC; *FTX*: Five prime to Xist.

dysregulated lncRNAs have been identified as participating in the initiation and progression of HCC. Here, we briefly summarize seven well-documented lncRNAs in HCC: *H19*, *HOTAIR*, *HULC*, *HOTTIP*, *MALAT1*, *MVIH*, and *MEG3*. *FTX*, a novel lncRNA associated with HCC, is also discussed. Up-regulated expression of lncRNAs in HCC is thought to have an oncogenic function, whereas a few lncRNAs exhibiting down-regulated expression in HCC may act as tumor suppressors (Table 1).

H19

The human *H19* gene (*H19*) is a paternally imprinted gene located on human chromosome 11p15.5, a locus that contains several imprinted genes, such as insulin-like growth factor 2 (*IGF2*) and *H19*. Although *H19* has been investigated for years, its role in tumorigenesis is still controversial. Increasing evidence suggests that *H19* is highly expressed in many human cancers^[73,85-88], indicating that it acts as an oncogene and that its activation may play a critical role in tumorigenesis. Nonetheless, several studies have shown that *H19* functions as a tumor suppressor^[89-92]. Apparently, *H19* has a dual role in tumorigenesis, reflecting the complexity of *H19* function. According to the literature, *H19* function in HCC is seemingly much more complicated than that in other types of cancers; indeed, its function in hepatocarcinogenesis is largely debated. Numerous investigations have shown that the *H19* gene behaves as an oncogene, with its activation contributing to hepatocarcinogenesis. For example, hypoxia induces *H19* expression in HCC cells both *in vitro* and *in vivo*. Furthermore, silencing *H19* expression attenuates tumor growth *in vivo*, suggesting that *H19* behaves as an oncogene and enhances the tumorigenic potential of HCC cells *in vivo*^[93]. A mechanism by which *H19* exerts its oncogenic activity in hepatocarcinogenesis has been proposed. Alterations in gene expression at the *H19/IGF2* locus are associated with malignancies^[87]. In

particular, *H19* is a precursor of *miR-675*, and *H19* and *miR-675* are increasingly described as having key roles in the progression and metastasis of cancers of different tissue origins^[94]. Recent data indicate that *H19*-derived *miR-675* favors tumor progression in HCC by repressing expression of twist-related protein 1^[95], and *miR-675* up-regulates *H19* by activating *EGR1* in human liver cancer^[96]. These findings suggest that the oncogenic role of *H19* is mediated through *miR-675*. Aflatoxin B1 (AFB1) presents another mechanism related to the oncogenic function of *H19*. AFB1 induces expression of transcriptional factor *E2F1* (*E2F1*), and AFB1-induced *E2F1* up-regulates the expression of *H19* in HCC HepG2 cells, thereby promoting cellular growth and invasion^[97].

Regardless, current evidence supports a role of *H19* as a tumor suppressor. A study investigating the effect and mechanism of *H19* and *miR-675* on HCC cell migration and invasion reported that inhibition of *H19* and *miR-675* expression can promote the migration and invasion of HCC cells via the AKT/GSK-3 β /Cdc25A signaling pathway^[98]. This finding suggests that *H19* acts as a tumor suppressor in HCC cells. Intriguingly, recent data indicate that *H19* is down-regulated in intratumoral HCC tissues compared with peritumoral tissues^[99]. Additionally, *H19* plays a role in promoting tumor initiation but exerts its tumor-suppressive effect on subsequent tumor progression and metastasis in HCC^[99]. These findings suggest a tumor-promoting mechanism for *H19* in peritumoral HCC tissues and also indicate that *H19* has distinct roles at different stages of HCC development. Given the complexity of *H19* function in HCC, there is a need for further investigation to resolve the discrepancy.

In particular, a recent study found that up-regulation of *H19* has a statistically significant linear correlation with *AFP* mRNA levels in HCC tumor samples^[95], suggesting its role as a potential non-invasive diagnostic biomarker in HCC. Therefore, it should be feasible to detect both *AFP* and *H19* simultaneously to achieve

better performance in HCC management.

HOTAIR

HOX transcript antisense intergenic RNA (*HOTAIR*) is a human gene located on chromosome 12q13.13 that is co-expressed with *HOXC* genes. *HOTAIR* has been identified as regulating chromatin silencing of the adjacent *HOX* locus^[100]. Recent studies have revealed that *HOTAIR* functions as a molecular scaffold to link polycomb repressive complex 2 (*PRC2*) and lysine-specific demethylase 1/REST corepressor 1/RE1-silencing transcription factor (*LSD1/CoREST/REST*) complexes and direct them to specific gene sites, leading to altered histone H3 lysine 27 (*H3K27*) methylation and *H3K4* demethylation and ultimately resulting in epigenetic gene silencing^[46,101]. Accumulating evidence demonstrates that *HOTAIR* is dysregulated in a variety of human cancers and that overexpression of *HOTAIR* is associated with cancer cell proliferation, apoptosis, invasion, progression, and metastasis as well as poor survival^[102-105].

It has been reported that *HOTAIR* expression in HCC tissues is significantly higher than that in adjacent non-cancerous tissues^[106,107]. In addition, the expression levels of *HOTAIR* in liver cancer cell lines were found to be higher than those in normal liver cell lines^[106]. These findings suggest that *HOTAIR* exhibits oncogenic activity in HCC. Thus far, several studies have investigated the clinical implications of *HOTAIR* in HCC. Patients with HCC that overexpress *HOTAIR* have an increased risk of recurrence following hepatectomy, and there is also a correlation between *HOTAIR* overexpression and increased risk of lymph node metastasis^[108]. A high level of *HOTAIR* expression has potential as a candidate biomarker for predicting HCC recurrence in liver transplantation (LT) patients^[106]. Furthermore, patients with high expression of *HOTAIR* have a significantly shorter recurrence-free survival than patients with low expression of *HOTAIR*^[109]. Taken together, these findings support the role of *HOTAIR* as a metastatic biomarker. Indeed, just as in most other types of cancer, *HOTAIR* is considered most valuable as a prognostic indicator in HCC, particularly as a metastatic biomarker rather than as a diagnostic biomarker^[110].

Various mechanisms have been proposed for the oncogenic activity of *HOTAIR* in HCC. For example, a regulatory network between *miR-218* and *HOTAIR* was elucidated, whereby *HOTAIR* inactivates P16 (Ink4a) and P14 (ARF) signaling by down-regulating *miR-218* expression in HCC via *EZH2* targeting of the *miR-218-2* promoter regulatory axis and enhancing *Bmi-1* expression, resulting in hepatocarcinogenesis^[111]. In addition, up-regulation of *HOTAIR* promotes proliferation, migration, and invasion of human HCC cells by activating autophagy^[112], by inhibiting RNA binding motif protein 38 (RBM38)^[113], or in part by modulating *miR-1*^[114].

HOTTIP

HOXA transcript at the distal tip (*HOTTIP*), which is transcribed from the 5' tip of the *HOXA* locus, has been observed to be up-regulated in various cancers, including HCC^[115]. For example, a recent meta-analysis demonstrated that a higher expression level of *HOTTIP* is correlated with positive lymph node metastasis (LNM) and poor overall survival (OS) in patients with diverse cancers^[116], suggesting that *HOTTIP* might be a potentially promising predictor of LNM and survival in human cancer.

Another recent study showed that *HOTTIP* expression is significantly up-regulated in HCC tissues compared with adjacent non-neoplastic tissues^[115]. Patients with higher levels of *HOTTIP* and homeobox protein Hox-A13 (*HOXA13*) showed increased metastasis formation and decreased OS. Moreover, knockdown of *HOTTIP* inhibited the proliferation of liver cancer-derived cell lines^[115]. These findings indicate that *HOTTIP* might serve as a potential predictor of LNM and survival in patients with HCC. Intriguingly, these authors have also observed marked up-regulation of *HOXA13* in HCC, with *HOTTIP* and *HOXA13* having a highly positive correlation. In addition, knock-down of *HOTTIP* expression led to a reduction in *HOXA13* expression in HCC cell lines^[115], suggesting that *HOTTIP* may serve as a transcriptional regulator of *HOXA13* in HCC cells. *HOTTIP* is located at the 5' end of the *HoxA* cluster, and can enhance expression of upstream *HoxA* genes, most prominently *HOXA13*^[117]. Furthermore, *HOXA13* has been shown to play a critical role in hepatocarcinogenesis. In a recent study, *HOXA13* expression was found to be significantly up-regulated in HCC tissues compared with corresponding paracarcinomatous tissues, and all *HOXA13*-positive paracarcinomatous tissues exhibited different levels of atypical hyperplasia. Moreover, *HOXA13* overexpression may be associated with tumor angiogenesis in HCC^[118]. These findings indicate that *HOXA13* may play a crucial role in hepatocyte carcinogenesis. Another study found that *HOXA13* was the only *HOX* network gene to be constitutively overexpressed in all tested HCCs, independently of stage^[119], suggesting its involvement in the tumorigenic process of HCC. These authors speculated that *HOXA13* deregulation is involved in HCC, possibly through nuclear export of eIF4E-dependent transcripts^[119]. In addition, overexpression of *HOXA13* was shown to rescue the phenotype of *HOTTIP* knock-down HCC cells, further supporting that up-regulation of *HOTTIP* in HCC may enhance expression of *HOXA13* and eventually mediate HCC carcinogenesis^[120]. Overall, *HOTTIP* exerts its oncogenic functions in hepatocarcinogenesis at least partly by modulating *HOXA13*. Additionally, the *HOTTIP/HOXA13* axis may represent a predictor of prognosis in patients with HCC and a potential therapeutic target for this fatal disease.

Increasing evidence reveals that lncRNAs can

interact with miRNAs. Indeed, lncRNAs can act as miRNA sponges, reducing their regulatory effect; in turn, miRNAs may directly interact with lncRNAs and silence their expression^[121,122]. *MiR-125b* has been shown to be a post-transcriptional regulator of *HOTTIP* in HCC, whereby loss of *miR-125b* expression might contribute to the frequent up-regulation of *HOTTIP*^[120]. In another recent study, the authors found that both *miR-192* and *miR-204* function as tumor suppressors to reduce *HOTTIP* expression via the Argonaute2-mediated RNA interference pathway in HCC. Furthermore, glutaminase has been identified as a potential downstream target of the *miR-192/-204-HOTTIP* axis in HCC^[123].

In summary, the afore-mentioned results suggest the existence of a complex regulatory interaction between *HOTTIP* and *HoxA* genes or miRNAs. Up-regulation of *HOTTIP* contributes to hepatocarcinogenesis at least partly by regulating expression of *HoxA* genes, especially *HOXA13*, and interacting with miRNAs. Further studies are required to determine whether the regulatory loop between *HOTTIP* and *HOXA13* or miRNAs may serve as potential therapeutic targets for HCC.

HULC

Expression of the highly up-regulated in liver cancer (*HULC*) gene, which is located on chromosome 6p24.3, is increased in HCC^[124], and several recent studies have helped shed light on the factors that contribute to its aberrant up-regulation. For example, research has found that expression of *HULC* can be enhanced by the transcription factor CREB (cAMP response element-binding protein) through interaction with *miR-372*^[125]. In addition, up-regulation of *HULC* by the hepatitis B virus (HBV) X protein promotes the proliferation of hepatoma cells through down-regulation of the tumor suppressor p18^[126]. Furthermore, it has been shown that *HULC* might function as an miRNA sponge for *miR-372* in HCC and may thereby regulate gene expression at the post-transcriptional level^[125].

As an oncogene, *HULC* is implicated in hepatocarcinogenesis via regulation of multiple biological processes. *HULC* promotes the proliferation of HCC cells by regulating tumor cell proliferation-associated genes, especially cell cycle-related genes to alter the cell cycle in HCC cells^[127]. *HULC* also contributes to HCC growth by acting mechanistically to deregulate lipid metabolism through a signaling pathway involving *miR-9*, peroxisome proliferator-activated receptor alpha (*PPARA*), and acyl-CoA synthetase long chain family member 1 (*ACSL1*)^[128]. In addition, *HULC* is responsible for perturbations in the circadian rhythm by up-regulating the circadian oscillator *CLOCK* (clock circadian regulator) in hepatoma cells, resulting in the promotion of hepatocarcinogenesis^[129]. Other biological processes, such as angiogenesis, alterations in cell metabolism, activation of a precursor cell

compartment, and tissue remodeling, as well as survival, invasion and migration^[124,130], may also contribute to hepatocarcinogenesis. Furthermore, *HULC* functions as a ceRNA to activate the epithelial-mesenchymal transition, stimulating HCC progression and metastasis through the *miR-200a-3p/ZEB1* signaling pathway^[130]. A recent study provides new insight into the molecular mechanisms underlying the functions of *HULC* in hepatocarcinogenesis. The authors demonstrate that *HULC* specifically binds to Y-box protein-1 (YB-1) to promote its phosphorylation through ERK kinase and in turn regulates the interaction of YB-1 with certain oncogenic mRNAs, consequently accelerating the translation of these oncogenic mRNAs in hepatocarcinogenesis^[131]. All of these findings indicate that *HULC* might be involved in the pathogenesis and progression of HCC.

However, there are conflicting data in the literature regarding whether *HULC* in HCC is associated with a favorable or an unfavorable prognosis. According to a recent study from China, high *HULC* expression is significantly associated with higher clinical stage and probability of intrahepatic metastasis, and HCC patients with high expression of *HULC* had worse survival than those with low or no *HULC* expression^[130]. Conversely, two recent studies from South Korea and Germany, propose that high *HULC* expression is significantly associated with a low stage and grade and less vascular invasion and that HCC patients with high *HULC* expression have better survival than those with low or no *HULC* expression^[132,133]. These conflicting findings might be largely due to the inclusion of different racial and regional groups. Future studies with larger patient cohorts and various geographic and etiologic backgrounds are needed to confirm the prognostic value of *HULC* in HCC.

Compared with healthy controls, the plasma level of *HULC* was found to be dramatically increased in a large cohort of HCC patients, and higher *HULC* expression was significantly associated with larger tumor size, and no tumor encapsulation^[134], as well as higher Edmondson grades and HBV-positive status^[135]. Therefore, plasma *HULC* might act as a potential noninvasive biomarker for predicting the growth, progression and metastasis in HCC.

In summary, the afore-mentioned findings suggest that *HULC* may contribute to the carcinogenesis and progression of HCC. Therefore, *HULC* may act as a potential noninvasive biomarker for predicting the growth, progression, metastasis, and prognosis of HCC.

MALAT1

Metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) is also known as non-coding nuclear-enriched abundant transcript 2. The *MALAT1* locus at 11q13.1 has been reported to harbor chromosomal translocation breakpoints, deletions, translocations, and

point mutations linked to cancer^[136,137]. These studies have suggested that patients with these phenotypes are more susceptible to cancer.

Nonetheless, the molecular mechanism of *MALAT1* in cancer is currently uncertain. Previous cell culture studies have shown that *MALAT1* is specifically retained in nuclear speckles to regulate alternative splicing of pre-mRNAs by modulating the functional levels of serine/arginine (SR) splicing proteins^[138,139]. Moreover, a recent study suggests that *MALAT1* function is only apparent in particular cell types, such as metastatic cancer cells^[140]. These studies indicate that aberrant *MALAT1* expression promotes tumor metastasis by modulating alternative pre-mRNA splicing. However, another study has suggested a mechanism of gene regulation^[141]. Two molecular functions of *MALAT1* in cell-based models, contributing to its association with tumor metastasis, have been proposed: regulation of gene expression and alternative splicing^[142-144]. For example, regulation of expression of metastasis-associated genes, rather than alternative splicing, is the critical function of *MALAT1* in lung cancer metastasis^[145]. Although alternative splicing is critical for regulating gene expression, it may not be a major mechanism for modulating gene expression, and alternative splicing alone cannot explain the role of *MALAT1* in some cancer cell lines or tissues. Overall, *MALAT1* functions as a regulator of alternative splicing or gene expression, governing the hallmarks of cancer metastasis.

Increasing evidence shows that *MALAT1* is frequently up-regulated in both liver cancer cell lines and human HCC tissue samples^[146], suggesting that it plays an oncogenic role in HCC. A few studies to date have investigated the roles and clinical implications of *MALAT1* in HCC. In one study, *MALAT1* expression was found to be significantly up-regulated in HCC tumor tissues compared with corresponding non-tumor tissues. Furthermore, *MALAT1* was found to act as a marker with high sensitivity for human HCCs at both early and late stages^[147], suggesting that the gene can serve as a potential diagnostic tool for HCC. In another study, patients with high expression levels of *MALAT1* had a significantly increased risk of tumor recurrence after LT, and silencing *MALAT1* with siRNA in HepG2 cells effectively reduced cell viability, motility, and invasiveness and also increased susceptibility to apoptosis^[148]. These findings suggest that *MALAT1* may play a critical role in HCC progression and serve as a potential predictor of HCC recurrence after LT. Importantly, inhibition of *MALAT1* may be a potential therapeutic target for treatment of HCC.

A recent study investigated the role of specificity protein 1/3 (Sp1/3) in the regulation of *MALAT1* transcription in HCC cells, and the authors found that Sp1 and Sp3 play roles in up-regulating *MALAT1* expression^[149]. Several potential mechanisms linking *MALAT1* with HCC oncogenesis have been proposed.

For instance, *MALAT1* was found to be up-regulated in HCC and to act as a proto-oncogene to promote HCC cell growth through Wnt pathway activation and induction of oncogenic serine/arginine-rich splicing factor 1 (SRSF1). In addition, inhibition of SRSF1 expression or mTOR activity abolished the oncogenic properties of *MALAT1*, and the authors concluded that *MALAT1* promotes HCC development through SRSF1 up-regulation and mTOR activation^[150]. Nevertheless, the molecular mechanisms underlying the biological functions of *MALAT1* in HCC remain largely elusive and require further investigation.

MVIH

The lncRNA microvascular invasion in hepatocellular carcinoma (*MVIH*) is located in the intron of the *RPS24* gene, which encodes a protein belonging to the S24E family of ribosomal proteins^[151]. *MVIH* functions as a tumor promoter and is thus up-regulated in many human cancers. Furthermore, *MVIH* has been shown to activate angiogenesis^[152]. Thus far, only a few studies have shown that *MVIH* is involved in the pathogenesis and progression of HCC, and the function and mechanism of *MVIH* in HCC still need to be fully investigated.

A recent study found that *MVIH* expression was significantly increased in HCC tissues and cells and that *MVIH* promoted HCC cell growth and inhibited apoptosis by inhibiting *miR-199a* expression *in vitro* and *in vivo*^[153]. Taken together, these findings provide evidence that *MVIH* acts as an *miR-199a* sponge, linking regulation of gene expression in HCC pathogenesis. In addition to its role in HCC pathogenesis, *MVIH* has also been shown to activate angiogenesis. A previous study demonstrated that *MVIH* is generally overexpressed in HCC and plays a key role in activating angiogenesis; consequently, dysregulation of *MVIH* might serve as a predictor of poor recurrence-free survival of HCC patients after hepatectomy^[154]. It is well-known that pathological angiogenesis is essential for oncogenesis, tumor invasion and metastasis. The above-mentioned results suggest that blocking *MVIH* function might inhibit tumor angiogenesis. Thus, *MVIH* might serve as a promising therapeutic target for HCC antiangiogenic therapy.

MEG3

Maternally expressed gene 3 (*MEG3*) is an imprinted gene located at chromosome 14q32.3; imprinting of this gene is controlled by the upstream intergenic differentially methylated region (IG-DMR)^[155]. Although *MEG3* is expressed in many normal tissues, its expression is lost in various human cancers or cancer cell lines. Numerous studies have verified the functional role of *MEG3* as a tumor suppressor in many human cancers^[156-158]. Therefore, loss of *MEG3* expression may contribute to tumor pathogenesis in

a wide range of tissues of different origin. In recent years, hypermethylation of the *MEG3* promoter or the *MEG3*-3IG-DMR has been shown to contribute to loss of *MEG3* expression in human cancer cells^[159-161], and increasing evidence shows that hypermethylation of the *MEG3* promoter plays an important role in loss of *MEG3* expression in tumors^[156,158,162-165]. Overall, hypermethylation in specific *MEG3* regions might result in permanent gene transcriptional silencing and the consequent loss of its antiproliferative function, thus contributing to oncogenesis^[159].

MEG3 expression was found to be markedly reduced in HCC tissues and cell lines compared with that in adjacent normal liver tissues and normal hepatocytes^[79,166]. Furthermore, ectopic expression of *MEG3* in hepatoma cells significantly inhibits proliferation and induces apoptosis^[166,167], and forced expression of *MEG3* in HCC cells significantly decreases both anchorage-dependent and -independent growth and induces apoptosis^[79,160]. These data therefore indicate that *MEG3* functions as a tumor suppressor in hepatoma cells and plays an important role in hepatocarcinogenesis. Several studies have investigated the mechanism underlying loss of or reduction in *MEG3* expression in HCC. Similar to many other cancers, it has been revealed that loss of *MEG3* expression in HCC is associated with hypermethylation of its promoter region^[79,160,167,168].

It has been proven that *MEG3* can inhibit cell proliferation and promote apoptosis through a p53-related pathway^[169]. Several studies have also confirmed that overexpression of *MEG3* results in an increase in p53 protein and stimulates its transactivational activity in HCC cells^[166,170,171]. Further investigation showed that *MEG3* functions as a tumor suppressor in hepatoma cells by interacting with p53 to enhance p53-mediated transcriptional activity and influence the expression of partial p53 target genes^[166]. In addition, dysregulated tissue-specific expression of *miR-29a* in HCC epigenetically modulates *MEG3* expression through promoter hypermethylation^[79].

Kaplan-Meier analysis demonstrated that patients with low *MEG3* expression have worse overall and relapse-free survival compared with those with high expression of *MEG3*, and Cox proportional hazard analyses showed *MEG3* expression to be an independent prognostic factor for HCC patients^[171]. These findings suggest that decreased expression of *MEG3* contributes to HCC development and progression. Overall, *MEG3* may serve as a useful molecular diagnostic marker and a potential therapeutic target for HCC.

FTX

The gene five prime to *XIST* (*FTX*) is located upstream of *XIST*, within the X-inactivation center (XIC). *FTX* is thought to positively regulate the expression of *XIST*, which is essential for the initiation and spread of X-inactivation^[172], and recent studies have indicated

the pro-oncogenic potential of *FTX* in several types of cancer, including renal cell carcinoma^[173] and glioma^[174].

Surprisingly, there are two opposite findings regarding the role of *FTX* in HBV-related HCC in a Chinese population. In one study, *FTX* and *FTX*-derived *miR-545* were found to be up-regulated in HCC tissues compared with matched tumor-adjacent tissues, and patients with high *FTX* expression exhibited poor survival^[175], indicating that *FTX* functions as an oncogenic lncRNA in HCC. Conversely, in another study, *FTX* was found to be significantly down-regulated in HCC tissues compared with that in normal liver tissues, and patients with higher *FTX* expression exhibited longer survival, suggesting that *FTX* acts as a tumor suppressor in HCC^[176]. There are several possible explanations for these two contradictory findings. First, *FTX* might play distinct roles in HCC because it can function as a precursor for miRNAs and as an endogenous miRNA sponge (also termed ceRNA). *FTX* can encode a related cluster of miRNAs (*miR-374a* and *miR-545*) in most mammalian species^[177]. Accordingly, in HCC, *FTX* can function as an oncogene when it serves as the precursor of *miR-545*, with which it is co-transcribed, or as a tumor suppressor when it acts as a microRNA sponge for *miR-374a* to inhibit the binding of *miR-374a* to its targets. Second, in two studies, *FTX* was either up-regulated or down-regulated in HCC compared with non-tumor liver samples, suggesting a high *FTX* variability across different cohorts of patients. Third, different levels of *FTX* distribution at different sites of the HCC nodule may exist, and inadequate tumor sampling may also be a factor. Fourth, different methods were used to detect *FTX* in these two studies, with the former using quantitative reverse transcription-quantitative polymerase chain reaction, and the latter *in situ* hybridization.

PROBLEMS AND PERSPECTIVES

In this review, we summarize the recent progress regarding the functional roles of lncRNAs associated with HCC, including *H19*, *HOTAIR*, *HULC*, *HOTTIP*, *MALAT1*, *MV1H*, *MEG3*, and *FTX*. As potent gene regulators, these HCC-related lncRNAs are involved in diverse biological functions, such as cell proliferation, apoptosis, migration, invasion, metastasis, and angiogenesis, thereby contributing to the initiation and progression of HCC. In addition, these HCC-related lncRNAs may serve as potential diagnostic or prognostic biomarkers and also as therapeutic targets for HCC.

Intriguingly, due to their highly specific expression patterns in particular types of cancer^[178], efficient detection in the bodily fluids of patients (e.g., blood, plasma, and urine) and relatively stable local secondary structures, lncRNAs have the potential to serve as novel noninvasive biomarkers^[13]. For example, *HULC* is detected with a higher frequency in the

plasma of HCC patients than in healthy controls^[135], suggesting the possibility of using *HULC* as a potent circulating biomarker to facilitate early diagnosis of HCC. Nevertheless, further investigations in larger patient cohorts are necessary to validate the diagnostic effectiveness of circulating *HULC* in HCC.

Despite the importance of lncRNAs in HCC, our current understanding of HCC-related lncRNAs remains rather limited. First, the behavioral characteristics and mechanisms underlying HCC-related lncRNAs contributing to HCC remain largely unclear. Second, “driver lncRNAs” associated with tumorigenesis and progression of HCC have not yet been identified. To gain insight into lncRNA functions and mechanisms of action in HCC, several major issues need to be addressed: (1) technological advances in high-throughput RNA-Seq and high-resolution imaging of RNAs are required. In addition, computational algorithm analysis and integrated datasets are also essential; (2) rather than acting alone, the regulatory role of lncRNAs typically occurs through a large complex network that involves mRNAs, miRNAs, DNA, and proteins^[179]. Therefore, it is critical to understand how lncRNAs interact with RNA, DNA, and proteins and how aberrant crosstalk may be regulated in HCC; and (3) most of the previous studies concerning lncRNAs have been retrospective single-center analyses with a relatively small sample size. Thus, a multicenter prospective cohort study with a large sample is needed to gain a deeper understanding of the explicit roles of lncRNAs in HCC in various ethnic populations^[185].

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Nano albumin bound-paclitaxel in pancreatic cancer: Current evidences and future directions

Guido Giordano, Massimo Pancione, Nunzio Olivieri, Pietro Parcesepe, Marianna Velocci, Tania Di Raimo, Luigi Coppola, Giuseppe Toffoli, Mario Rosario D'Andrea

Guido Giordano, Marianna Velocci, Tania Di Raimo, Mario Rosario D'Andrea, Medical Oncology Unit, San Filippo Neri Hospital, 00135 Roma, Italy

Guido Giordano, CRO Aviano National Cancer Institute, 33081 Aviano, Italy

Massimo Pancione, Department of Sciences and Technologies, University of Sannio, 82100 Benevento, Italy

Massimo Pancione, Department of Biochemistry and Molecular Biology II, Faculty of Pharmacy, Complutense University, 28040 Madrid, Spain

Nunzio Olivieri, Department of Biology, University of Naples, Federico II, Via Mezzocannone, 80134 Napoli, Italy

Pietro Parcesepe, Department of Pathology and Diagnostics, University of Verona Strada, 37134 Verona, Italy

Luigi Coppola, Anatomic Pathology Unit, San Filippo Neri, 00135 Roma, Italy

Giuseppe Toffoli, Experimental and Clinical Pharmacology Unit, CRO-National Cancer Institute Via F, 33081 Aviano (Pordenone), Italy

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Correspondence to: Guido Giordano, MD, Medical Oncology Unit, San Filippo Neri Hospital, ASL Roma 1, Via Giovanni Martinotti 20, 00135 Roma, Italy. giordano.guido81@gmail.com
Telephone: +39-6-33062437
Fax: +39-6-33062445

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Abstract

Pancreatic cancer (PDAC) is an aggressive and chemo-resistant disease, representing the fourth cause of cancer related deaths in western countries. Majority of patients have unresectable, locally advanced or metastatic disease at time of diagnosis and the 5-year survival rate in these conditions is extremely low. For more than a decade gemcitabine has been the cornerstone of metastatic PDAC treatment, although survival benefit was very poor. PDAC cells are surrounded by an intense desmoplastic reaction that may create a barrier to the drugs penetration within the tumor. Recently PDAC stroma has been addressed as a potential therapeutic target. Nano albumin bound (Nab)-paclitaxel is an innovative molecule depleting

tumor stroma, through interaction between albumin and secreted protein acidic and rich in cysteine. Addition of nab-paclitaxel to gemcitabine has showed activity and efficacy in metastatic PDAC first-line treatment improving survival and overall response rate *vs* gemcitabine alone in the MPACT phase III study. This combination represents one of the standards of care in advanced PDAC therapy and is suitable to a broader spectrum of patients compared to other schedules. Nab-paclitaxel is under investigation as a backbone of chemotherapy in novel combinations with target agents or immunotherapy in locally advanced or metastatic PDAC. In this article, we provide an updated and critical overview about the role of nab-paclitaxel in PDAC treatment based on the latest advances in preclinical and clinical research. Furthermore, we focus on the use of nab-paclitaxel within the context of metastatic PDAC treatment landscape and we discuss about future implications in the light of current clinical ongoing trials.

Key words: Nano albumin bound-paclitaxel; Pancreatic cancer; Metastatic disease; Gemcitabine; Folfirinox

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Core tip: In this article, we provide an updated and critical overview about the role of nano albumin bound (Nab)-paclitaxel in pancreatic cancer (PDAC) treatment based on the latest advances in preclinical and clinical research. Furthermore, we focus on the use of Nab-paclitaxel within the context of metastatic PDAC treatment landscape and we discuss about future implications in the light of current clinical ongoing trials.

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INTRODUCTION

Pancreatic cancer (PDAC) is a lethal disease with an incidence rate nearly equal to its mortality and it is the fourth cause of cancer-related death worldwide^[1,2]. Five-year survival rate is about 5% and it increases to 20% in patients receiving radical surgery, chemotherapy and radiation^[3-5]. The majority of patients have locally advanced or metastatic disease at the time of diagnosis and chemotherapy represents the only curative chance, even if prognosis remains very poor^[6]. For more than a decade, gemcitabine has been the cornerstone of treatment in metastatic setting, despite of a small advantage in terms of survival^[7]. Recently, PDAC stroma has been addressed as a possible target

for novel molecules^[8,9]. PDAC is characterized by an intense desmoplastic reaction that may explain its high chemoresistance. Tumor stroma contributes to poor vascularization and high intratumoral pressure resulting in reduced drugs penetration within cancer cells^[10,11]. Among stromal components, secreted protein acidic and rich in cysteine (SPARC) has been investigated as a potential therapeutic target because of its involvement in PDAC cells proliferation, migration, metastasization and escape mechanisms^[12-15]. Nano albumin bound (Nab)-paclitaxel is an innovative molecule obtained by the combination of paclitaxel with nano-particles of albumin^[16]. SPARC binds albumin and it has been postulated that "nab-technology" may enhance selective delivery and uptake of paclitaxel in cancer cells^[17]. Following the results of the MPACT phase III study, nab-paclitaxel in combination with gemcitabine has become a standard of care in metastatic PDAC first-line therapy^[18]. Accordingly, this doublet represents also a valid backbone for the new developing schedules. In this paper, we will focus on molecular structure, mechanism of action, preclinical data, clinical studies and future perspectives regarding nab-paclitaxel in PDAC treatment.

NAB-PACLITAXEL: MOLECULAR STRUCTURE AND MECHANISM OF ACTION

Nab-paclitaxel (ABI 007 or Abraxane®; Celgene) is a 130-nm, albumin-bound, formulation of paclitaxel without any solvents or ethanol^[19]. The Cremophor® EL free formulation reduces the incidence of infusion adverse reactions and makes premedication with steroids not necessary^[19]. This agent is prepared by homogenization of human serum albumin at 3%-4% concentration with paclitaxel^[20]. Nab-paclitaxel particles have a reduced diameter that enhances intracellular paclitaxel delivery and thus higher antitumor activity^[21]. This molecule has a greater distribution volume, higher concentration and a faster clearance than conventional paclitaxel^[22]. After administration of equal doses of nab-paclitaxel and radiolabeled paclitaxel-cremophor to MX-1 xenograft athymic mice, nab-paclitaxel had a more rapid tumor uptake. This resulted in overall increase in the area under the curve and a more effective intratumoral accumulation of nab-paclitaxel compared to paclitaxel-cremophor^[23]. Preclinical studies have shown that albumin facilitates transport of paclitaxel across endothelial cells through the gp60 albumin receptor/caveolin-1 pathway (Figure 1). Notably, endothelial transcytosis of nab-paclitaxel was inhibited by methyl β-cyclodextrin, a gp60/caveolin-1 transport inhibitor. *In vitro*, a significant 9.9 times increase of nab-paclitaxel binding to the endothelial cells of human umbilical vein compared to conventional paclitaxel was recorded. Accordingly, a 4.2 times increased endothelial transcytosis of paclitaxel linked

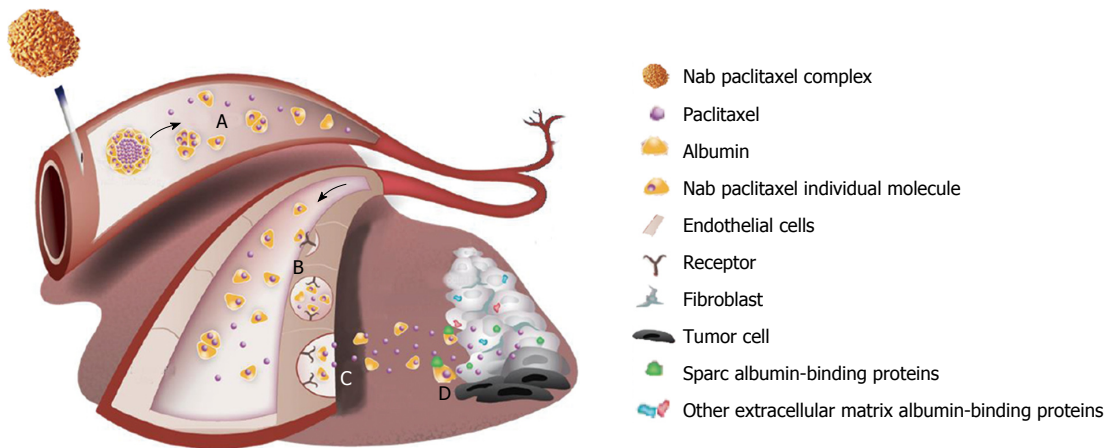


Figure 1 Nab-paclitaxel mechanism of action. A: After intravenous administration nab-paclitaxel is carried through bloodstream; B: Nab-paclitaxel binds gp60 albumin receptor on the endothelial cell; C: Endothelial transcytosis of nab-paclitaxel through the gp60 albumin receptor/caveolin - 1 pathway; D: Interaction between SPARC and nab-paclitaxel both on the pancreatic cancer cells and stromal fibroblasts. SPARC: Secreted protein acidic and rich in cysteine.

to nano-particles of albumin was demonstrated^[22,24]. Furthermore, passive transport through permeable peritumoral vessels could have a role in paclitaxel delivery to cancer cells^[25]. Peculiar and tumor-selective mechanism of action may be partially elucidated by interactions between SPARC and albumin (Figure 1). SPARC is an albumin-binding glycoprotein, also known as osteonectin, overexpressed in different types of tumor such as breast, lung, PDAC and melanoma^[26]. SPARC is expressed both in PDAC stroma and tumor cells, representing a potential target for nab-paclitaxel mechanism of action^[26]. Nevertheless, preclinical data on engineered mouse models (KPC models) demonstrated that SPARC had no role in nab-paclitaxel internalization in tumor cells^[27]. Consistently, tumor delivery of nab-paclitaxel was not related to SPARC expression in patient derived xenograft (PDX) PDAC murine models^[28].

DEVELOPMENT OF NAB-PACLITAXEL IN PDAC PRECLINICAL MODELS

In the context of a phase I - II study by Von Hoff *et al.*^[29], PDAC PDX murine models were randomly assigned to control, gemcitabine 100 mg/kg intraperitoneally on days 1 and 5 weekly for 4 wk, nab-paclitaxel 30 mg/kg per day intravenously for 5 consecutive days, and gemcitabine plus nab-paclitaxel in the preceding regimens for 4 wk. This study aimed to evaluate: (1) tumor progression; (2) changes in the pancreatic stroma; and (3) intratumoral drug penetration. Tumor regression was observed in 18%, 36% and 64% of xenografts treated with gemcitabine, nab-paclitaxel alone and both in combination, respectively. A depleted desmoplastic stroma, combined with dilated blood vessels, was found in each of nab-paclitaxel treatment group. Nab-paclitaxel plus gemcitabine resulted in 2.8 fold increased intratumor concentration of gemcitabine, compared to

gemcitabine alone^[29]. Another study investigated nab-paclitaxel, gemcitabine, docetaxel or control in PDAC cell-lines AsPC-1, BxPC-3, MIA PaCa-2 and Panc-1a. Addition of docetaxel or nab-paclitaxel at IC25, reduced gemcitabine IC50. Tumor growth inhibition after gemcitabine, docetaxel or nab-paclitaxel was 67%, 31% and 72%, respectively. Tumor stromal density, measured through reduction in α -smooth muscle actin, S100A4 and collagen 1 expression, was decreased by nab-paclitaxel better than docetaxel. In the same study, xenograft model was used to evaluate the efficacy of gemcitabine, docetaxel or nab-paclitaxel. Nab-paclitaxel followed by gemcitabine resulted in a longer median survival vs control or gemcitabine alone^[30]. Notably, combination of nab-paclitaxel and gemcitabine improved survival, reduced incidence of metastasis and increased intratumoral gemcitabine levels in KPC mouse models. Those effects were explained by the decrease of cytidine-deaminase, a gemcitabine-metabolizing enzyme^[31]. Engineered mice models of PDAC treated with nab-paclitaxel and gemcitabine, showed a distorted collagen with low cellular content in a preclinical-clinical study. Conversely, cancer-associated fibroblasts were increased in gemcitabine-treated mice. In the clinical section of this study, surgical specimen from patients with resectable PDAC, receiving preoperative treatment with two cycles of nab-paclitaxel and gemcitabine were studied to determine the collagen content and cancer associated fibroblasts density. Tumor samples of patients treated with nab-paclitaxel plus gemcitabine, showed a less abundant fibrillar collagen matrix around the tumor. Consistently, cancer associated fibroblasts were decreased in number, without effects on their activity^[32]. Recently, a preclinical study definitively demonstrated that nab-paclitaxel was superior to conventional paclitaxel in blocking primary tumor progression, depletion of tumor stroma and resulted in a longer survival in PDAC murine models^[33].

NAB-PACLITAXEL AND GEMCITABINE: A STANDARD OF CARE IN METASTATIC PDAC TREATMENT

Combination of nab-paclitaxel and gemcitabine in metastatic PDAC patients was investigated in an open label phase I - II study conducted in four United States centers. In the phase I part of this trial, primary endpoint was to identify the maximum tolerated dose (MTD) and dose-limiting toxicities (DLTs) of gemcitabine 1000 mg/m² followed by nab-paclitaxel 100, 125 or 150 mg/m² administered intravenously on day 1, 8 and 15 of a 28 d cycle. DLTs were sepsis and neutropenia and the MTD was fixed at level 2 (nab-paclitaxel 125 mg/m²). Phase II part of this study continued accrual at MTD to evaluate activity and safety of this combination. A cohort of 67 patients was enrolled and 44 received treatment at MTD. Median overall survival (OS) and progression free survival (PFS) in patients treated at MTD were 12.2 and 7.9 mo, respectively. Overall response rate (ORR) was 48% and overall disease control rate (DCR) was 68%. Most common grade ≥ 3 toxicities were represented by neutropenia (67%), leukopenia (44%), thrombocytopenia (23%), fatigue (21%) and neuropathy (15%). SPARC levels analysis was performed by immunohistochemistry in 36 tumor samples and patients were classified into high-SPARC and low-SPARC. High-SPARC was related to longer OS than low-SPARC (17.8 mo vs 8.1 mo, $P = 0.0431$) and it was significant predictor for OS at multivariate Cox regression model ($P = 0.041$)^[29]. Phase I - II studies have been performed also in Asian patients. In Japanese population, no differences were observed in grade 3 or higher toxicities compared to the American phase I - II study, using the same schedule^[34]. A Chinese phase I - II trial evaluated a different schedule of nab-paclitaxel plus gemcitabine. MTD was fixed at nab-paclitaxel 120 mg/m² in combination with gemcitabine 1000 mg/m² on day 1 and 8 every 3 wk^[35].

MPACT phase III trial

The MPACT trial was an international, multicenter, open label, randomized phase III study involving 11 countries worldwide. Eligible patients were randomly assigned 1:1 to receive either nab-paclitaxel 125 mg/m² plus gemcitabine 1000 mg/m² on days 1, 8, 15 every 4 wk or gemcitabine alone weekly for 7 of 8 wk during first cycle and days 1, 8, 15 every 4 wk in subsequent cycles. Treatment was administered until disease progression or unacceptable toxicity. Eight-hundred sixty-one patients (431 in the experimental arm) were enrolled and stratified according Karnofsky Performance Status (KPS), presence of liver metastases and geographic region. Primary end-point was OS; secondary objectives were PFS and ORR.

Age, gender, race, region and KPS were comparable in both study arms. No significant differences in pancreatic tumor site, location of metastases, number of metastatic sites, median CA19.9 levels, previous surgical or medical treatment and biliary stent implantation were observed between nab-paclitaxel plus gemcitabine and single agent gemcitabine groups. In the experimental arm, median duration of treatment was 3.9 mo and median dose intensity was 81% for nab-paclitaxel and 75% for gemcitabine. Addition of nab-paclitaxel to gemcitabine increased significantly median OS over gemcitabine alone (8.5 mo, 95%CI: 7.89-9.53 vs 6.7 mo, 95%CI: 6.01-7.23) with a 28% reduced risk of death (HR = 0.72, 95%CI: 0.62-0.82, $P < 0.001$). One and two-year survival rates were significantly higher in nab-paclitaxel and gemcitabine group compared to gemcitabine alone (35% vs 22%, $P < 0.001$ and 9% vs 4%, $P = 0.02$, respectively). Median PFS was prolonged by combination schedule (5.5 mo, 95%CI: 4.5-5.9 vs 3.7 mo, 95%CI: 3.6-4.0; HR = 0.69, 95%CI: 0.58-0.82, $P < 0.001$) with a significantly higher 12 mo- PFS rate than single agent gemcitabine (16% vs 9%). ORR was increased by addition of nab-paclitaxel to gemcitabine vs gemcitabine alone as assessed both by independent and investigator review (23% vs 7%, $P < 0.001$ and 29% vs 8%, $P < 0.001$, respectively). Notably, DCR, as evaluated by independent review, was higher in combination than single agent arm (48% vs 33%). Nab-paclitaxel and gemcitabine combination benefit was consistent across the different subgroups in terms of OS and PFS. Patients with more advanced disease, poorer PS, presence of liver metastases, more than 3 metastatic sites and CA19.9 higher than 59 times the upper limit of the normal range had the highest reduction in risk of death. Most common grade 3-4 hematologic events in the experimental arm were neutropenia (38%), leukopenia (31%), thrombocytopenia (13%) and most common non-haematological events were fatigue (17%) and peripheral neuropathy (17%)^[18]. An updated analysis of MPACT trial with a longer median follow-up (13.9 mo) confirmed the superior OS in nab-paclitaxel plus gemcitabine vs single agent gemcitabine group (8.7 mo vs 6.6 mo, respectively) and identified 10%, 4% and 3% of long survivors in the combination arm at 24, 36 and 42 mo, respectively^[36].

MPACT study: post hoc analyses

Nab-paclitaxel plus gemcitabine remained an independent predictor of improved OS and PFS also after correction for prognostic factors. In fact, MPACT investigators evaluated the effect of treatments according stratification factors and known prognostic factors (age, sex, KPS, peritoneal carcinomatosis, primary tumor location, pulmonary metastases, liver metastases, Whipple procedure, biliary stent implantation, stage at diagnosis, CA19.9 level).

Combination regimen was more effective than gemcitabine in a larger spectrum of patients^[37]. In addition, an elevated neutrophil to lymphocytes ratio (NLR ≥ 5) at baseline resulted as an independent prognostic factor related to a worse OS in an updated multivariate analysis^[36]. Nab-paclitaxel plus gemcitabine combination showed activity both on primary and metastatic lesions in terms of tumor shrinkage benefit in a further analysis^[38]. In MPACT trial, baseline CA19.9 levels were not a significant predictor of survival; conversely, CA19.9 decrease was related to better outcomes in both arms. An exploratory analysis confirmed the CA19.9 decrease at 8 wk as an early marker of treatment efficacy in terms of OS and ORR^[39]. A pre-specified analysis evaluated the role of metabolic response detected by PET scan and compared metabolic response rate with treatment efficacy. Metabolic response rates at 8 wk as well as best metabolic response were higher in nab-paclitaxel plus gemcitabine arm. Metabolic response appeared as predictor of longer OS in both study arms^[40]. Nab-paclitaxel plus gemcitabine was related to 54% of any grade neuropathy. Majority of patients experiencing grade 3 neuropathy, improved to grade 1 or less within one-month. Patients who developed peripheral neuropathy received a longer treatment and obtained a longer OS. Survival benefit increased according to the grade of peripheral neuropathy^[41]. An exploratory analysis was performed in order to gain insight the role of SPARC expression as prognostic or predictive factor of survival. Neither baseline plasma SPARC levels, nor changes from baseline were predictors of OS. Stromal and tumor levels of SPARC, measured by immunohistochemistry had no correlation with OS^[42]. Nab-paclitaxel plus gemcitabine increased OS, PFS and ORR, compared to gemcitabine alone in patients treated until disease progression. Accordingly, more than 50% of patients treated until progression received a second line therapy^[43]. In the MPACT study 41% of patients had a nab-paclitaxel dose reduction, 71% had a dose delay, mostly after two cycles of treatment. In nab-paclitaxel plus gemcitabine arm, those modifications led to a longer treatment exposure and OS respect of patients who did not receive reductions or delays^[44]. Forty-percent of patients treated in nab-paclitaxel and gemcitabine group received a second line treatment, mostly (74%) with fluoropyrimidines in combination regimens. Second line therapies were related to a significant benefit in OS in both study arms compared to patients without second line therapy. Notably, OS was significantly increased in patients treated with first line nab-paclitaxel plus gemcitabine vs gemcitabine alone (12.8 mo vs 9.0 mo, HR = 0.76, $P = 0.015$)^[45].

NOVEL INVESTIGATIONS WITH NAB-PACLITAXEL IN PDAC

Hypoxia represents a common feature of several

malignancies and it correlates with a worse prognosis and resistance to chemo- and radio-therapy^[46,47]. TH-302 is a bromo-isophosphoramidate mustard that works under conditions of hypoxia. The triplet containing nab-paclitaxel, gemcitabine and TH-302 exhibited superior efficacy in reducing stromal density and cell proliferation compared to TH-302 alone or nab-paclitaxel plus gemcitabine, in human PDAC xenograft models^[48]. Combination of nab-paclitaxel with the oral fluoropyrimidine S-1 demonstrated to inhibit proliferation in PDAC cell lines and to decrease relative tumor volume in murine xenograft models^[49,50]. An important pathway involved in PDAC stem cells maintenance is represented by Notch signaling^[51]. Recently, the oncolytic adenovirus AdNuPARmE1A has showed to interfere with Notch pathway. In PDAC PDX mouse models, administration of nab-paclitaxel and gemcitabine combined with AdNuPARmE1A resulted in a higher therapeutic response than nab-paclitaxel plus gemcitabine combination and AdNuPARmE1A alone^[52]. Tumor microenvironment plays a crucial role in PDAC aggressiveness and drug-resistance^[53,54]. In this context, pancreatic stellate cells have been addressed as responsible for fibrosis and they have similar characteristics to the monocyte-macrophage lineage. Pamidronate and zoledronic acid are nitrogen-containing bisphosphonates that have showed *in vitro* anti-proliferative and pro-apoptotic activity on macrophages. *In vivo*, antitumor effect of nab-paclitaxel is enhanced by the combination with bisphosphonates in PDAC PDX mouse models^[55]. Depending on predominant signals within the tumor microenvironment, macrophages can adopt a variety of functional states. In particular, macrophages with M2 phenotype acquire immuno-suppressive and tumor-promoting features^[56]. *In vitro* and *in vivo* studies showed that nab-paclitaxel is internalized by macrophages *via* macro-pinocytosis, leading to a polarization towards a potentially tumoricidal M1 phenotype^[57]. Insulin-like growth factor (IGF) signaling proteins are overexpressed in PDAC, predicting poor prognosis and higher aggressiveness. A small IGF-1 receptor and Insulin receptor reversible inhibitor (BMS-754807) reduced PDAC relative volume when combined with nab-paclitaxel in PDX mouse models^[58].

Phase I-II studies

Several clinical trials in locally advanced or metastatic PDAC treatment included nab-paclitaxel in new therapeutic schedules. A phase I study has investigated the combination of FOLFOX plus nab-paclitaxel in a cohort of 35 patients, aiming to establish the nab-paclitaxel MTD. A dose of 150 mg/m² every 2 wk was identified as MTD and a 20% reduction in oxaliplatin dose was recommended in patients who developed grade 2 neuropathy. This schedule showed a promising activity with 60% response rate and median survival of 15 mo^[59]. The four-drug schedule combining

cisplatin, nab-paclitaxel, capecitabine and gemcitabine (PAXG) has been tested in unresectable or borderline resectable PDAC patients. The PAXG regimen was investigated in a phase I trial on 24 patients with the objective to define the recommended phase 2 dose (RP2D) of nab-paclitaxel. A dose of 150 mg/m² every 2 wk resulted as RP2D and major grade 3-4 toxicities were represented by neutropenia (31%), anemia (12%), fatigue (19%), hand-foot syndrome (12%). Disease control was obtained in all 21 patients and 67% of them showed a partial response. Six- and twelve-months PFS rates were 96% and 50% respectively^[60]. This novel combination was evaluated in a randomized phase II study in which PAXG was compared to standard nab-paclitaxel plus gemcitabine. Fifty-four patients with unresectable or borderline resectable PDAC were randomized and 26 received PAXG. Primary endpoint of the study was resectability rate and each regimen would have been considered active if at least 4 patients per arm underwent to surgical resection. Both schedules resulted to be active and 5 patients in each arm underwent to surgery^[61]. Combination of nab-paclitaxel and gemcitabine has been tested as a therapeutic backbone with target agents. A phase Ib trial evaluated the addition of erlotinib to nab-paclitaxel and gemcitabine in previously untreated advanced PDAC patients. The triplet was not feasible at standard dose of each single agent and it was necessary to reduce both nab-paclitaxel and erlotinib doses to 75 mg/m² and 75 mg/d, respectively. Due to the small number of patients treated at dose level 3, no valid information about activity outcomes were obtained^[62]. Recently, combination of nab-paclitaxel with both oral and intravenous fluoropyrimidines has been investigated. A single arm phase II trial evaluated the combination of capecitabine 825 mg/m² orally bis in die on days 1-15 and nab-paclitaxel 125 mg/m² intravenously on days 1 and 8 every 3 wk as a first line treatment in 30 PDAC patients. In patients without relevant adverse reaction after first cycle, the nab-paclitaxel dose was escalated to 100 mg/m² on days 1, 8, 15 every 4 wk. ORR and DCR were 41.4% and 76%, respectively. Major grade 3 toxicities were peripheral neuropathy (23%), neutropenia (17%), hand-foot syndrome (13%), and phototoxic skin reaction (10%)^[63]. A randomized phase II study compared the combination of nab-paclitaxel 125 mg/m², leucovorin 400 mg/m², 5-fluorouracil 400 mg/m² bolus, followed by 2400 mg/m² given as 46 h continuous infusion on day 1 and 15 every 4 wk (NABFU schedule) vs standard nab-paclitaxel plus gemcitabine. One hundred-fourteen patients were included with 2:1 randomization in favor of experimental arm. PFS at 4 mo was the primary endpoint. No differences were observed between the experimental arm and the standard treatment (55% vs 54%). NABFU regimen resulted in a higher median OS, as well as higher OS rates at 12 and 18 mo vs

nab-paclitaxel plus gemcitabine (11.4 mo vs 9.2 mo, 48% vs 41% and 34% vs 31% respectively). Safety profile was similar in both study arms, except for grade 3 mucositis and diarrhea that were higher in the NABFU treatment group, reflecting the fluorouracil-related toxicities^[64,65].

PUTTING NAB-PACLITAXEL IN PDAC

LANDSCAPE: PAST, PRESENT...

Despite of promising preclinical and “early” clinical activity, paclitaxel and docetaxel have never showed superiority compared to gemcitabine in PDAC therapy^[66]. Conversely, nano-technology improved bioavailability and intratumoral delivery of paclitaxel as well as avoided effects due to cremophor and therefore premedication with steroids. The albumin-bound formulation, acts on tumor microenvironment and depletes stroma through interaction between SPARC and albumin. This mechanism of action favors gemcitabine increased intratumoral concentration, making nab-paclitaxel an ideal partner for this drug. Nab-paclitaxel plus gemcitabine was the first combination to increase survival and response rates in metastatic PDAC treatment compared to gemcitabine alone. This schedule represents one of the standards of care in advanced PDAC first line treatment together with FOLFIRINOX and single agent gemcitabine^[7,67,68]. Among these schedules, the most appropriate therapeutic choice should be guided by patient-related features (age, performance status, comorbidities, preferences), disease characteristics (tumor burden, metastatic sites, biliary stent implantation, bilirubin levels), also taking into consideration previous therapies and cost-effectiveness. In this context, FOLFIRINOX, according to the ACCORD/PRODIGE phase II - III study, could be an option in young, healthy and good performance status patients. Single agent gemcitabine could be used in unfit patients, with poor performance status and few comorbidities where a doublet or a triplet would not be feasible. Nab-paclitaxel and gemcitabine combination appears suitable for a broader spectrum of patients. In fact, MPACT trial was performed on a larger and more heterogeneous population than FOLFIRINOX and gemcitabine pivotal trials (Table 1). This study demonstrated that nab-paclitaxel plus gemcitabine improved survival in metastatic PDAC patients regardless to age, gender, geographic region, KPS, primary tumor location, presence of liver metastases, number of metastatic sites and baseline CA19.9 PDAC incidence is higher in elderly patients although this population is scarcely included in clinical trials^[69]. Patients aged ≥ 75 years were not enrolled in the ACCORD/PRODIGE study; conversely they represented 10% of the MPACT population. Nab-paclitaxel plus gemcitabine showed efficacy and improved survival regardless age at a cut-off of 65 years; nevertheless data about “truly elderly”

Table 1 Patients characteristics in metastatic pancreatic cancer pivotal trials

	Gemcitabine	FOLFIRINOX	Nab-paclitaxel + gemcitabine
Trial (yr)	Gemcitabine <i>vs</i> 5-fluorouracil (1997) ¹	FOLFIRINOX <i>vs</i> Gemcitabine (2011) II-III	Nab-paclitaxel + Gemcitabine <i>vs</i> Gemcitabine (2013) III
Phase			
Countries	United states- Canada- United Kingdom	France	Worldwide
Patients	126	342	861
Median age	61.5	61	62
Elderly (≥ 75 yr)	Yes		Yes
ECOG PS 0-1	Yes	Yes	Yes
ECOG PS 2	Yes		Yes
Biliary Stent	Yes	Yes	Yes
Multiple metastatic sites	Yes	Yes	Yes

¹Phase not specified in the full paper. ECOG PS: Eastern Cooperative Oncology Group Performance Status.

patients, aged over 75 years were available only on a small proportion of subjects. Real life experiences, with the limitation of retrospective series, suggest that the combination schedule was feasible, active, effective and well tolerated in elderly patients aged more than 70 and 75^[70,71]. No data are available about the use of FOLFIRINOX in patients with poor performance status, in fact only subjects with ECOG PS 0-1 were included in the pivotal trial. Addition of nab-paclitaxel to gemcitabine did not improve survival in the KPS 70 (corresponding to ECOG PS of 2) compared to single agent gemcitabine, although these results were limited only to the 8% of MPACT study population. In order to make the best therapeutic choice in this population, physicians should differentiate between ECOG PS 2 due to "tumor burden" or due to comorbidities. In fact, as stated by European Society for Medical Oncology Guidelines for PDAC treatment, nab-paclitaxel plus gemcitabine could be considered an option in patients with ECOG PS 2 due to "heavy tumor load" for best chance of response^[72]. Feasibility and flexibility of nab-paclitaxel plus gemcitabine combination allow to treat patients until disease progression. Data from MPACT study post-hoc analysis confirm that the higher exposure to both drugs correlates to better outcomes. Metastatic PDAC subjects treated until disease progression as well as receiving dose delays or modifications within the study, had a longer OS than patients who did not. Introduction of new, active schedules in PDAC first line treatment let to higher proportion of patients to benefit also from further lines of therapy. In fact, in ACCORD/PRODIGE and MPACT study population, 46% and 40% of patients received a second line chemotherapy in the FOLFIRINOX and nab-paclitaxel plus gemcitabine arm, respectively. Real

life data show that this number is even higher in nab-paclitaxel and gemcitabine treated subjects, reaching more than 50%^[73]. Second-line options that have demonstrated efficacy are oxaliplatin plus fluoro-folate (OFF schedule) and nano-liposomal irinotecan plus fluoro-folate according to CONKO-003 and NAPOLI-1 phase III trials results, respectively^[74-76]. The choice of nab-paclitaxel as a first-line regimen allows the use of a non-cross-resistant second-line chemotherapy both with oxaliplatin and nano-liposomal irinotecan, whereas FOLFIRINOX does not. Finally, the optimal first-line therapeutic decision should take into account treatment-related costs. Recent data show that patient who initiated first line therapy with nab-paclitaxel plus gemcitabine has similar treatment duration but lower all-cause costs than FOLFIRINOX^[77].

CONCLUSION

Innovative technology, peculiar mechanism of action and clinical activity of nab-paclitaxel have made this molecule an intriguing weapon for further studies in PDAC treatment. Several phase I, II and III trials are actually recruiting patients in PDAC therapy in order to investigate nab-paclitaxel based chemotherapy plus immunotherapy or target agents in the metastatic setting (Table 2). Furthermore, recent data from a randomized phase II trial suggest that sequential administration of nab-paclitaxel on day 1, 8, 15 and gemcitabine day 2, 9, 16 every 4 wk as first-line therapy in metastatic PDAC, may be more effective than a concomitant schedule and this approach warrants further studies^[78]. Notably, combination of nab-paclitaxel and gemcitabine is under investigation in adjuvant setting (Table 3). APACT is a phase III, multicenter, open label, randomized study of nab-paclitaxel plus gemcitabine *vs* gemcitabine alone in subjects with surgically resected PDAC. This trial planned to enroll 846 patients evaluating the disease free survival as primary endpoint and study results are expected in the next future. Several studies are ongoing in the locally advanced, borderline resectable or unresectable PDAC (Table 3). In particular, LAPACT is a phase II, international, multicenter, open label, single-arm study in subjects with unresectable, locally advanced PDAC who have not received prior treatment. Nab-paclitaxel and gemcitabine was administered for a maximum of six cycles as induction therapy. Only in patients without disease progression or unacceptable toxicity, the investigator could choose among different options: (1) continuing chemotherapy with the same schedule; (2) chemo-radiation; and (3) surgical resection. Primary end-point was the time to treatment failure (time from the first day of treatment and disease progression or death). An interim analysis evaluated preliminary safety and efficacy data on 47 of 110 planned patients. Induction phase was completed in 60% of patients and 40% discontinued treatment,

Table 2 Principal clinical trials with nab-paclitaxel in metastatic pancreatic cancer

Study ID	Setting	Study drugs	Phase	Status
NCT02993731	Metastatic	Nab-paclitaxel + Gemcitabine +/- Napabucasin	III	Recruiting
NCT02101021	Metastatic	Nab-paclitaxel + Gemcitabine +/- Momelotinib	III	Active not recruiting
NCT02715804	Metastatic	Nab-paclitaxel + Gemcitabine +/- PEGPH20	III	Recruiting
NCT02436668	Metastatic	Nab-paclitaxel + Gemcitabine +/- Ibrutinib	II - III	Recruiting
NCT02827201	Metastatic	Sequential Nab-paclitaxel + Gemcitabine / FOLFIRI	II	Active not recruiting
NCT02767557	Metastatic	Nab-paclitaxel + Gemcitabine +/- tocilizumab	II	Recruiting
NCT03086369	Metastatic	Nab-paclitaxel + Gemcitabine +/- Olaratumab	II	Not yet recruiting
NCT02879318	Metastatic	Nab-paclitaxel + Gemcitabine +/- Durvalumab + Tremelimumab	II	Recruiting
NCT02399137	Metastatic	Nab-paclitaxel + Gemcitabine + MM-141	II	Recruiting
NCT02340117	Metastatic	Nab-paclitaxel + Gemcitabine + SGT-53	II	Recruiting
NCT02124317	Metastatic	Nab-paclitaxel + S-1	II	Active not recruiting
NCT03076216	Metastatic	Nab-paclitaxel + Gemcitabine + ONCOSil	II	Not yet recruiting
NCT02905578	Metastatic	Nab-paclitaxel + Gemcitabine + High-dose ascorbate	II	Not yet recruiting
NCT02732938	Metastatic	Nab-paclitaxel + Gemcitabine + PF-04136309	II	Recruiting
NCT02551991	Metastatic	Nal-Iri or Gemcitabine + Nab-paclitaxel	II	Recruiting
NCT03009058	Metastatic	Nab-paclitaxel + Gemcitabine + IMM 101	I - II	Not yet recruiting
NCT02559674	Metastatic	Nab-paclitaxel + Gemcitabine + ALT-803	I - II	Recruiting
NCT02705196	Metastatic	Nab-paclitaxel + Gemcitabine + LOAd703 oncolytic virus	I - II	Recruiting
NCT01834235	Metastatic	Nab-paclitaxel + Gemcitabine + NPC-1C	I - II	Active not recruiting
NCT01730222	Metastatic	Nab-paclitaxel + Gemcitabine + Capecitabine + Cisplatin	I - II	Recruiting
NCT02620800	Metastatic	Nab-paclitaxel + 5-fluorouracil + Leucovorin + Bevacizumab + Oxaliplatin	I - II	Recruiting
NCT01506973	Metastatic	Nab-paclitaxel + Gemcitabine + Hydroxychloroquine	I - II	Recruiting
NCT02504333	Metastatic	Nab-paclitaxel + Gemcitabine followed by FOLFFOX	I - II	Recruiting
NCT02050178	Metastatic	Nab-paclitaxel + Gemcitabine + OMP-54F28	I	Active not recruiting
NCT02309177	Metastatic	Nab-paclitaxel + Nivolumab +/- Gemcitabine	I	Recruiting
NCT02514031	Metastatic	Nab-paclitaxel + Gemcitabine + ARQ-761	I	Recruiting
NCT01934634	Metastatic	Nab-paclitaxel + Gemcitabine + LCL161	I	Active not recruiting
NCT02101580	Metastatic	Nab-paclitaxel + Gemcitabine + ADI-PEG 20	I	Active not recruiting
NCT02138383	Metastatic	Nab-paclitaxel + Gemcitabine + Enzalutamide	I	Active not recruiting
NCT02975141	Metastatic	Nab-paclitaxel + Gemcitabine + Afatinib	I	Recruiting
NCT02227940	Metastatic	Nab-paclitaxel + Gemcitabine + Ceritinib	I	Recruiting
NCT02231723	Metastatic	Nab-paclitaxel + Gemcitabine + BBI608	I	Recruiting
NCT02501902	Metastatic	Nab-paclitaxel + Palbociclib	I	Recruiting

Nal-Iri: Nanoliposomal irinotecan.

due to adverse events in the majority of cases. DCR was 80% and 13% of patients underwent to surgical resection. Major grade 3-4 toxicities were neutropenia (45%), anemia (13%) and fatigue (11%)^[79]. Clinical trials in neo-adjuvant treatment or in locally advanced disease are evaluating integrated strategies containing nab-paclitaxel with both intensity modulated, image guided and stereotactic radiotherapy (Table 3). Actually there are no predictive biomarkers that may guide therapeutic choice in PDAC treatment and may help clinicians to choose the best regimen for each patient. An intense research is ongoing in order to investigate the potential role of micro-RNA as predictive of sensitivity and resistance to the drugs included in the FOLFIRINOX and nab-paclitaxel plus gemcitabine schedules^[80].

Introduction of nab-paclitaxel in Oncologist's portfolio of drugs has represented a very important step forward in PDAC treatment. Nab-paclitaxel plus gemcitabine is one of the standards of care in advanced PDAC therapy and this combination is suitable for a wide spectrum of patients with different features and clinical presentations. This scenario could change rapidly as the result of nab-paclitaxel anticipation in "early settings" and further studies could be required in order to investigate a "re-challenge" in the metastatic disease.

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Table 3 Principal clinical trials with nab-paclitaxel in pancreatic cancer “early settings”

Study ID	Setting	Study drugs	Phase	Status
NCT01964430	Adjuvant	Nab-paclitaxel + Gemcitabine <i>vs</i> Gemcitabine	III	Recruiting
NCT02506842	Adjuvant	Nab-paclitaxel + Gemcitabine <i>vs</i> OFF	III	Recruiting
NCT02023021	Adjuvant	Nab-paclitaxel + Gemcitabine	II	Recruiting
NCT02125136	Neoadjuvant	Nab-paclitaxel + Gemcitabine <i>vs</i> FOLFIFINOX	II	Recruiting
NCT02243007	Neoadjuvant	Nab-paclitaxel + Gemcitabine <i>vs</i> FOLFIFINOX	II	Recruiting
NCT02717091	Neoadjuvant/borderline resectable	Nab-paclitaxel + Gemcitabine <i>vs</i> FOLFIFINOX	II	Recruiting
NCT02481635	NEO/adjuvant	Nab-paclitaxel + Gemcitabine + radiotherapy	I - II	Recruiting
NCT01431794	Neoadjuvant	Nab-paclitaxel + Gemcitabine + LDE-225	I - II	Active not recruiting
NCT02588443	NEO/adjuvant	Nab-paclitaxel + Gemcitabine + RO7009789	I	Recruiting
NCT02272738	Neoadjuvant	Nab-paclitaxel + Gemcitabine + radiotherapy	I	Recruiting
NCT02506803	Neoadjuvant	Nab-paclitaxel + Gemcitabine	I	Recruiting
NCT02427841	Resectable	Nab-paclitaxel + Gemcitabine followed by radiotherapy	II	Recruiting
NCT02550327	Resectable	Nab-paclitaxel + Gemcitabine + Cisplatin + Anakinra	I	Recruiting
NCT02210559	Locally advanced	Nab-paclitaxel + Gemcitabine + FG-3019	I - II	Recruiting
NCT02394535	Locally advanced	Nab-paclitaxel + Capecitabine + radiotherapy	I	Recruiting
NCT02124369	Borderline unresectable	Nab-paclitaxel + Gemcitabine	II	Recruiting
NCT02043730	Unresectable locally advaced	Nab-paclitaxel + Gemcitabine	II	Recruiting
NCT02207465	Unresectable locally advanced	Nab-paclitaxel + Gemcitabine + radiotherapy	I	Recruiting
NCT02272738	Unresectable locally advanced	Nab-paclitaxel + Gemcitabine	I	Recruiting
NCT01978184	Preoperative	Nab-paclitaxel + Gemcitabine + Hydroxychloroquine	II	Recruiting
NCT02427841	Preoperative	Nab-paclitaxel + Gemcitabine + radiotherapy	II	Recruiting
NCT02723331	Preoperative	Nab-paclitaxel + Gemcitabine followed by SBRT	II	Recruiting
NCT02930902	Preoperative	Nab-paclitaxel + Gemcitabine + Pembrolizumab + Paricalcitol	I	Recruiting

SBRT: Stereotactic body radiotherapy; OFF: Oxaliplatin plus fluoro-folate.

paclitaxel mechanism of action from their educational slides kit.

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Basic Study

Comparison between tocotrienol and omeprazole on gastric growth factors in stress-exposed rats

Mohd Fahami Nur Azlina, Hj Mohd Saad Qodriyah, Kien Hui Chua, Yusof Kamisah

Mohd Fahami Nur Azlina, Hj Mohd Saad Qodriyah, Yusof Kamisah, Department of Pharmacology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Kuala Lumpur 56000, Malaysia

Kien Hui Chua, Department of Physiology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Kuala Lumpur 56000, Malaysia

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Correspondence to: Mohd Fahami Nur Azlina, DVM, MMed-SC, PhD, Associate Professor, Department of Pharmacology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Kuala Lumpur 56000, Malaysia. nurazlinamf@ukm.edu.my
Telephone: +603-91459574
Fax: +603-91459547

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Abstract

AIM

To investigate and compare the effects of tocotrienol and omeprazole on gastric growth factors in rats exposed to water-immersion restraint stress (WIRS).

METHODS

Twenty-eight male Wistar rats were randomly assigned to four groups of seven rats. The two control groups were administered vitamin-free palm oil (vehicle) and the two treatment groups were given omeprazole (20 mg/kg) or tocotrienol (60 mg/kg) by oral gavage. After 28 d of treatment, rats from one control group and both treated groups were subjected to WIRS one time for 3.5 h. Gastric lesions were measured and gastric tissues were obtained to measure vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and transforming growth factor- α (TGF- α) mRNA expression.

RESULTS

Rats exposed to WIRS for 3.5 h demonstrated the presence of considerable ulcers in the form of gastric erosion. The lesion index in the stressed control (S) group was increased ($P < 0.001$) compared to the tocotrienol treated and omeprazole treated groups. Stress led to a decrease in gastric VEGF ($P < 0.001$), bFGF ($P < 0.001$) and TGF- α ($P < 0.001$) mRNA levels and caused an increase in EGF mRNA ($P < 0.001$) that was statistically significant compared to the non-stressed control group. Although both treatment agents

exerted similar ulcer reducing ability, only treatment with tocotrienol led to increased expression of VEGF ($P = 0.008$), bFGF ($P = 0.001$) and TGF- α ($P = 0.002$) mRNA.

CONCLUSION

Tocotrienol provides gastroprotective effects in WIRS-induced ulcers. Compared to omeprazole, tocotrienol exerts a similar protective effect, albeit through multiple mechanisms of protection, particularly through up-regulation of growth factors that assist in repair of gastric tissue injuries.

Key words: Tocotrienol; Omeprazole; Restraint-stress; Gastric ulcers; Growth factors

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Core tip: During the process of ulcer healing, growth factors such as epidermal growth factor, transforming growth factor- α , basic fibroblast growth factor and vascular endothelial growth factor acts by activating the migration of cells from the edge of the ulcer and cell proliferation together with the formation of granulation tissue and angiogenesis. Rats exposed to stress develop gastric mucosal ulcers and changes in expression of these growth factors surrounding the ulcers had been reported. Tocotrienol effects on gastric mucosal growth factors were compared to those by omeprazole in this study. The findings suggest that in contrast with omeprazole, tocotrienol has a protective effect on the gastric mucosa through its effect on these growth factors.

Nur Azlina MF, Qodriyah HMS, Chua KH, Kamisah Y. Comparison between tocotrienol and omeprazole on gastric growth factors in stress-exposed rats. *World J Gastroenterol* 2017; 23(32): 5887-5894 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i32/5887.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i32.5887>

INTRODUCTION

Stress ulcers often occur in critically ill patients as a result of major stressful events, such as trauma, shock, surgery, sepsis and burns. The responses to stress are both psychological and physiological. Physiological responses include neurohormonal and immunological activation, which includes release of corticotropin-releasing factor^[1], while the psychological responses include anxiety, depression, feeling of helplessness, fear, *etc.*

The pathological basis for the development of stress ulcers is multifactorial and includes changes in gastric acid secretion^[2], oxidative stress^[3-5], impaired gastric blood flow^[6], reduced gastric prostaglandin synthesis^[7,8], inflammation^[9,10], and inhibition of

mucosal growth and proliferation. Growth factors, by contrast, play pivotal roles in prevention and repair of stress-induced gastric ulcers^[11]. This is particularly true during recovery of the mucosa after stress-induced injuries. Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor- α (TGF- α) and epidermal growth factor (EGF) are crucial for reconstruction of damaged mucosal structures.

Among the growth factors involved, VEGF and bFGF are important factors because of their effects on angiogenesis. These growth factors are produced by endothelial cells, fibroblasts, macrophages and smooth muscle cells, and are involved in the regulation of physiological and pathological angiogenesis^[12]. Angiogenesis and growth factors such as bFGF and VEGF play important roles in the repair of gastric ulcers caused by disturbances in the balance between factors that damage and factors that protect the stomach^[13].

In animal studies, angiogenesis has been shown to play a role in the process of supplying oxygen and nutrients to ulcers of affected areas^[14]. Malara *et al.*^[15] demonstrated a role for VEGF and angiogenesis in the repair of gastric ulcers caused by stress induced in rats. Research has also shown that a significant increase in the expression of VEGF protein followed by the formation of new blood vessels occurred as early as 1 d after formation of the ulcers^[14]. Exogenous bFGF was found to assist in repair of gastric ulcers and other stress, and angiogenesis was also found to reduce gastric acid secretion^[13]. Ernst *et al.*^[13] reported that there was a reduction in stress-induced endogenous bFGF and that this resulted in reduced gastric micro-circulation, which plays an important role in the repair of ulcers. It is unknown if transcription of these growth factors are activated in response to stress-induced gastric injury.

Tocotrienol has been shown to prevent gastric ulcer development in rats exposed to noxious stimuli including ethanol, non-steroidal anti-inflammatory drugs (NSAIDs) and stress^[8,16]. Tocotrienol, in comparison to tocopherol, was reported to be a more potent antioxidant^[17]. Other than its antioxidant capabilities, tocotrienol has also been shown to have anti-inflammatory effects^[10], which may play an effective role in reducing damage to the gastric mucosa due to stress.

The gastric ulcer formation is complex in nature and involves multiple pathways that play a role in the prevention and repair^[18]. The ulcer model that was used in this study was water-immersion restraint stress (WIRS). This experimental model was chosen because of its reproducibility, reliability and validity^[15,19]. This model had been used to mimic clinical acute gastric ulcers formation in critically ill patients and is widely accepted for research involving the mechanism of stress-induced gastric ulcers^[10,12]. The present study evaluated the limited information on the gastro-protective activity of palm-derived tocotrienol with

relation to anti-ulcer properties and gastric growth factors. The purpose of the study is to contribute a better understanding of the pathophysiology of stress-induced gastric ulcers. In this study, tocotrienol was compared to omeprazole, one of the widely used drugs for peptic-ulcer disease in clinical settings.

MATERIALS AND METHODS

Male Wistar rats ($n = 28$) were divided into four equally sized groups. Two control groups were fed a normal rat diet (non-stressed: NS and stressed: S), while the treatment groups received the same diet but with oral supplement of tocotrienol or omeprazole at 60 mg/kg or 20 mg/kg body weight, respectively, for 28 d. The tocotrienol dose was chosen based on our previous studies that demonstrated a protective effect on stress-induced gastric lesions^[10,20]. The tocotrienol and omeprazole were diluted in vitamin-free palm oil, acting as vehicle, and administered by oral gavage using an 18G gavage needle. Both S and NS control groups were administered vitamin-free palm oil.

At the end of the treatment period, rats from the S control group and both treated groups were exposed to WIRS, by placing them in individual plastic restrainers measuring approximately 17 cm × 5 cm and immersing them in water neck deep one time for 3.5 h, as previously described by Aziz Ibrahim *et al.*^[16]. Following the restraining procedure, rats were sacrificed by exsanguination under anesthesia. Stomachs were then dissected along the greater curvature. The dissected stomachs were taken for evaluation of gastric ulcers and mRNA expression of gastric EGF, bFGF, VEGF, and TGF- α . Gastric tissues were homogenized using an Omni Bead Ruptor machine at 25 °C with the speed of 8 m/s for 20 s. Homogenates were centrifuged at 1500 × *g* for 5 min at 4 °C. Supernatants were then used for mRNA analysis.

All rats were kept on a regular night/day cycle, with natural light for a period of 12 h (0700 to 1900 h). Throughout the feeding period, all rats were habituated to handling to reduce stress-related disturbances. Rats were housed in large cages with wide wire-mesh floors to prevent coprophagy. Food and water were given *ad libitum* throughout the experiment. Ethical approval was obtained from Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC). Humane methods of euthanasia were practiced (*i.e.*, exsanguination under anesthesia) by following guidelines of and with approval from UKMAEC. The anesthetic agent used was a combination of ketamine and xylazine (1:1 ratio).

Assessment of stress-induced gastric lesions

The macroscopic assessment of stress-induced gastric lesions in the gastric mucosa was performed by two independent examiners who were blinded to the treatment. The assessment of lesions was done according to a semi-quantitative scale. Lesion size

(mm) was determined by measuring each lesion area. Five petechial lesions were equal to 1 mm lesion. The total lesion area in each group of rats were averaged and expressed as the lesion index; this method was modified as previously described by Aziz Ibrahim *et al.*^[16].

Analysis of EGF, bFGF, VEGF and TGF- α mRNA expression

mRNA levels of EGF, bFGF, VEGF and TGF- α from gastric tissues were assayed according to the manufacturer's instructions using the standard QuantiGene Plex 2.0 assay kit (Genospectra, Fremont, CA, United States). Briefly, tissue lysates were transferred to a capture well in the presence of the gene-specific probe set and then hybridized at 53 °C overnight. Wells were washed twice with bDNA wash buffer and then incubated at 46 °C sequentially with an amplifier and an alkaline phosphatase-labeled probe, with a wash step in between incubations. After the final wash step, addition of streptavidin phycoerythrin generated a signal that was proportional to the amount of target RNA present in the sample. The luminescence signal was detected using a Luminex instrument. The protocol was followed as previously described by Zhang *et al.*^[21].

Statistical analysis

Statistical analyses were performed using PRISM software version 6.00 (Graphpad, San Diego, CA, United States). The results are expressed as the mean ± SE of the mean. Statistical significance ($P < 0.05$) was determined by ANOVA and Tukey's post-hoc test.

RESULTS

Macroscopic observation

Exposure to WIRS for 3.5 h caused the formation of ulcers in the form of gastric mucosal erosion and ulcers which were confined to the corpus of the stomach. The gastric lesion index (area in mm²) in the stressed-exposed (S) group was increased (11.92 ± 2.0 mm²; $P = 0.001$) compared to the tocotrienol-treated group (0.94 ± 0.30 mm²) and the omeprazole-treated group (2.44 ± 0.7 mm²), as shown in Figure 1A. Rats not exposed to stress did not develop any gastric lesions.

Quantitative changes in gastric VEGF mRNA expression in response to stress

Figure 1B shows that VEGF mRNA expression in stressed control rats was decreased by 45% compared to NS rats ($P < 0.0001$). Pre-treatment with tocotrienol caused a statistically significant increase in VEGF expression compared to the stressed control group ($P = 0.0075$). However, pre-treatment with omeprazole did not enhance VEGF expression compared to the S control group ($P = 0.0593$).

Quantitative changes in gastric EGF mRNA expression in response to stress

Stress exposure caused an increase in EGF gene

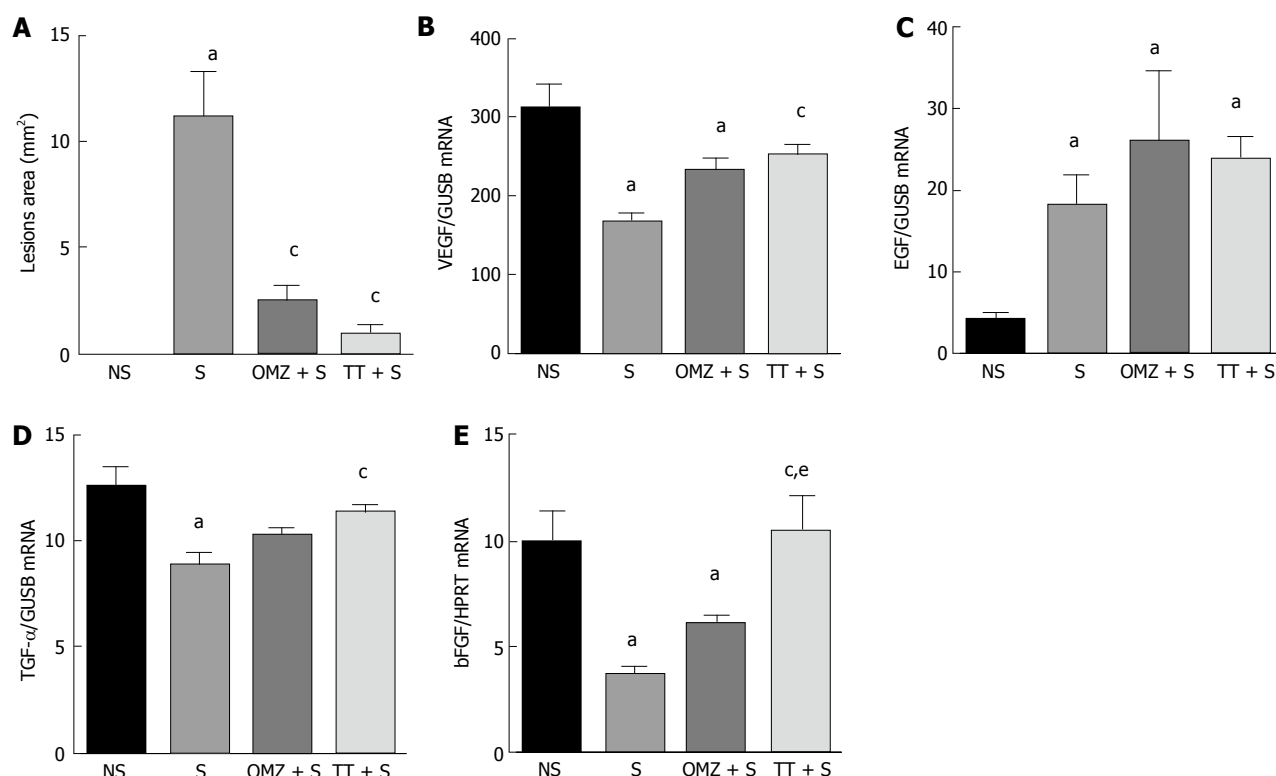


Figure 1 Effects of pre-treatment with tocotrienol (60 mg/kg body weight) or omeprazole (20 mg/kg body weight) on gastric lesions formation (A), and gastric vascular endothelial growth factor (B), epidermal growth factor (C), transforming growth factor- α (D) and basic fibroblast growth factor (E) mRNA expression in rats exposed to water immersion restraint stress ($n = 7$). ^a $P < 0.05$ vs NS non-stressed control (NS); ^c $P < 0.05$ vs Stressed control (S); ^e $P < 0.05$ vs Omeprazole (OMZ + S). bFGF: Basic fibroblast growth factor; BW: Body weight; EGF: Epidermal growth factor; NS: Non-stressed; OMZ: Omeprazole; S: Stressed; TGF- α : Transforming growth factor-alpha; TT: Tocotrienol; VEGF: Vascular endothelial growth factor.

expression ($P = 0.0001$). Expression was increased in all groups exposed to stress and no differences were observed between the three stressed groups (S, tocotrienol-treated and omeprazole-treated) (Figure 1C), suggesting that tocotrienol and omeprazole had no effect on EGF mRNA levels in stress-exposed rats.

Quantitative changes in gastric TGF- α mRNA expression in response to stress

Exposure to immobilization stress caused a decrease in expression of TGF- α in gastric tissue (Figure 1D). Stress caused a significant reduction in the gastric expression as shown. Pre-treatment with tocotrienol caused an increase in TGF- α gene expression compared to the S control group ($P = 0.0024$). Similar levels of TGF- α were observed in the NS control group. This result suggests that tocotrienol had a protective effect by preventing the stress-induced decrease of TGF. By contrast, TGF- α gene expression in the omeprazole-treated rats was decreased to levels similar to those observed in the S control group ($P > 0.999$).

Quantitative changes in gastric bFGF mRNA expression in response to stress

Stress caused a 63% decrease in bFGF gene expression ($P = 0.0022$), as compared to NS rats. bFGF expression in the tocotrienol-treated group was increased compared

to stressed control rats ($P = 0.0013$) and there was no statistically significant change in bFGF levels when compared with the NS control rats. This suggests that pre-treatment with tocotrienol protects stress-induced rats by preventing a decrease in the level of bFGF to a level that was similar to that observed in the NS controls. bFGF expression in the tocotrienol-treated group was increased by 43% compared to the omeprazole-treated group ($P = 0.0492$). Omeprazole treatment had no protective effect when the bFGF levels were similar to those observed in S rats, as shown in Figure 1E.

DISCUSSION

Various genes are regulated at different rates and times during and after gastric mucosal injury. Most data regarding gene expression after mucosal injury models are taken from experimental animals. Together with the formation of granulation tissue, blood vessel and angiogenesis, growth factors act to enable the migration of cells from the edge of the ulcer and induce cell proliferation during the process of ulcer healing^[7,22]. For example, EGF mRNA is detected immediately after ulcer induction, peaks during day 3, and continues to decrease 10 d after the induction of ulcers, whereas TGF- α mRNA expression increases 6 d after injury^[23].

VEGF is a growth factor that helps tissue healing

by stimulating angiogenesis, and is also important for the formation of connective tissue^[24,25]. Antonisamy *et al.*^[26] showed that injury to the gastric mucosa through administration of indomethacin caused a striking decrease in VEGF content in the gastric mucosa. Findings from this study also showed that exposure to WIRS caused a significant decrease in VEGF gene expression in gastric tissue compared to rats that were not exposed to stress. In another study, pre-treatment with a single dose of oral VEGF protected the stomach against damage due to acute ethanol administration^[27]. Furthermore, gastric ulcer healing was prolonged and angiogenesis was decreased in response to a reduction in expression of VEGF^[28]. Finally, up-regulation of VEGF has been shown to play an important role in the healing of acute gastric injury^[29].

Our findings show that tocotrienol led to increased VEGF expression in stress-induced rats. Studies that evaluate the effect of tocotrienols on growth factor expression in gastric ulcers are limited. δ -tocotrienol has been shown to decrease the expression of VEGF in tumor cells, thereby reducing angiogenesis in these cells^[30-32]. The tocotrienol administered in this study contained less than 4% δ -tocotrienol and consisted mostly of other isomers, the most being isomeric γ -tocotrienol (approximately 50%). The effect obtained from this study leads us to assume that the other isomers in the tocotrienol mixture used in this study might assist in the healing process of ulcers or provide gastric mucosal protection against injury by promoting the process of granulation tissue formation and angiogenesis through VEGF expression.

Unlike tocotrienol, omeprazole administration did not decrease VEGF expression in stress-induced rats. This is inconsistent with results from a study by Kobayashi *et al.*^[30] that showed that administration of lansoprazole (another proton pump inhibitor) led to increased expression of the VEGF gene in rats with gastric ulcers induced with acetic acid. These results suggest that proton pump inhibitors have an additional impact on protection of gastric ulcer formation (*i.e.*, regulation of growth factor expression) other than prevention of gastric acid secretion^[30]. However, this effect was not observed in our study using omeprazole, suggesting that not all proton pump inhibitors have the same effect on growth factors. Abdul-Aziz *et al.*^[27] reported similar results, *i.e.*, omeprazole reduced gastric ulcers but did not enhance VEGF expression levels.

In addition to VEGF, the growth of the mucosa is under the influence of various other growth factors, such as EGF and polyamines, which play important roles in tissue maintenance and repair. EGF is secreted into the intestinal lumen and into the bloodstream after being produced in saliva and pancreatic gland, and excreted *via* the urine as urogastrone^[33]. It is well established that EGF is necessary for the maintenance of mucosal integrity. Furthermore, accumulation of EGF in the region of gastric mucosal injury promotes

the local lesion healing process^[1].

Milani *et al.*^[24] showed that expression of growth factors detected in gastric mucosal cells, constantly fluctuates even under normal conditions. In the absence of induction of lesions, vitamin E does not significantly affect the function of growth factors where a decline in immunoreactivity of the EGF receptor (EGF-R) has been reported^[34]. The results of this study also showed that rats that were not exposed to stress had low levels of EGF gene expression. Exposure to NSAIDs causes gastric ulcers to form and leads to decreased levels of EGF in gastric mucosa^[26]. Increased expression of both EGF and EGF-R in the ulcer area also contributes to the repair process^[35]. This reaction may occur in response to the sharp decline of this growth factor during gastric injury.

Exposure to stress led to formation of gastric lesions and caused an increase in gene expression of EGF that was statistically significant compared to the non-stressed control group. Increased expression of EGF has been reported to accelerate healing of gastroduodenal ulcers by increasing gastric mucin production and reducing gastric acid secretion^[26]. This indicates that expression of EGF levels increase in response to injury of the gastric mucosa in order to restore the tissue back to its original state.

In this study, however, pre-treatment with tocotrienol or omeprazole did not change gastric tissue EGF expression; expression of EGF remained elevated in stress-exposed rats compared to rats that were not exposed to stress. This may have occurred due to the presence of gastric tissue injury in the treatment group that could have led to increased expression of EGF, which accelerates the recovery of gastric tissue caused by stress. Studies by Qodriyah *et al.*^[34] showed that EGF levels were increased compared to the control group under normal conditions after 8 wk of palm vitamin E (PVE) treatment. The results of their study suggest that, under normal circumstances, vitamin E also enhances expression of EGF. While in a state of gastric injury due to NSAIDs, expression of EGF remained elevated after pre-treatment with PVE^[34], consistent with the results of this study.

EGF released from the salivary glands and TGF- α from the gastric mucosa act to maintain mucosal integrity and recovery during gastric mucosal injuries. Both these growth factors produce the same biological activities during recovery. For example, TGF- α and bFGF levels change when an injury occurs in the gastric mucosa. In this study, exposure to stress caused a significant decrease in bFGF gene expression. bFGFs have been shown to play a role in both angiogenesis and recovery of gastric ulcers in rats^[36,37]. bFGF activation occurs in response to injury of the gastric mucosa, as demonstrated by the increased expression observed near ulcers^[38]. Administration of bFGF (100 ng) locally into ulcers or systemically caused significant recovery in acetic acid-induced gastric ulcers^[37]. bFGFs are also known to stimulate synthesis of prostaglandins

locally^[30,39], leading to increased formation of blood vessels^[14,40] as well as proliferation of endothelial cells^[41], sustaining and assisting the recovery of gastric tissues in the event of injury.

Pre-treatment with tocotrienol in this study led to increased expression of bFGF in rats that were exposed to stress. Vitamin E at 150 mg/kg has been shown to improve bFGF expression in mice that developed gastric mucosal injury due to NSAID exposure^[34,42]. Rashid *et al.*^[42] showed that tocotrienol increased bFGF levels, thus reducing the formation of scar tissue. However, our study found that omeprazole had no effect on bFGF gene expression, which is in contrast to the tocotrienol-treated group. Studies that examine bFGF expression in response to omeprazole treatment are limited. Tsuji *et al.*^[43] found that administration of lansoprazole, also a proton pump inhibitor, helped repair gastric ulcers by increasing bFGF levels at the edge of the ulcer border. Pantoprazole helped promote angiogenesis in gastric lesions induced by NSAIDs through increased expression of bFGF^[44]. This effect, however, was not observed with pre-treatment of omeprazole in this study.

Growth factors such as EGF, TGF- α , bFGF and VEGF activate migration and proliferation of cells at the edge of the ulcer and promote the formation of granulation tissue and angiogenesis during the process of ulcer repair^[22]. EGF is required to maintain the integrity of gastric mucosa and promotes healing of injured tissue^[26]. TGF- α accelerates replacement of the epithelium and regulates regeneration of epithelial cells in gastric tissues^[33]. VEGF assists in repair of ulcers by stimulating angiogenesis and remodeling of connective tissues^[24], while bFGF has been known to stimulate synthesis of local prostaglandins, which ultimately leads to increased formation of blood vessels^[14,40] and endothelial cell proliferation^[41]. This assists in maintenance and recovery of gastric tissue in the event of injury. The results of this study suggest that in contrast with omeprazole, tocotrienol has a protective effect on the gastric mucosa through regulation of these growth factors.

Here, we show that tocotrienol provides a gastro-protective effect in WIRS-induced ulcers and exerts similar effectiveness when compared to omeprazole. However, it displays a more diverse mechanism of protection, particularly through increased expression of bFGF, TGF- α and VEGF in a stress-induced gastric ulcer rat model in comparison to omeprazole. Thus, the effect of tocotrienol might be of therapeutic interest for the prevention and repair of gastric mucosal injuries due to other mechanisms.

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COMMENTS

Background

Stress is well known to induce gastric ulcers. Although the proposed pathogenesis is multifactorial, a common entity observed in peptic ulcer diseases is oxidative stress which overwhelms the endogenous antioxidant system. Thus, prevention and treatment using an antioxidant like tocotrienol is a logical therapeutic approach. This study focuses on the therapeutic ability of tocotrienol on reducing stress-induced gastric ulcers and its effects on gastric growth factors which plays an important role in the prevention and repair of ulcers.

Research frontiers

Few studies had investigated the effect of tocotrienol from palm source on gastric growth factors.

Innovations and breakthrough

Tocotrienol provides gastroprotective effect in water-immersion restraint stress-induced ulcers. Although tocotrienol provides similar effectiveness as compared to omeprazole, it has a more diverse mechanism of protection, particularly through up-regulation of basic fibroblast growth factor, transforming growth factor- α and vascular endothelial growth factor in a stress-induced gastric ulcer model.

Applications

Tocotrienol as therapeutic agent for the prevention and enhancing the repair of gastric mucosa against injuries.

Terminology

Stress ulcers can occur as a result of major stressful events, such as trauma, shock, surgery, sepsis and burns. Tocotrienol prevents gastric ulcer development in rats exposed to noxious stimuli including ethanol, non-steroidal anti-inflammatory drugs and stress.

Peer-review

The research is well conducted and the paper is well written. The series of experiments conducted were able to answer the objective of the study and the statistical tests used were scientifically sound.

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Basic Study

(-)-Epigallocatechin-3-gallate enhances poly I:C-induced interferon- λ 1 production and inhibits hepatitis C virus replication in hepatocytes

Yi-Zhong Wang, Jie-Liang Li, Xu Wang, Ting Zhang, Wen-Zhe Ho

Yi-Zhong Wang, Ting Zhang, Department of Infectious Diseases, Shanghai Children's Hospital, Shanghai Jiao Tong University, Shanghai 200040, China

Jie-Liang Li, Xu Wang, Wen-Zhe Ho, Department of Pathology and Laboratory Medicine, Temple University Lewis Katz School of Medicine, Philadelphia, PA 19140, United States

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Correspondence to: Ting Zhang, MD, PhD, Department of Infectious Diseases, Shanghai Children's Hospital, Shanghai Jiao Tong University, 355 Luding Road, Shanghai 200040, China. zhangt@shchildren.com.cn

Telephone: +86-21-52976338

Fax: +86-21-52976338

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Abstract

AIM

To investigate the effect of (-)-epigallocatechin-3-gallate (EGCG) on polyinosinic-polycytidylic acid (poly I:C)-triggered intracellular innate immunity against hepatitis C virus (HCV) in hepatocytes.

METHODS

A cell culture model of HCV infection was generated by infecting a hepatoma cell line, Huh7, with HCV JFH-1 strain (JFH-1-Huh7). Poly I:C with a high molecular weight and EGCG were used to stimulate the JFH-1-Huh7 cells. Real-time reverse transcription-polymerase chain reaction was used to detect the expression levels of intracellular mRNAs and of intracellular and extracellular HCV RNA. Enzyme-linked immunosorbent assay was used to evaluate the interferon (IFN)- λ 1 protein level in the cell culture supernatant. Immunostaining was used to examine HCV core protein expression in Huh7 cells.

RESULTS

Our recent study showed that HCV replication could impair poly I:C-triggered intracellular innate immune responses in hepatocytes. In the current study, we

showed that EGCG treatment significantly increased the poly I:C-induced expression of Toll-like receptor 3 (TLR3), retinoic acid-inducible gene I, and IFN- λ 1 in JFH-1-Huh7 cells. In addition, supplementation with EGCG increased the poly I:C-mediated antiviral activity in JFH-1-Huh7 cells at the intracellular and extracellular HCV RNA and protein levels. Further investigation of the mechanisms showed that EGCG treatment significantly enhanced the poly I:C-induced expression of IFN-regulatory factor 9 and several antiviral IFN-stimulated genes, including *ISG15*, *ISG56*, myxovirus resistance A, and 2'-5'-oligoadenylate synthetase 1, which encode the key antiviral elements in the IFN signaling pathway.

CONCLUSION

Our observations provide experimental evidence that EGCG has the ability to enhance poly I:C-induced intracellular antiviral innate immunity against HCV replication in hepatocytes.

Key words: (-)-Epigallocatechin-3-gallate; Toll-like receptor 3; Retinoic acid-inducible gene I; IFN- λ 1; Hepatitis C virus; IFN-stimulated genes

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Core tip: The interactions between hepatitis C virus (HCV) and the host immune system in the liver play a key role in the immunopathogenesis of HCV-induced diseases. We showed here that (-)-epigallocatechin-3-gallate (EGCG) treatment could significantly increase the poly I:C-induced expression of TLR3, RIG-I and interferon (IFN)- λ 1 in JFH-1-Huh7 cells. In addition, supplementation with EGCG enhanced poly I:C-mediated viral inhibition in JFH-1-Huh7 cells at both RNA and protein levels. Further investigation of the mechanisms showed that EGCG treatment significantly enhanced the poly I:C-induced expression of IFN-regulatory factor 9 and several IFN-stimulated genes. It would be interesting to investigate the possible use of EGCG in combination with current antiviral drugs for HCV therapy.

Wang YZ, Li JL, Wang X, Zhang T, Ho WZ. (-)-Epigallocatechin-3-gallate enhances poly I:C-induced interferon- λ 1 production and inhibits hepatitis C virus replication in hepatocytes. *World J Gastroenterol* 2017; 23(32): 5895-5903 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i32/5895.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i32.5895>

INTRODUCTION

Hepatitis C virus (HCV) is a hepatotropic virus, which holds a single-stranded, positive-sense RNA genome of 9.6 kb in length^[1]. Currently, around 150 million people are chronically infected with HCV worldwide^[2].

HCV infection causes liver diseases ranging from mild to moderate and severe, such as hepatitis, cirrhosis, and hepatocellular carcinoma^[3]. The host immune responses play pivotal roles in the pathogenesis of viral infection, and the interplays of host antiviral immunity and HCV determine the outcomes of HCV infection^[4]. It has been demonstrated that host immune responses are triggered by HCV, however, it is less effective in clearing the virus and leading a high rate of chronic infection in the majority of cases^[5]. Acute HCV infection is accompanied by a spontaneous viral clearance in only about 20% to 30% of the subjects^[2]. Recent studies showed that HCV has evolved several strategies to escape the attacks mediated by the host antiviral immunity^[6]. Nonstructural (NS) 3/4A protease encoded by HCV genome has been showed to be able to impair the host antiviral innate immunity by disrupting the signaling of several pattern recognition receptors (PPRs), such as Toll-like receptor 3 (TLR3) and retinoic acid-inducible gene I (RIG-I)^[7-9]. HCV NS4B blocks interferon (IFN) production by disrupting the interaction of STING with mitochondrial antiviral signaling protein (MAVS) and TBK1^[10,11]. Furthermore, host genetic factors play important roles in controlling HCV infection. Studies have shown that polymorphisms located in the IFN- λ region are related with the rate of viral clearance in HCV-infected individuals without treatment, as well as the sustained viral response (SVR) rate to the IFN- α -based treatment^[12-15]. To date, tremendous progress has been made in understanding the biology of HCV and its related disease. Important advances in characterizing the HCV life cycle have led to the discovery of direct-acting antivirals (DAAs), which were developed for the effective treatment of chronic HCV infection^[16,17]. However, it is still challenging to dramatically decrease the incidence of HCV infection in the near future. High costs, low barrier to resistance-associated mutations, viral reinfection, failure of the DAAs in HCV-induced liver diseases, and lack of effective vaccines are obstacles to overcoming HCV infection globally^[2].

(-)-Epigallocatechin-3-gallate (EGCG), the most abundant and bioactive catechin in green tea, possesses various physiological and pharmacological benefits to human health. Previous studies^[18-20] revealed that EGCG exhibits abilities against viral infection and can prevent cardiovascular diseases, metabolic syndrome, neurodegenerative diseases, and cancer. It has been shown that EGCG can inhibit the replication of several viruses, such as human immunodeficiency virus^[19], herpes simplex virus^[21], as well as influenza virus^[22]. Recent studies^[23,24] have revealed that EGCG can act as an inhibitor of HCV entry and limit intercellular spread of HCV. EGCG is a potent antioxidant that has both anti-inflammatory and anti-atherogenic properties^[25-27]. Our previous study^[27] indicated that EGCG inhibits lipopolysaccharide-induced inflammatory cytokine expression in microvascular

Table 1 Primers used for real-time reverse transcription-polymerase chain reaction

Primer	Orientation	Sequences (5'-3')
GAPDH	Forward	GGTGGTCTCCTCTGACTTCAACA
	Reverse	GTGTCTGTAGCCAAATTCGTGT
IFN- λ 1	Forward	CTTCCAAGCCACCCCAACT
	Reverse	GGCCTCCAGGACCTTCAGC
TLR3	Forward	AGCCACCTGAAGTTGACTCAGG
	Reverse	CAGTCAAATTCGTGCAGAAGGC
RIG-I	Forward	CTTGGCATGTTACACAGCTGAC
	Reverse	GCTTGGGATGTGGTCTACTCA
IRF-9	Forward	GCATCAGGCAGGGCAGCTGCACCCG
	Reverse	GCCTGCATGTTTCCAGGGAATCCGG
ISG15	Forward	GGCTGGGAGCTGACGGTGAAG
	Reverse	GCTCCGCCCGCCAGGCTCTGT
ISG56	Forward	TTCGGAGAAAGGCATTAGA
	Reverse	TCCAGGCTTCATTCATAT
MxA	Forward	GCCGGCTGTGGATATGCTA
	Reverse	TTTATCGAAACATCTGTGAAAGCAA
OAS-1	Forward	AGAAGGCAGCTACGAAACC
	Reverse	CCACCACCCAAGTTCTCTGTA
HCV	Forward	RAYCACTCCCTGTGAGGAAC
	Reverse	TGRTGCACGGTCTACGAGACCTC

HCV: Hepatitis C virus; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IFN: Interferon; TLR3: Toll-like receptor 3; RIG-I: Retinoic acid-inducible gene I.

endothelial cells. In addition, EGCG exhibits anticancer functions through inhibition of proteasome activity and induction of endoplasmic reticulum stress^[28,29]. Furthermore, it was also shown that EGCG increases the formation of lipid droplets and inhibits the secretion of very low-density lipoproteins in human hepatocytes^[30].

Our recent study showed that HCV replication could impair the polyinosinic-polycytidylic acid (poly I:C)-triggered intracellular innate immune signaling pathway in hepatocytes^[31]. Furthermore, we showed that EGCG possesses the ability to increase HCV dsRNA intermediate-induced expression of IFN- λ 1 and IFN-stimulated genes (ISGs)^[32]. In this study, we investigated the effect of EGCG on poly I:C-induced IFN pathway activation and its antiviral activities.

MATERIALS AND METHODS

EGCG

EGCG (purity, $\geq 95\%$) was purchased from SIGMA-ALDRICH, St. Louis, MO, United States (CAS#: 989-51-5; Cat# E4143). EGCG stock solution was prepared with sterile double distilled water at a concentration of 20 mmol/L.

Cells, virus, and reagents

The hepatoma cell line (Huh7), provided by Dr. Charles Rice (Rockefeller University, NY, United States), was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL). The generation of infectious HCV JFH-1 and infection of Huh7 cells (multiplicity of infection

of 0.01) were carried out as previously described^[33]. HCV JFH-1 infection of Huh7 cells was analyzed by immunostaining with mouse anti-HCV core antibody or by real-time reverse transcription-polymerase chain reaction (RT-PCR) for HCV RNA. LyoVec transfection reagent and poly I:C with a high molecular weight were purchased from InvivoGen (San Diego, CA, United States). An enzyme-linked immunosorbent assay (ELISA) kit for IFN- λ 1 was purchased from eBioscience Inc. (San Diego, CA, United States). Mouse antibody against the HCV core antigen was purchased from ABR Affinity BioReagents, Thermo Scientific (Rockford, IL, United States). Hoechst 33342 was purchased from Molecular Probes (Carlsbad, CA, United States).

EGCG treatment and poly I:C stimulation

The JFH-1-infected Huh7 cells (72 h post-infection) were treated with EGCG (1-10 μ mol/L) for 1 h prior to poly I:C (1 μ g/mL) stimulation with LyoVec transfection reagent. The cells were collected for total RNA extraction after 24 h or 48 h of stimulation, and the supernatant (SN) was collected for ELISA after 48 h of stimulation. As a negative control of the transfection experiment, cells were incubated with the LyoVec transfection reagent without poly I:C.

RNA extraction and real-time RT-PCR

Total RNA from the cultured cells or SN was extracted with TRI Reagent (Molecular Research Center, Cincinnati, OH, United States) and subjected to reverse transcription (RT) using the RT system (Promega, Madison, WI, United States) with random primers for 1 h at 42 °C. The reaction was terminated by incubating the reaction mixture at 99 °C for 5 min, and the mixture was kept at 4 °C. The resulting cDNA was used as a template for quantitative real-time PCR. Real-time PCR was performed with 1/10 of the cDNA with the iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, United States). The amplified products were visualized and analyzed using the software MyiQ provided with the thermocycler (iCycler iQ real-time PCR detection system; Bio-Rad Laboratories). The oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, United States), and the sequences are shown in Table 1. The cDNA was amplified by PCR, and the products were measured using SYBR green I (Bio-Rad Laboratories, Inc., Hercules, CA, United States). The data were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and presented as the change in induction relative to that of the untreated control cells.

ELISA

IFN- λ 1 protein expression was evaluated by ELISA. SN collected from EGCG and/or poly I:C-treated Huh7 cell cultures was directly tested for IFN- λ 1 protein levels by ELISA, which was performed according to the

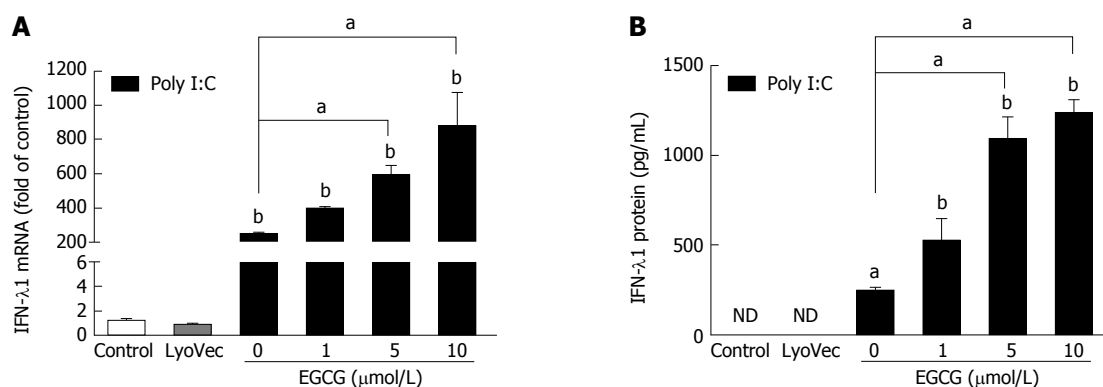


Figure 1 (-)-Epigallocatechin-3-gallate enhances poly I:C-induced interferon-λ1 expression in JFH-1-Huh7 cells. JFH-1-infected Huh7 cells (72 h post-infection) were treated with EGCG at the indicated concentrations for 1 h prior to poly I:C (1 μg/mL) stimulation. Total RNA extracted from cells after 24 h of stimulation was subjected to real-time reverse transcription-polymerase chain reaction for the determination of IFN-λ1 and glyceraldehyde-3-phosphate dehydrogenase mRNA levels. The data are expressed as IFN-λ1 mRNA (A) levels relative (fold) to the control (vehicle only, which is defined as 1). After 48 h of stimulation, the supernatant (SN) was collected from the cell cultures for the determination of IFN-λ1 protein levels by enzyme-linked immunosorbent assay (B). The results shown are the mean ± SD of triplicate measurements representative of three experiments (Poly I:C vs LyoVec, ^a*P* < 0.05, ^b*P* < 0.01). IFN: Interferon; EGCG: (-)-Epigallocatechin-3-gallate.

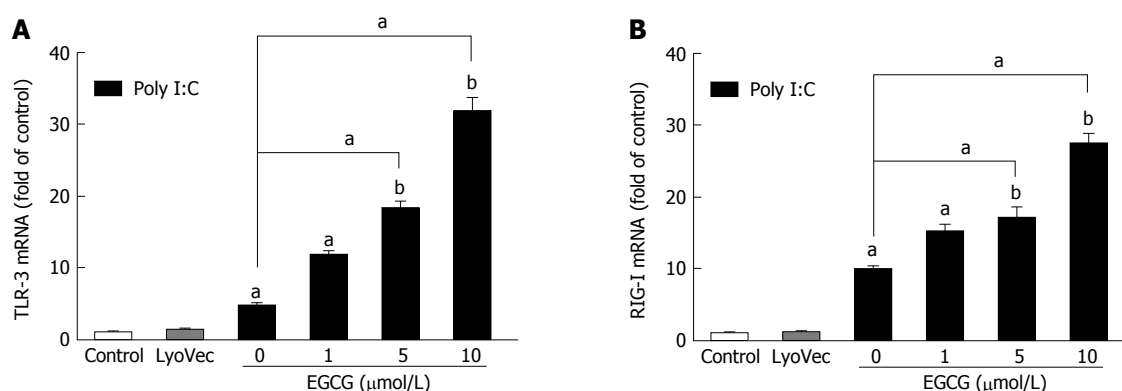


Figure 2 (-)-Epigallocatechin-3-gallate increases poly I:C-induced *TLR3* and *RIG-I* mRNA expression in JFH-1-infected Huh7 cells. JFH-1-infected Huh7 cells (72 h post-infection) were treated with (-)-epigallocatechin-3-gallate (EGCG) at the indicated concentrations for 1 h prior to poly I:C (1 μg/mL) stimulation. Total RNA extracted from the cells after 24 h of stimulation was evaluated for *TLR3* (A) and *RIG-I* (B) gene expression by real-time RT-PCR. The results shown are the mean ± SD of triplicate measurements representative of three experiments (Poly I:C vs LyoVec, ^a*P* < 0.05, ^b*P* < 0.01). TLR3: Toll-like receptor 3; RIG-I: Retinoic acid-inducible gene I.

manufacturer's instructions.

Statistical analysis

Student's *t*-test was used to evaluate the significance of difference between the groups, and multiple comparisons were performed by regression analysis and one-way analysis of variance. All data are presented as the mean ± SD. Statistical analyses were performed with SPSS 11.5 for Windows. Statistical significance was defined as *P* < 0.05.

RESULTS

EGCG enhances poly I:C-induced IFN-λ1 expression in JFH-1-Huh7 cells

In order to test the effect of EGCG on poly I:C-induced IFN-λ1 expression, we treated JFH-1-Huh7 cells (72 h post-infection) with EGCG (1–10 μmol/L) for 1 h before poly I:C treatment. The data showed that EGCG could significantly increase poly I:C-mediated IFN-λ1 mRNA expression (Figure 1A), as well as IFN-λ1 protein

production (Figure 1B), in a dose-dependent manner, whereas EGCG treatment alone had a negligible effect on IFN-λ1 expression in JFH-1-infected Huh7 cells^[32].

EGCG enhances poly I:C-induced TLR3 and RIG-I mRNA expression in JFH-1-Huh7 cells

TLR3 and RIG-I are major cellular receptors that recognize pathogen-associated molecular patterns (PAMPs) during RNA virus infections. While EGCG (at a concentration lower than 10 μmol/L) treatment alone showed little effect on TLR3 and RIG-I expression^[32], EGCG significantly increased the poly I:C-induced mRNA expression of *TLR3* (Figure 2A) and *RIG-I* (Figure 2B) in JFH-1-Huh7 cells.

EGCG contributes to poly I:C-mediated inhibition of HCV replication

Our previous study^[32] showed that when we treated JFH-1-Huh7 cells with EGCG (1–10 μmol/L) alone, EGCG could not inhibit viral replication. We found that poly I:C treatment also had limited antiviral effect on

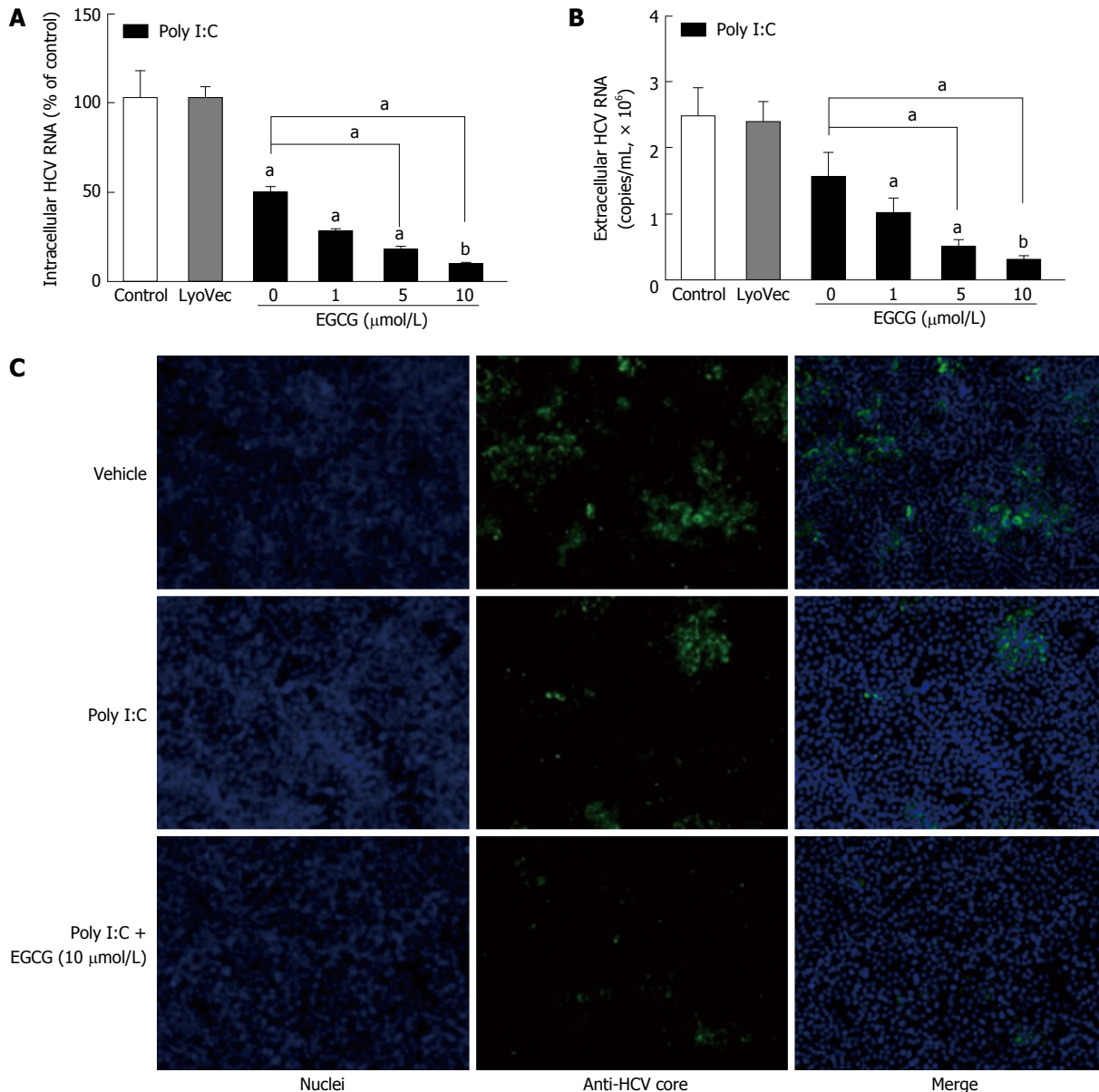


Figure 3 (-)-Epigallocatechin-3-gallate contributes to poly I:C-mediated inhibition of hepatitis C virus replication. JFH-1-infected Huh7 cells (72 h post-infection) were treated with EGCG at the indicated concentrations for 1 h prior to poly I:C (1 μg/mL) stimulation. Intracellular (A) and extracellular (B) RNA was extracted from the JFH-1-infected Huh7 cells or culture SN after 48 h of stimulation and subjected to real-time reverse transcription-polymerase chain reaction for HCV and GAPDH RNA quantification. The intracellular hepatitis C virus (HCV) RNA level is expressed as the HCV RNA level relative (%) to the control (vehicle only, which is defined as 100%). Extracellular RNA levels are expressed as copies/mL. The results shown are the mean ± SD of triplicate cultures representative of three experiments (Poly I:C vs LyoVec, ^a*P* < 0.05, ^b*P* < 0.01). HCV core protein expression (C) was determined by immunofluorescence staining with an antibody against the HCV core protein (green) after 48 h of stimulation. The nuclei were stained with Hoechst 33342 (blue). One representative experiment is shown (original magnification: × 200). EGCG: (-)-Epigallocatechin-3-gallate.

the HCV-infected Huh7 cells (Figure 3). When the JFH-1-Huh7 cells were treated with EGCG 1 h before poly I:C stimulation, EGCG enhanced the poly I:C-mediated HCV inhibition in Huh7 cells in a dose-dependent manner (Figure 3). The inhibition of HCV by EGCG and poly I:C was confirmed by examining the intracellular (Figure 3A) and extracellular levels (Figure 3B) of HCV RNA and the level of HCV core protein (Figure 3C).

EGCG enhances poly I:C-induced *IRF-9* expression in JFH-1-Huh7 cells

As *IRF-9* plays a critical role in the induction of antiviral

ISGs, we further investigated the impact of EGCG on poly I:C-induced *IRF-9* mRNA expression. While EGCG alone had little impact on *IRF-9* mRNA expression (data not shown), EGCG pretreatment could significantly enhance poly I:C-induced *IRF-9* mRNA expression in JFH-1-Huh7 cells (Figure 4).

EGCG enhances poly I:C-induced expression of ISGs in JFH-1-Huh7 cells

EGCG dose-dependently enhanced the poly I:C-induced expression of *ISG15* (Figure 5A), *ISG56* (Figure 5B), *MxA* (Figure 5C), and *OAS-1* (Figure 5D) in JFH-1-

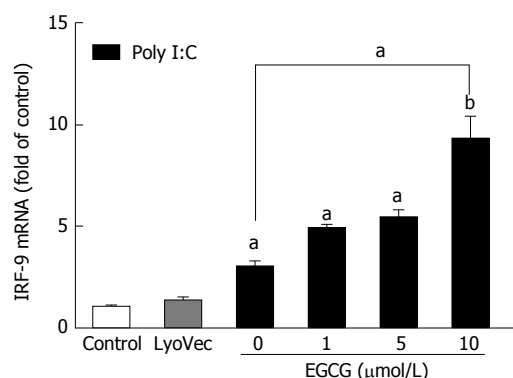


Figure 4 (-)-Epigallocatechin-3-gallate increases poly I:C-induced IRF-9 expression in JFH-1-Huh7 cells. JFH-1-infected Huh7 cells (72 h post-infection) were treated with EGCG at the indicated concentrations for 1 h prior to poly I:C (1 μ g/mL) stimulation. Total RNA extracted from the cells after 24 h of stimulation was subjected to real-time reverse transcription-polymerase chain reaction for the analysis of *IRF-9* gene expression. The results shown are the mean \pm SD of triplicate measurements representative of three experiments (Poly I:C vs LyoVec, ^a $P < 0.05$, ^b $P < 0.01$). EGCG: (-)-Epigallocatechin-3-gallate.

infected cells. However, EGCG treatment alone showed a negligible impact on the expression of those ISGs (data not shown).

DISCUSSION

In the current study, we showed that EGCG could enhance the poly I:C-induced innate immune responses in hepatocytes, which contributed to poly I:C-mediated HCV inhibition. Although poly I:C could induce the expression of *IFN- λ 1* and several antiviral ISGs in JFH-1-infected Huh7 cells, the expression level was restricted by existing virus^[31]. Our data indicated that EGCG significantly enhanced the poly I:C-induced expression of *IFN- λ 1*, *TLR3*, *RIG-I*, *ISG15*, *ISG56*, *MxA*, and *OAS-1* in JFH-1-infected Huh7 cells. More importantly, pretreatment with EGCG enhanced poly I:C-mediated viral inhibition in JFH-1-infected cells. It has been demonstrated that *IFN- λ* could inhibit HCV replication, and recombinant *IFN- λ 1* was used as an antiviral drug in HCV treatment trials^[34,35]. The basic level of *IFN- λ 1* is very low in human hepatocytes, but it could be triggered by viral infections through activation of PPRs, such as *TLR3*^[31,36]. Our previous study^[31] indicated that poly I:C treatment induces *IFN- λ 1* expression; however, HCV replication could impair the poly I:C-triggered *TLR3* signaling pathway and decrease *IFN- λ 1* induction in Huh7 cells. In addition, HCV induced *IFN- λ 1* expression in primary human hepatocytes could resist against HCV^[36]. Thus, the anti-HCV effect mediated by EGCG and poly I:C combination treatment in JFH-1-infected cells was likely through the enhancement of intracellular *IFN- λ 1* expression.

The interplays of HCV and the host antiviral immunity play important roles in the pathogenesis of HCV-related diseases. The innate immunity is the

front line of host defenses against pathogen infections. TLRs and RIG-I-like receptors (RLRs) are major cellular receptors that recognize PAMPs during viral infections^[37]. Among these TLR members, *TLR3* is triggered by dsRNAs from the genome of some RNA viruses or intermediates formed during viral genome replication, such as HCV dsRNA intermediates^[38,39]. In addition to *TLR3*, *RIG-I* has been demonstrated to recognize HCV genome, inducing immune antiviral responses through the type I IFN signaling activation^[40]. Our data showed that EGCG enhanced poly I:C-induced *TLR3* and *RIG-I* expression in HCV-infected hepatocytes, which may contribute to the activation of IFN signaling to inhibit viral replication.

In order to maintain a persistent infection, HCV evolves several strategies to escape host antiviral immune responses^[6]. It has been shown that a protease of HCV, NS3/4A, could disrupt both *TLR3* and *RIG-I* activation through cleaving the cellular adaptor molecules, TRIF and MAVS, respectively^[7-9]. HCV NS4B blocks IFN production by disrupting the interaction of STING with MAVS and TBK1^[10,11]. Studies also revealed that HCV impairs *IFN- λ 7* translocation, and inhibits both *IFN- α* and *IFN- λ 1* expression in hepatocytes^[31,41,42]. Therefore, how to rescue the host antiviral immunity impaired by existing viruses is very important for the HCV eradication. In the current study, we found that EGCG enhanced poly I:C-induced *IFN- λ 1*, *TLR3*, *RIG-I* and ISGs expression in JFH-1-Huh7 cells, showing that EGCG could improve intracellular antiviral immune responses in viral-infected cells.

In recent years, the development of effective DAAs has greatly increased the opportunity to cure HCV and achieve a milestone of HCV therapy. Following the approval of high effective DDAs in 2013, all-oral, IFN-free regimens were available for chronic HCV management^[43]. Clinical applications of DAAs including inhibitors of NS3/4A, NS5A, and NS5B nucleotide or non-nucleotide inhibitors can achieve a high SVR rate of 90% to 100%^[2,44]. However, challenges still exist in the future management of HCV infection. DAAs are unavailable in poor areas and most of developing countries due to the high prices. It is also important to investigate the clinical effects of DAAs on advanced or decompensated liver diseases caused by HCV, as well as the clinical use of DAAs in HCV-infected children and pregnant women^[45]. In addition, the majority of current DAAs have a low barrier to resistance, which leads to a high risk of selection of drug-resistant viral strains^[46]. In the absence of an effective vaccine against HCV, reinfection is probable in cured patients under continuous HCV exposure^[16].

In summary, EGCG enhances both poly I:C- and HCV dsRNA intermediate-induced innate immune responses in hepatocytes^[32]. It would be interesting to investigate the possible use of EGCG in combination with current antiviral drugs for HCV therapy in the future.

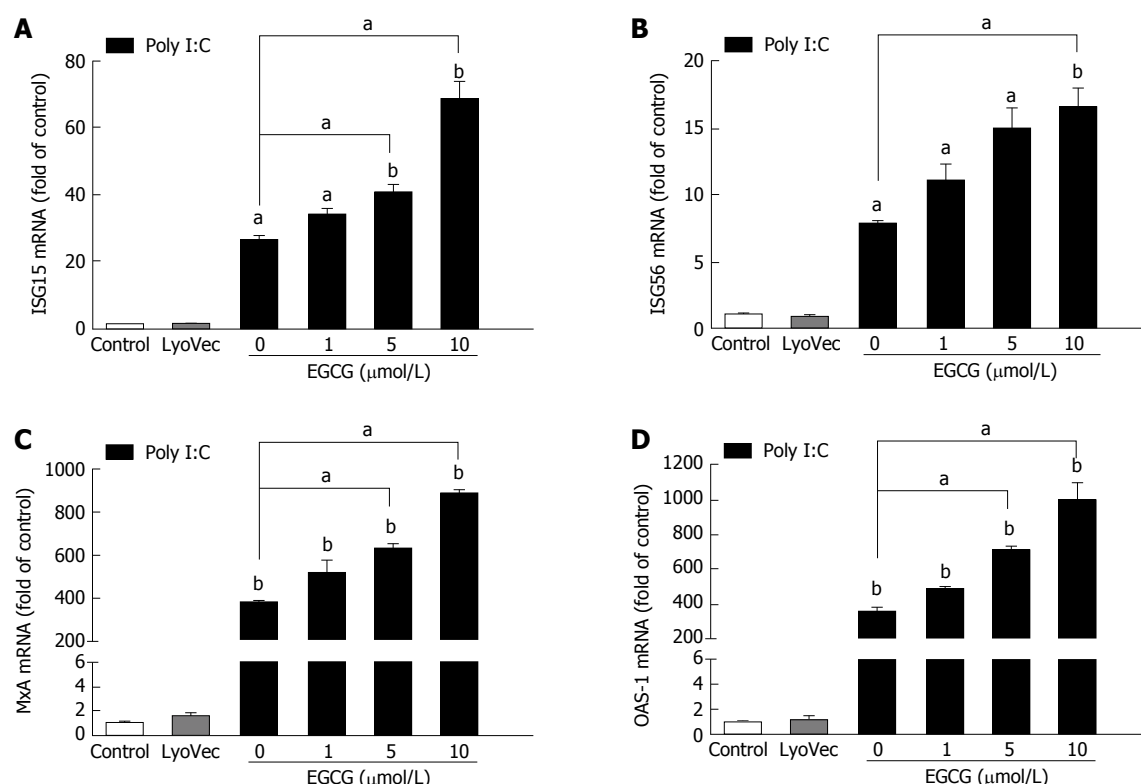


Figure 5 Effect of (-)-epigallocatechin-3-gallate on poly I:C-induced ISGs expression in JFH-1-infected Huh7 cells. JFH-1-infected Huh7 cells (72 h post-infection) were treated with EGCG at the indicated concentrations for 1 h prior to poly I:C (1 μg/mL) stimulation. Total RNA extracted from the cells after 24 h of stimulation was subjected to real-time reverse transcription-polymerase chain reaction for the determination of ISG and glyceraldehyde-3-phosphate dehydrogenase mRNA levels. The data are expressed as ISG15 (A), ISG56 (B), MxA (C), and OAS-1 (D) mRNA levels relative (fold) to the control (vehicle only, which is defined as 1). The results shown are the mean \pm SD of triplicate measurements representative of three experiments (Poly I:C vs LyoVec, ^a $P < 0.05$, ^b $P < 0.01$). EGCG: (-)-Epigallocatechin-3-gallate.

COMMENTS

Background

Chronic hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. New direct-acting antivirals (DAAs) therapies are able to achieve a sustainable virological response of up to 90% against the most prevalent HCV genotypes. However, continued efforts are still needed due to the high cost, low barrier to viral resistance, and the inability to treat HCV-related diseases of the DAAs as well as the lack of an effective HCV vaccine.

Research frontiers

The authors previously reported that HCV replication can impair the poly I:C-triggered innate immune response in hepatocytes. They also showed that epigallocatechin-3-gallate (EGCG) enhances HCV dsRNA intermediate-induced expression of IFN- λ 1 and ISGs in hepatocytes. Effects of EGCG on the poly I:C-mediated expression of antiviral factors and inhibition of HCV were investigated in the present study. They found that EGCG increased poly I:C-induced IFN- λ 1 and ISG expression and contributed to poly I:C-mediated HCV inhibition.

Innovations and breakthroughs

The present study showed for the first time that EGCG can increase poly I:C-induced IFN- λ 1 and ISG expression and contribute to poly I:C-mediated HCV inhibition.

Applications

The authors demonstrated the effects of EGCG on poly I:C-triggered innate immune responses in hepatocytes. However, our observations only provided *in vitro* evidence due to the lack of *in vivo* data. It would be interesting to investigate the possible use of EGCG in combination with current antiviral drugs

for HCV therapy in the future.

Terminology

EGCG is the most abundant and bioactive catechin in green tea and has been considered to have a number of physiological and pharmacological health benefits. Polyinosinic-polycytidylic acid (poly I:C) is a synthetic analog of dsRNA, a molecular pattern associated with viral infection. The average size of HMW poly I:C ranges from 1.5 kb to 8 kb.

Peer-review

This paper reports a well detailed investigation of the effect of EGCG on poly I:C-triggered intracellular innate immunity against HCV in hepatocytes. The authors demonstrated that EGCG had the ability to enhance poly I:C-induced innate immune responses in hepatocytes, which contributed to poly I:C-mediated HCV inhibition. Thus, it would be interesting to investigate the possible use of EGCG in combination with current antiviral drugs for HCV therapy in the future.

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Basic Study

Effects and mechanism of adenovirus-mediated phosphatase and tension homologue deleted on chromosome ten gene on collagen deposition in rat liver fibrosis

Shu-Rui Xie, Jun-Yan An, Li-Bo Zheng, Xiao-Xia Huo, Jian Guo, David Shih, Xiao-Lan Zhang

Shu-Rui Xie, Jun-Yan An, Li-Bo Zheng, Xiao-Xia Huo, Jian Guo, Xiao-Lan Zhang, Department of Gastroenterology, The Second Hospital of Hebei Medical University, Hebei Key Laboratory of Gastroenterology, Hebei Institute of Gastroenterology, Shijiazhuang 050000, Hebei Province, China

Shu-Rui Xie, Department of Gastroenterology, Xingtai People's Hospital, Xingtai 054031, Hebei Province, China

David Shih, Inflammatory Bowel and Immunobiology Research Institute, F. Widjaja Foundation, Cedars-Sinai Medical Center, Los Angeles, CA 90048, United States

Author contributions: Xie SR and An JY contributed equally to this work; Xie SR and An JY performed the majority of the experiments; Zheng LB and Guo J assisted with various experiments and helped to analyze the data; Huo XX and Zhang XL drafted and edited the manuscript; Shih D performed critical revision of the manuscript.

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Correspondence to: Xiao-Lan Zhang, MD, PhD, Professor, Department of Gastroenterology, The Second Hospital of Hebei Medical University, Hebei Key Laboratory of Gastroenterology, Hebei Institute of Gastroenterology, 215 West Heping Road, Shijiazhuang 050000, Hebei Province, China. xiaolanzh@hb2h.com
Telephone: +86-311-66007370
Fax: +86-311-66007370

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Abstract

AIM

To evaluate the effects of phosphatase and tension homologue deleted on chromosome ten (*PTEN*) gene on collagen metabolism in hepatic fibrosis and the underlying mechanisms.

METHODS

Rat primary hepatic stellate cells (HSCs) and human

LX-2 cells were transfected with adenovirus containing cDNA constructs encoding wild-type *PTEN* (Ad-PTEN), *PTEN* mutant *G129E* gene (Ad-G129E), and RNA interference constructs targeting the *PTEN* sequence. PTEN short hairpin RNA to up-regulate and down-regulate the expression of *PTEN*. HSCs were assayed using fluorescent microscopy, real-time polymerase chain reaction, and western blotting. Moreover, a CCl₄-induced rat hepatic fibrosis model was established to investigate the *in vivo* effects. Hematoxylin and eosin, and Masson's trichrome were used to assess the histological changes. The expression of collagen I and III was assessed using immunohistochemistry and western blot analysis.

RESULTS

Elevated expression of *PTEN* gene reduced serum levels of alanine transaminase and aspartate transaminase, decreased collagen deposition in the liver, and reduced hepatocyte necrosis. In contrast, knockdown of *PTEN* expression had an opposite effect, such as increased collagen deposition in the liver, and was molecularly characterized by the increased expression of matrix metalloproteinase (MMP)-13 ($P < 0.01$) and MMP-2 ($P < 0.01$), as well as decreased expression of the tissue inhibitor of metalloproteinase (TIMP)-1 ($P < 0.01$) and TIMP-2 ($P < 0.01$).

CONCLUSION

These data indicated that gene therapy using recombinant adenovirus encoding PTEN might be a novel way of treating hepatic fibrosis.

Key words: Collagen metabolism; Hepatic stellate cells; Phosphatase and tension homologue deleted on chromosome ten; PTEN; Gene therapy; Hepatic fibrosis

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Core tip: Phosphatase and tension homologue deleted on chromosome ten (PTEN) has a negative relation with the activation and proliferation of hepatic stellate cells (HSCs), which is the central event in liver fibrogenesis as HSCs are the major source of collagens and matrix metalloproteinases in fibrotic liver. In this study, adenoviruses containing cDNA constructs encoding wild-type PTEN (Ad-PTEN) and *PTEN* mutant *G129E* gene (Ad-G129E) were constructed to over-express the *PTEN* gene in both rat primary HSCs and human LX-2 cells as well as in the CCl₄-induced rat liver fibrosis model. The adenovirus-mediated over-expression of the *PTEN* gene attenuated extracellular matrix (ECM) synthesis (collagens I and III) and promoted ECM degradation, representing a possible novel anti-fibrosis therapy.

Xie SR, An JY, Zheng LB, Huo XX, Guo J, Shih D, Zhang XL. Effects and mechanism of adenovirus-mediated phosphatase and tension homologue deleted on chromosome ten gene on collagen

deposition in rat liver fibrosis. *World J Gastroenterol* 2017; 23(32): 5904-5912 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i32/5904.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i32.5904>

INTRODUCTION

Cirrhosis, with its manifestation of liver fibrosis, represents a major medical problem worldwide^[1,2]. Hepatic stellate cells (HSCs) are one of the cell types that play a critical role in the development and maintenance of liver fibrosis. Under fibrogenic conditions, HSCs undergo a complex activation process with morphological and phenotypic changes from quiescent vitamin A-storing cells to activated myofibroblast-like cells under fibrogenic conditions, resulting in increased synthesis and deposition of extracellular matrix (ECM) components, such as collagen I^[3,4].

Phosphatase and tension homologue deleted on chromosome ten (*PTEN*) is the first tumor-suppressing gene found to inhibit the proliferation and promote the apoptosis of tumor cells^[5,6]. PTEN has pleiotropic effects including pulmonary fibrosis, renal fibrosis, and cardiac interstitial fibrosis^[7-10]. The absence of PTEN in specific hepatic cells leads to not only hepatocellular carcinoma but also nonalcoholic steatohepatitis, which has been found to be associated with hepatic fibrosis^[11].

A previous study showed that the expression of PTEN was decreased in rat fibrotic liver tissues and HSCs induced by bile duct ligation *in vivo*^[12]. During the reversal of liver fibrosis, pretreated *PTEN* mRNA and protein expression normalized, showing the relationship between PTEN and the severity of rat hepatic fibrosis^[13]. The study presented herein investigated the *in vitro* and *in vivo* effects of PTEN on liver fibrosis using adenoviral transduction of wild-type *PTEN* (Ad-PTEN), mutant *PTEN* (Ad-G129E), and *PTEN* short hairpin RNA (PTEN shRNA) to better characterize the molecular mechanisms of PTEN in liver fibrosis.

MATERIALS AND METHODS

Animals

Adult male Wistar rats weighing 350-400 g were obtained from the Experimental Animal Center of Hebei Medical University, Hebei Province, China. The study was performed in compliance with the national ethical guidelines for the care and use of laboratory animals, following the internationally accepted principles for laboratory animal use and care as found in the United States' guidelines (National Institutes of Health publication #85-23, revised in 1985).

Isolation of rat primary HSCs and cell culture

Rat primary HSCs were isolated from normal healthy

male Wistar rats using *in situ* recirculating perfusion technology, as described in a previous study^[14]. Then, Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum was used for cell culture; passages 2-3 were used in this study. Human LX-2 cells were obtained from Mount Sinai School of Medicine, authorized by Dr. Friedman.

Recombinant adenovirus and transfection

Adenovirus containing cDNA constructs encoding wild-type *PTEN* (Ad-PTEN) with green fluorescent protein (GFP), *PTEN* mutant G129E gene (Ad-G129E) with GFP, and the empty virus control (Ad-GFP) were kindly provided by Prof. Junshan Zhu from the Third Military Medical University in China. RNA interference targeting *PTEN* sequence shRNA with enhanced GFP was established by Wuhan Genesil Biotechnology Co., Ltd (Wuhan, China). The transfection was performed as described in a previous study^[15].

The rat primary HSCs and human LX-2 cells were divided into five groups: (1) control group, with serum-free antibiotic-free DMEM; (2) Ad-GFP group, with Ad-GFP transfection; (3) Ad-PTEN group, with Ad-PTEN transfection; (4) Ad-G129E group, with Ad-G129E transfection; and (5) *PTEN* shRNA group, with *PTEN* shRNA transfection.

Real-time polymerase chain reaction assay

A real-time polymerase chain reaction assay was performed using a previously established protocol^[13,16]. Primer Express 5.0 was used to design the following primers: *PTEN* (rat), forward 5'-GGAAAGGACGGACTGGTGTA-3' and reverse 5'-GGAAAGGACGGACTGGTGTA-3'; *PTEN* (human), forward 5'-ACCGCCAAATTTAATTGCAG-3' and reverse 5'-GGGTCCTGAATTGGAGGAA T-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (rat), forward 5'-GGCAAGTTCACGGCAG-3' and reverse 5'-CGCCAGTAGACTCCACGACAT-3'; and GAPDH (human), forward 5'-ACTTTGGTATCGTGAAGGACT-3' and reverse 5'-GTAGAGGCAGGGATGATGTTCT-3'. The primers were synthesized by SBS Genetech Co., Ltd (Beijing, China). The mRNA expression of genes was normalized to GAPDH.

Western blot assay

Western blotting was performed as described in a previous study^[12]. Anti-PTEN, anti- α -smooth muscle actin, anti-collagen I, anti-collagen III, anti-matrix metalloproteinase (anti-MMP)-13, anti-MMP-2, anti-tissue inhibitor of metalloproteinase (anti-TIMP)-1 and anti-TIMP-2 antibodies (1:200), and anti-GAPDH antibody (1:500) were used as primary antibodies.

Animal model

The CCl₄-induced rat hepatic fibrosis model was established as described in a previous study^[13]. Rats were randomly divided into pretreatment and treatment groups. Pretreatment with recombinant

adenovirus (2×10^9 pfu/100 μ L/rat) through tail vein injection was conducted on rats once a week by administering CCl₄ for 7 wk. Treatment with adenovirus (2×10^9 pfu/100 μ L/rat) through tail vein injection was performed on rats once a week starting in the fourth week postadministration of CCl₄ for 4 wk. Recombinant adenoviruses used were Ad-GFP, Ad-PTEN, Ad-G129E, and *PTEN* shRNA.

Pathology and immunohistochemical and immunofluorescent staining on liver tissue

Hematoxylin and eosin (H&E) staining and Masson's trichrome (MT) staining were performed to assess the histological changes and fibrosis in liver tissues. Immunohistochemical staining was used to further check the deposition of collagens I and III in the fibrotic liver; the procedure was performed as described in a previous study^[12]. Immunofluorescent staining was also performed on frozen liver sections as described in a previous study to check the changes in the expression of *PTEN* in liver tissue^[13].

RESULTS

Establishment of adenoviral transfection to modulate the expression of *PTEN*

Adenoviral transfection using Ad-GFP was performed initially to establish the feasibility to modulate the expression of *PTEN* in rat primary HSCs and human LX-2 cells. This study showed that an adenovirus multiplicity of infection of 50 for 72 h gave the best transfection in rat primary HSCs (94.46% efficiency; Figure 1A) and human LX-2 cells (89.89% efficiency). At 72 h posttransfection, the mRNA and protein expression of *PTEN* in both rat primary HSCs and human LX-2 cells significantly increased in the Ad-PTEN group (mRNA: 1.698, 1.547 and protein: 1.91 ± 0.09 , 2.13 ± 0.01 , respectively) and Ad-G129E group (mRNA: 1.624, 1.479 and protein: 1.74 ± 0.08 , 1.98 ± 0.12 , respectively) compared with the Ad-GFP group (mRNA: 0.994, 0.998 and protein: 1.15 ± 0.04 , 1.21 ± 0.14 , respectively) ($P < 0.01$), and significantly decreased in the *PTEN* shRNA group (mRNA: 0.357, 0.548 and protein: 0.56 ± 0.04 , 0.58 ± 0.13 , respectively) (Figure 1B and C).

PTEN negatively regulated collagen metabolism

Collagen deposition in fibrotic liver tissues mainly comprised collagens I and III. The protein expression of collagens I and III in rat primary HSCs was found to be significantly decreased in the Ad-PTEN (0.32 ± 0.05 and 0.18 ± 0.02 , respectively) and Ad-G129E groups compared with the CCl₄ control and Ad-GFP groups (Figure 2A and B). In contrast, the expression of collagens I and III significantly increased in the *PTEN* shRNA group compared with the CCl₄ control and Ad-GFP groups. A similar tendency was also found in experiments using human LX-2 cells (Figure 2A and C).

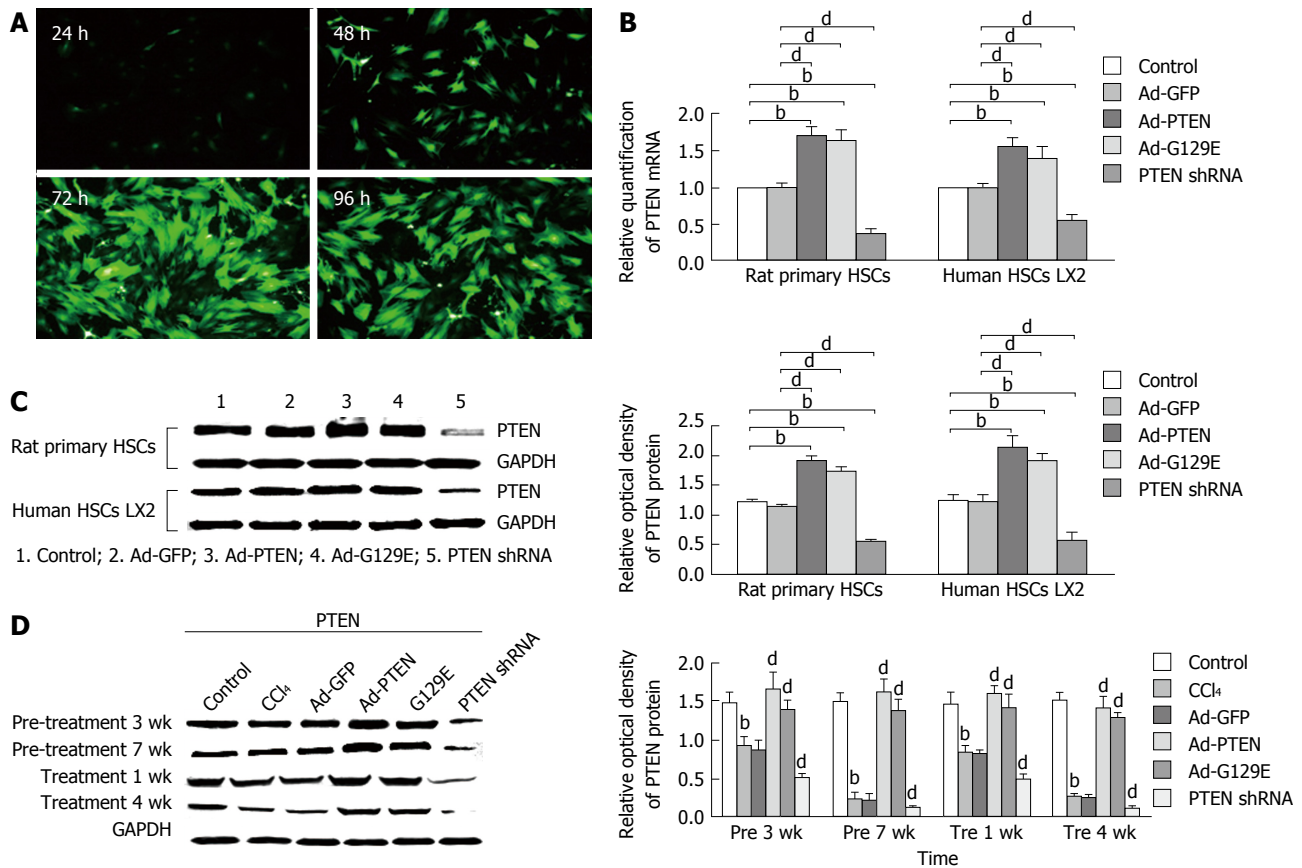


Figure 1 Effective modulation of the expression of phosphatase and tension homologue deleted on chromosome ten *in vitro* and *in vivo*. A: Representative immunofluorescent image showing adenoviral transfection efficiency using Ad-GFP in rat primary HSCs; B: The mRNA expression of PTEN was quantitated for rat primary HSCs (left panel) and human LX-2 cells (right panel). Data are expressed as a relative value, and the error bars represent SE; C: Representative western blot showing the protein expression of PTEN (left panel), quantitated and expressed as relative optical density. The error bars represent SE. ^b $P < 0.01$ vs the control group; ^d $P < 0.01$ vs the Ad-GFP group; D: Representative western blot showing that the protein expression of PTEN in rat livers treated with Ad-PTEN was significantly enhanced in pre 1 wk, pre 3 wk, pre 5 wk, and pre 7 wk, compared with the Ad-GFP and control CCl₄ groups. $n = 3$ for all experiments. GFP: Green fluorescent protein; HSCs: Hepatic stellate cells; PTEN: Phosphatase and tension homologue deleted on chromosome ten.

MMP-13 and MMP-2 play a critical role in the metabolism of collagens I and III. In rat primary HSCs, a significantly higher expression of MMP-13 and MMP-2 was found in the Ad-PTEN and Ad-G129E groups compared with the PTEN shRNA and CCl₄ control groups. As expected, the expression of MMP-13 and MMP-2 was significantly reduced in the PTEN shRNA group compared with the control and Ad-GFP groups (Figure 2A and B). Similar regulation of MMP-13 and MMP-2 was also found using human LX-2 cells (Figure 2A and C).

The degree of collagen deposition was due to the balance between collagenases and their inhibitors. The inhibitors of MMP-13 and MMP-2 were additionally measured by western blotting at 72 h posttransfection in both rat primary HSCs and human LX2 HSCs. The expression of TIMP-1 and TIMP-2 was significantly down-regulated by Ad-PTEN and Ad-G129E compared with the CCl₄ control and Ad-GFP groups (Figure 2A and B). Transfection of PTEN shRNA up-regulated these inhibitors of collagenases compared with the control and Ad-GFP groups (Figure 2).

PTEN had a protective effect on CCl₄-induced rat liver fibrosis

The increased expression of PTEN gene in the Ad-PTEN and Ad-G129E groups was significantly reduced, whereas the reduced PTEN gene expression in the PTEN shRNA group was increased, along with the serum levels of alanine transaminase and aspartate transaminase compared with the CCl₄ control and Ad-GFP groups, indicating improved liver function (Tables 1 and 2).

H&E and MT staining confirmed that the increased expression of PTEN in the Ad-PTEN and Ad-G129E groups led to reduced hepatocyte necrosis and collagen deposition in liver tissue compared with that in the control groups (Figure 3). Immunofluorescent staining for PTEN was performed to see whether the improved pathology was associated with changes in the expression of the PTEN gene. It was found that the expression of the PTEN gene significantly increased with Ad-PTEN and Ad-G129E recombinant adenovirus in both the prevention and treatment groups (Figure 3).

Moreover, the total protein was isolated from liver

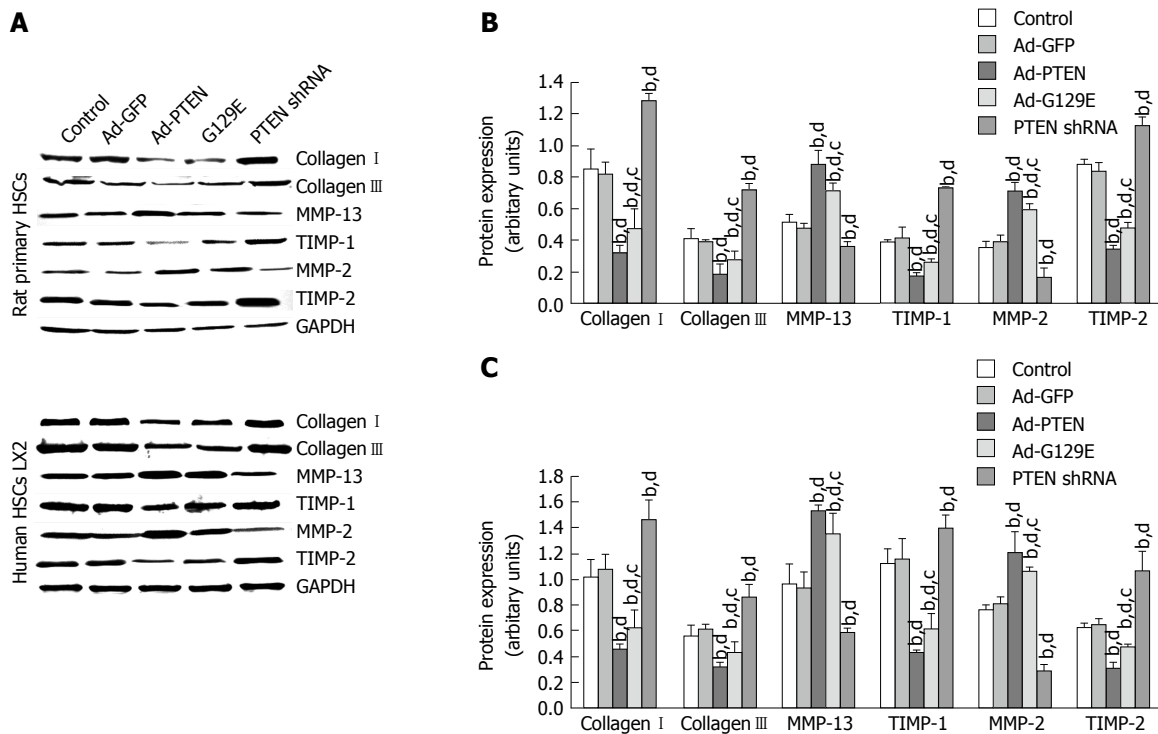


Figure 2 Phosphatase and tension homologue deleted on chromosome ten has a negative effect on collagen deposition *in vitro*. A: Representative western blots of factors involved in the metabolism of fibrosis at 72 h posttransfection are shown in (A) and quantitated for rat primary HSCs in (B) and for human HSC LX2 cells in (C). Data in (B) and (C) are represented as mean \pm SE. $n = 3$ for all groups. ^b $P < 0.01$ vs the control group; ^c $P < 0.05$ vs Ad-PTEN group; ^d $P < 0.01$ vs the Ad-GFP group. HSC: Hepatic stellate cell; PTEN: Phosphatase and tension homologue deleted on chromosome ten.

Table 1 Impact of adenovirus-mediated phosphatase and tension homologue deleted on chromosome ten on liver function in rat liver fibrosis induced by CCl₄ in prevention groups at different time points

Group	ALT (U/L)	AST (U/L)	ALB (g/L)	TBIL (μ mol/L)	DBIL (μ mol/L)
Pre 1 wk					
Control	58.09 \pm 6.54	147.23 \pm 10.25	28.95 \pm 2.14	2.15 \pm 0.54	1.12 \pm 0.32
CCl ₄	414.45 \pm 29.37	339.12 \pm 45.37	27.20 \pm 2.35	2.12 \pm 0.38	1.70 \pm 0.21
Ad-GFP	426.24 \pm 30.76	289.37 \pm 20.76	27.92 \pm 3.12	2.13 \pm 0.31	1.56 \pm 0.12
Ad-PTEN	404.23 \pm 26.78	283.76 \pm 23.45 ^a	29.12 \pm 4.25	1.98 \pm 0.34	1.42 \pm 0.31
Ad-G129E	409.98 \pm 43.24	289.76 \pm 35.43 ^a	28.82 \pm 3.23	2.00 \pm 0.34	1.50 \pm 0.21
PTEN shRNA	2970.11 \pm 267.34 ^b	1690.25 \pm 200.34 ^b	27.10 \pm 3.23	4.03 \pm 0.78 ^b	2.67 \pm 0.56 ^b
Pre 3 wk					
Control	60.71 \pm 5.34	150.54 \pm 12.34	27.65 \pm 3.28	2.34 \pm 0.23	1.28 \pm 0.77
CCl ₄	532.21 \pm 34.02	804.12 \pm 67.34	25.31 \pm 4.32	17.31 \pm 1.21	7.78 \pm 2.01
Ad-GFP	589.34 \pm 26.45	787.45 \pm 43.21	26.70 \pm 3.15	15.04 \pm 2.12	7.34 \pm 1.29
Ad-PTEN	422.37 \pm 34.23 ^a	478.43 \pm 50.34 ^a	27.31 \pm 2.43	5.78 \pm 1.01 ^a	3.24 \pm 0.78 ^a
Ad-G129E	506.45 \pm 57.32	526.43 \pm 32.98 ^a	26.59 \pm 1.37	10.54 \pm 2.76 ^a	6.21 \pm 2.37
PTEN shRNA	3440.32 \pm 342.32 ^b	3090.39 \pm 228.23 ^b	24.41 \pm 2.34 ^b	28.37 \pm 4.78 ^b	14.56 \pm 2.42 ^b
Pre 5 wk					
Control	59.22 \pm 7.34	145.38 \pm 11.32	28.37 \pm 2.56	2.34 \pm 0.78	1.43 \pm 0.22
CCl ₄	672.34 \pm 17.37	817.56 \pm 46.32	26.70 \pm 3.02	15.23 \pm 2.78	7.65 \pm 1.22
Ad-GFP	597.45 \pm 33.21	753.24 \pm 32.12	26.30 \pm 1.76	16.30 \pm 3.56	8.72 \pm 2.11
Ad-PTEN	456.43 \pm 32.12 ^a	566.76 \pm 53.21 ^a	28.54 \pm 2.12 ^a	9.31 \pm 2.12 ^a	4.50 \pm 0.89 ^a
Ad-G129E	492.37 \pm 40.65 ^a	705.43 \pm 63.21 ^a	27.21 \pm 1.35	10.56 \pm 1.23 ^a	5.61 \pm 1.23 ^a
PTEN shRNA	2060.21 \pm 325.34 ^b	2490.34 \pm 532.12 ^b	24.23 \pm 1.23 ^b	17.31 \pm 2.67 ^b	9.45 \pm 2.12 ^b
Pre 7 wk					
Control	60.21 \pm 3.19	152.12 \pm 15.34	28.34 \pm 1.15	2.38 \pm 0.59	1.48 \pm 0.26
CCl ₄	605.23 \pm 85.37	1110.32 \pm 215.32	22.76 \pm 3.21	15.26 \pm 1.23	8.65 \pm 0.32
Ad-GFP	623.45 \pm 56.34	1134.23 \pm 121.24	23.43 \pm 1.21	14.87 \pm 1.02	9.34 \pm 0.98
Ad-PTEN	456.45 \pm 32.12 ^a	183.23 \pm 34.23 ^a	26.73 \pm 2.23 ^a	4.32 \pm 0.97	2.62 \pm 0.42
Ad-G129E	545.87 \pm 43.21 ^a	694.32 \pm 25.34 ^a	25.63 \pm 1.23 ^a	4.56 \pm 0.32	2.54 \pm 0.23
PTEN shRNA	1825.23 \pm 28.23 ^b	1878.45 \pm 56.87 ^b	21.90 \pm 3.23 ^b	17.23 \pm 3.56 ^b	9.08 \pm 1.32 ^b

Data are presented as mean \pm SD for $n = 3$. ^a $P < 0.01$ vs CCl₄, Ad-GFP; ^b $P < 0.05$ vs CCl₄, Ad-GFP, Ad-PTEN. ALB: Albumin; ALT: Alanine transaminase; AST: Aspartate transaminase; DBIL: Direct bilirubin; PTEN: Phosphatase and tension homologue deleted on chromosome ten; TBIL: Total bilirubin.

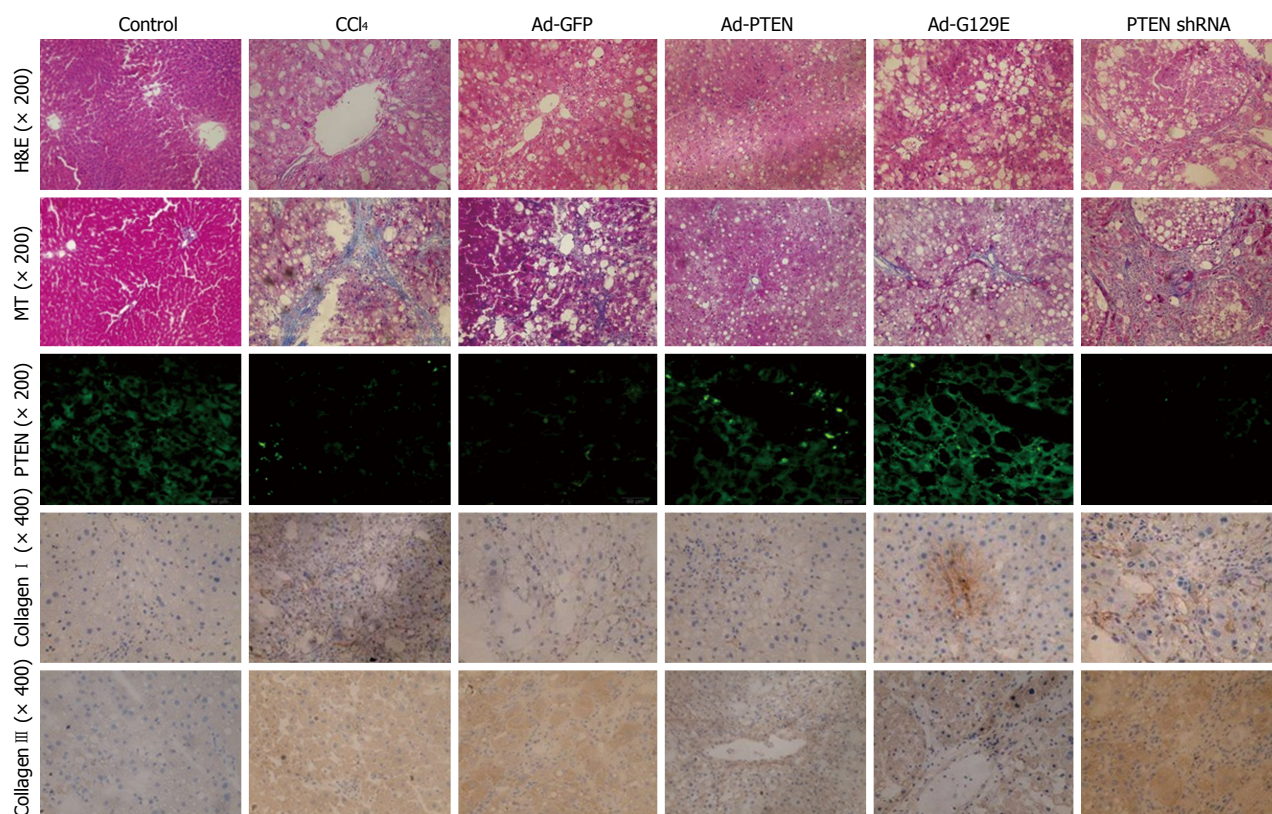


Figure 3 Phosphatase and tension homologue deleted on chromosome ten has protective effects on CCl₄-induced rat hepatic fibrosis *in vivo*. H&E stain (× 200) and MT stain (× 200) showed reduced hepatic cell necrosis and collagen deposition in liver tissue by over-expressed PTEN gene in Ad-PTEN and Ad-G129E groups. Immunofluorescent staining for PTEN (green) showed increased PTEN expression with Ad-PTEN and Ad-G129E recombinant adenovirus in both the prevention group or the treatment group. Immunohistochemical staining (bottom 2 rows) showed decreased collagen I and collagen III expression in hepatic tissues (× 400) with over-expression of PTEN induced by exogenous wild-type PTEN or G129E gene, whereas collagen I and collagen III expressions was reduced by PTEN shRNA. H&E: Hematoxylin and eosin; MT: Masson's trichrome; PTEN: Phosphatase and tension homologue deleted on chromosome ten.

tissues in each group, and western blot analysis of the expression of PTEN was performed. Consistent with the findings from previous studies^[13], the protein expression of PTEN was significantly decreased in rats treated with CCl₄. The protein expression of PTEN was significantly increased in rats treated with Ad-PTEN in the pretreatment and treatment groups compared with the control group (Figure 1D).

Then, western blot analysis was performed to check the protein expression of collagens I and III in the rat liver tissue at each time point. Compared with the Ad-GFP group, the expression of collagens I and III significantly decreased with the enhanced expression of PTEN in the Ad-PTEN group at pre 3 wk, pre 7 wk, pre 1 wk, and pre 4 wk (Figure 4). In contrast, the reduced expression of PTEN with *PTEN* shRNA significantly increased the expression of collagens I and III (Figure 4).

DISCUSSION

Hepatic fibrosis is the accumulation of ECM in response to chronic liver injury that ultimately leads to cirrhosis^[1,2]. Cirrhosis is associated with increased morbidity and mortality and results in substantial

economic and social costs. At present, no effective therapy is available to treat or reverse hepatic fibrosis.

Under chronic injury, HSCs are activated to produce more ECM, mainly comprised of collagens I and III in the liver tissue. Moreover, HSCs also regulate the balance of MMPs and TIMPs, which determines the degree of collagen deposition in the liver^[1,17-19]. PTEN has been found to be involved in myocardial fibrosis, renal fibrosis, and lung fibrosis^[8-10]. A previous study found that higher expression of PTEN reduced the number of activated HSCs to negatively regulate fibrogenesis *in vivo*^[12,13]. This suggested that PTEN may also regulate the accumulation of ECM components in liver fibrosis because ECM is mainly produced from activated HSCs^[1].

The expression of *PTEN* was modulated in this study using recombinant adenovirus that either increased or reduced the expression of PTEN. This study showed that reducing the expression of *PTEN* conferred worsened liver fibrosis through its modulation of collagens I and III, MMP-13 and MMP-2, and TIMP-1 and TIMP-2. These PTEN-dependent changes in collagen, collagenases, and collagenase inhibitors reduced collagen deposition that was associated with CCl₄-induced liver fibrosis.

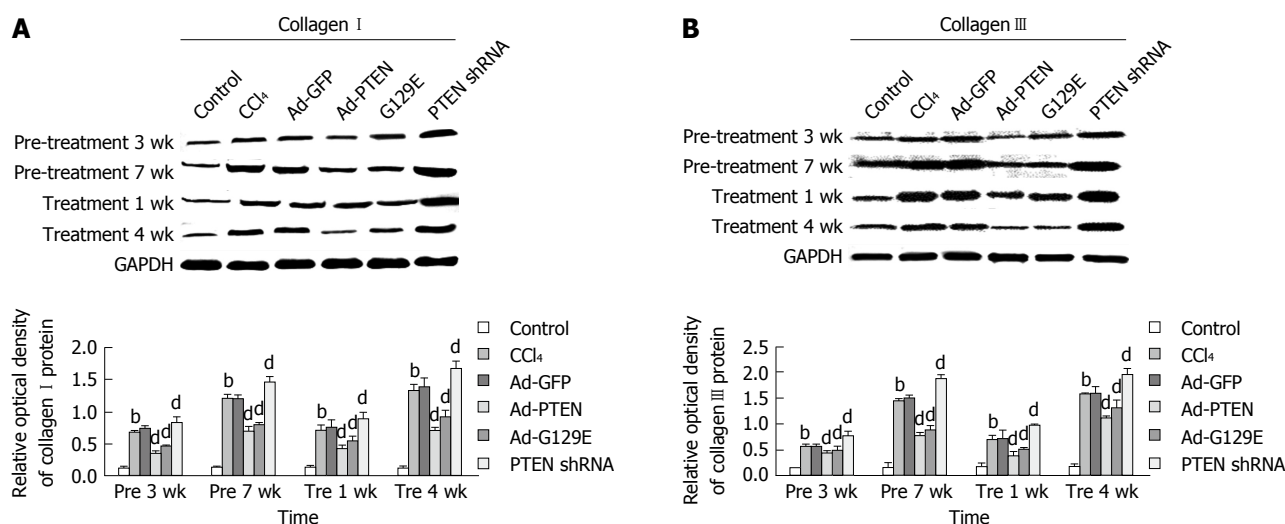


Figure 4 Phosphatase and tension homologue deleted on chromosome ten reduced collagen deposition in CCl₄-induced fibrotic hepatic tissues. Representative western blot of collagen I (A) and collagen III (B) expressions. Collagen I and collagen III expressions were quantitated in the bottom panels and represented as mean \pm SE. ^b*P* < 0.01 vs the control group; ^d*P* < 0.01 vs the Ad-GFP group. PTEN: Phosphatase and tension homologue deleted on chromosome ten.

Table 2 Impact of adenovirus-mediated phosphatase and tension homologue deleted on chromosome ten on liver function in rat liver fibrosis induced by CCl₄ in treatment groups at different time points

Group	ALT (U/L)	AST (U/L)	ALB (g/L)	TBIL (μ mol/L)	DBIL (μ mol/L)
Tre 1 wk					
Control	57.78 \pm 8.23	146.78 \pm 12.23	27.99 \pm 1.18	2.49 \pm 0.55	1.65 \pm 0.36
CCl ₄	684.32 \pm 78.34	840.32 \pm 32.25	25.26 \pm 3.25	15.45 \pm 3.23	6.56 \pm 1.12
Ad-GFP	645.65 \pm 34.26	832.12 \pm 40.32	24.54 \pm 1.21	14.31 \pm 1.21	7.21 \pm 2.12
Ad-PTEN	384.98 \pm 36.23 ^b	398.43 \pm 43.23 ^b	27.60 \pm 2.34 ^b	11.21 \pm 1.21 ^b	5.32 \pm 1.46 ^b
Ad-G129E	495.34 \pm 45.12 ^b	546.32 \pm 32.12 ^b	26.12 \pm 2.43	13.23 \pm 2.12	7.86 \pm 1.23 ^b
PTEN shRNA	2030.31 \pm 112.34 ^a	1730.54 \pm 87.34 ^a	24.12 \pm 2.34 ^a	34.21 \pm 5.34 ^a	17.23 \pm 2.12 ^a
Tre 2 wk					
Control	57.46 \pm 3.58	142.34 \pm 10.24	29.15 \pm 3.21	2.68 \pm 0.74	1.55 \pm 0.46
CCl ₄	712.34 \pm 34.54	843.23 \pm 54.32	26.32 \pm 1.34	14.32 \pm 5.32	8.32 \pm 2.12
Ad-GFP	697.45 \pm 45.76	876.34 \pm 33.32	25.79 \pm 2.32	13.34 \pm 2.13	7.86 \pm 1.23
Ad-PTEN	338.12 \pm 12.34 ^b	407.34 \pm 32.12 ^b	27.56 \pm 3.23 ^b	6.56 \pm 1.21 ^b	3.72 \pm 0.78 ^b
Ad-G129E	364.32 \pm 34.56 ^b	408.67 \pm 23.78 ^b	27.21 \pm 1.22 ^b	10.32 \pm 1.21 ^b	5.34 \pm 1.09 ^b
PTEN shRNA	2040.21 \pm 126.32 ^a	1860.32 \pm 210.32 ^a	25.60 \pm 3.12 ^a	27.12 \pm 4.21 ^a	14.78 \pm 2.12 ^a
Tre 3 wk					
Control	58.54 \pm 6.58	148.45 \pm 13.27	29.12 \pm 3.15	2.12 \pm 0.21	1.48 \pm 0.24
CCl ₄	1892.32 \pm 113.12	2018.32 \pm 123.45	22.14 \pm 1.23	8.18 \pm 1.10	5.21 \pm 0.65
Ad-GFP	1746.32 \pm 98.23	1879.23 \pm 119.34	23.10 \pm 2.13	9.12 \pm 1.23	5.72 \pm 0.54
Ad-PTEN	524.35 \pm 34.12 ^b	690.35 \pm 54.23 ^b	27.30 \pm 3.21 ^b	6.45 \pm 0.32 ^b	4.12 \pm 0.54 ^b
Ad-G129E	576.23 \pm 43.21 ^b	820.43 \pm 67.32 ^b	25.20 \pm 1.21 ^b	8.12 \pm 0.87 ^b	4.89 \pm 0.81 ^b
PTEN shRNA	1937.56 \pm 32.21 ^a	2390.32 \pm 112.23 ^a	21.34 \pm 2.34 ^a	10.76 \pm 1.23 ^a	6.10 \pm 0.54 ^a
Tre 4 wk					
Control	62.31 \pm 8.12	155.37 \pm 13.27	29.91 \pm 3.12	2.49 \pm 0.32	1.65 \pm 0.21
CCl ₄	594.39 \pm 32.18	1110.32 \pm 89.34	23.45 \pm 2.12	17.32 \pm 2.32	9.28 \pm 1.23
Ad-GFP	576.45 \pm 23.43	886.43 \pm 45.67	22.79 \pm 1.21	13.45 \pm 2.45	7.12 \pm 1.34
Ad-PTEN	490.32 \pm 32.23 ^b	528.34 \pm 32.12 ^b	25.31 \pm 3.21 ^b	7.34 \pm 1.21 ^b	4.65 \pm 0.98 ^b
Ad-G129E	530.23 \pm 22.56 ^b	628.43 \pm 45.34 ^b	25.12 \pm 1.23 ^b	10.54 \pm 2.32 ^b	6.54 \pm 1.21 ^b
PTEN shRNA	1148.32 \pm 54.32 ^a	1270.34 \pm 121.28 ^a	20.37 \pm 3.12 ^a	26.32 \pm 3.56 ^a	14.56 \pm 2.13 ^a

Data are presented as mean \pm SD for *n* = 3. ^a*P* < 0.05 vs CCl₄, Ad-GFP, Ad-PTEN; ^b*P* < 0.01 vs CCl₄, Ad-GFP. ALB: Albumin; ALT: Alanine transaminase; AST: Aspartate transaminase; DBIL: Direct bilirubin; PTEN: Phosphatase and tension homologue deleted on chromosome ten; TBIL: Total bilirubin.

The human homologue of rat MMP-13 is MMP-1 and is expressed by HSCs, fibroblasts, Kupffer cells, and so forth^[20]. MMP-13 remodels the surrounding tissue to clear room for deposition of newly synthesized ECM. The activity of MMP-13 can be inhibited by TIMP-1^[21]. MMP-2 mainly serves to degrade the collagen in

the basement membrane^[22]. TIMP-2 is essential for MMP-2 activation, as it can bind to pro-MMP-2 and then combine with MT1-MMP to activate pro-MMP-2. However, over-expressed TIMP-2 can inhibit MMP-2 activity, causing excessive collagen deposition^[20,23]. In this study, MMP-13 and MMP-2, the major collagenases

involved in collagen degradation^[24,25], increased with a high expression level of the *PTEN* gene, which also down-regulated the expression of TIMP-1 and TIMP-2. The lower expression of PTEN could invert the ratio of MMP-13/TIMP-1 and MMP-2/TIMP-2 and cause severe collagen deposition. These data suggested that PTEN might regulate hepatic collagen metabolism through regulation of MMP-13, MMP-2, TIMP-1, and TIMP-2.

Gene therapy using adenovirus vector has been shown to be effective in modulating the expression of a gene of interest^[26-28]. In this study, treatment with recombinant adenovirus carrying a highly expressed *PTEN* gene in rats with CCl₄-induced hepatic fibrosis improved the liver function and reduced the collagen deposition in liver tissue.

In conclusion, these data demonstrated that PTEN might affect collagen deposition in the liver through MMP-13, MMP-2, TIMP-1, and TIMP-2. Gene therapy using recombinant adenovirus encoding wild-type *PTEN* may represent a novel way for treating hepatic fibrosis.

COMMENTS

Background

Phosphatase and tension homologue deleted on chromosome ten (PTEN) plays an essential role in the activation of hepatic stellate cells (HSCs), which are the major source of collagens and matrix metalloproteinases in the fibrotic liver. Liver fibrosis results from the excessive deposition of extracellular matrix (ECM) components, mainly comprising collagens I and III, which are produced by HSCs.

Research frontiers

Previous studies have shown that PTEN had a negative relation with the activation and proliferation of HSCs, which is the central event in liver fibrogenesis. The collagen synthesis could be inhibited by over-expression of the *PTEN* gene. Adenoviruses containing cDNA constructs encoding wild-type *PTEN* (Ad-PTEN) and *PTEN* mutant G129E gene (Ad-G129E) were used in both rat primary HSCs and human LX-2 cells, as well as in the CCl₄-induced rat liver fibrosis model.

Innovations and breakthroughs

Recent reports highlighted the importance of collagen metabolism in HSCs. This novel study reported that the adenovirus-mediated over-expression of the *PTEN* gene could attenuate ECM synthesis and promote ECM degradation, which represents a potential tool for novel anti-fibrosis therapies.

Applications

The results of this study indicated that the over-expressed *PTEN* gene might represent a novel tool for the treatment and reversal of hepatic fibrosis.

Peer-review

It's an interesting topic about effects of the *PTEN* gene on collagen metabolism in hepatic fibrosis and the underlying mechanisms.

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Retrospective Study

Integrating *TYMS*, *KRAS* and *BRAF* testing in patients with metastatic colorectal cancer

Anastasios Ntavatzikos, Aris Spathis, Paul Patapis, Nikolaos Machairas, George Peros, Stefanos Konstantoudakis, Danai Leventakou, Ioannis G Panayiotides, Petros Karakitsos, Anna Koumarianou

Anastasios Ntavatzikos, Anna Koumarianou, Hematology-Oncology Unit, 4th Department of Internal Medicine, Medical School, National and Kapodistrian University of Athens, "ATTIKON" University Hospital, 12462 Athens, Greece

Aris Spathis, Danai Leventakou, Petros Karakitsos, Department of Cytopathology, National and Kapodistrian University of Athens, Medical School, "ATTIKON" University Hospital, 12462 Athens, Greece

Paul Patapis, Nikolaos Machairas, 3rd Department of Surgery, Medical School, National and Kapodistrian University of Athens, "ATTIKON" University Hospital, 12462 Athens, Greece

George Peros, Department of Surgery, Medical School, National and Kapodistrian University of Athens, Evgenideio Therapeutirio S.A., "I AGIA TRIAS", 11528 Athens, Greece

Stefanos Konstantoudakis, Ioannis G Panayiotides, 2nd Department of Pathology, University of Athens, Medical School, "ATTIKON" University Hospital, 12462 Athens, Greece

ORCID number: Anastasios Ntavatzikos (0000-0003-3343-3550); Aris Spathis (0000-0001-8867-3661); Paul Patapis (0000-0003-2349-769X); Nikolaos Machairas (0000-0003-3239-3905); George Peros (0000-0001-7401-2811); Stefanos Konstantoudakis (0000-0002-0839-5137); Danai Leventakou (0000-0001-8743-7843); Ioannis G Panayiotides (0000-0002-6394-117X); Petros Karakitsos (0000-0002-2957-6850); Anna Koumarianou (0000-0002-4159-2511).

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Correspondence to: Anastasios Ntavatzikos, MD, General Surgeon, Research Scientist, Hematology-Oncology Unit, 4th Department of Internal Medicine, Medical School, National and Kapodistrian University of Athens, "ATTIKON" University Hospital, Rimini 1, Haidari, 12462 Athens, Greece. dmaal2@yahoo.gr
Telephone: +30-210-5831687
Fax: +30-210-5326446

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Abstract

AIM

To investigate the impact of thymidylate synthase (*TYMS*), *KRAS* and *BRAF* in the survival of metastatic colorectal cancer (mCRC) patients treated with chemotherapy.

METHODS

Clinical data were collected retrospectively from records of consecutive patients with mCRC treated with fluoropyrimidine-based chemotherapy from 1/2005 to 1/2007. Formalin-fixed paraffin-embedded tissues were retrieved for analysis. *TYMS* genotypes were identified with restriction fragment analysis PCR, while *KRAS* and *BRAF* mutation status was evaluated using real-time PCR assays. *TYMS* gene polymorphisms of each of the 3' untranslated region (UTR) and 5'UTR were classified into three groups according to the probability they have for high, medium and low *TYMS* expression (and similar levels of risk) based on evidence from previous studies. Univariate and multivariate survival analyses were performed.

RESULTS

The analysis recovered 89 patients with mCRC (46.1% *de novo* metastatic disease and 53.9% relapsed). Of these, 46 patients (51.7%) had colon cancer and 43 (48.3%) rectal cancer as primary. All patients were treated with fluoropyrimidine-based chemotherapy (5FU or capecitabine) as single-agent or in combination with irinotecan or/and oxaliplatin or/and bevacizumab. With a median follow-up time of 14.8 mo (range 0-119.8), 85 patients (95.5%) experienced disease progression, and 63 deaths (70.8%) were recorded. The 3-year and 5-year OS rate was 25.4% and 7.7% while the 3-year progression-free survival rate was 7.1%. Multivariate analysis of *TYMS* polymorphisms, *KRAS* and *BRAF* with clinicopathological parameters indicated that *TYMS* 3'UTR polymorphisms are associated with risk for disease progression and death ($P < 0.05$ and $P < 0.03$ respectively). When compared to tumors without any del allele (genotypes ins/ins and ins/loss of heterozygosity (LOH) linked with high *TYMS* expression) tumors with del/del genotype (low expression group) and tumors with ins/del or del/LOH (intermediate expression group) have lower risk for disease progression (HR = 0.432, 95%CI: 0.198-0.946, $P < 0.04$ and HR = 0.513, 95%CI: 0.287-0.919, $P < 0.03$ respectively) and death (HR = 0.366, 95%CI: 0.162-0.827, $P < 0.02$ and HR = 0.559, 95%CI: 0.309-1.113, $P < 0.06$ respectively). Additionally,

KRAS mutation was associated independently with the risk of disease progression (HR = 1.600, 95%CI: 1.011-2.531, $P < 0.05$). The addition of irinotecan in 1st line chemotherapy was associated independently with lower risk for disease progression and death (HR = 0.600, 95%CI: 0.372-0.969, $P < 0.04$ and HR = 0.352, 95%CI: 0.164-0.757, $P < 0.01$ respectively).

CONCLUSION

The *TYMS* genotypes ins/ins and ins/LOH associate with worst prognosis in mCRC patients under fluoropyrimidine-based chemotherapy. Large prospective studies are needed for validation of our findings.

Key words: Thymidylate synthase; Polymorphisms; mCRC; Loss of heterozygosity; Survival; Chemotherapy; *KRAS*; *BRAF*; *TYMS*

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Core tip: The etiology of resistance to new targeted agents and chemotherapy is currently being investigated based upon the patients' genetic profile in order to develop a prognostic model that could lead to individualized treatment. In this context, we studied the effect of thymidylate synthase (*TYMS*) polymorphisms that have been described so far, taking into account the presence of *KRAS* and *BRAF* mutations in association with the treatment. *TYMS* 3' untranslated region polymorphism ins/ins and ins/loss of heterozygosity emerged as an independent factor that increases the risk of both disease progression and death. Regimens that included irinotecan had reduced risk of disease progression and death.

Ntavatzikos A, Spathis A, Patapis P, Machairas N, Peros G, Konstantoudakis S, Leventakou D, Panayiotides IG, Karakitsos P, Koumariannou A. Integrating *TYMS*, *KRAS* and *BRAF* testing in patients with metastatic colorectal cancer. *World J Gastroenterol* 2017; 23(32): 5913-5924 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i32/5913.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i32.5913>

INTRODUCTION

Metastatic colorectal cancer (mCRC) is the second and third leading cause of cancer-related death in Europe^[1] and the United States^[2] respectively, although important and constant overall survival (OS) improvements have been achieved^[3] in the last decades. Even though today there are more treatment options, including several basic chemotherapy regimens in combination with targeted agents^[4], it has been found that large variation exists in individual patient prognosis and response to chemotherapy, caused by molecular heterogeneity^[5]. As a result, treatment decisions are more complex and largely

empirical^[6]. This depicts our lack of understanding of the molecular background and the interplay of different oncogenic pathways, such as *RAS* and *BRAF*, with gene polymorphisms, such as thymidylate synthase (*TYMS*), that can be responsible for the heterogeneity of responses to treatments.

KRAS is a member of the *RAS* family of genes (*KRAS*, *NRAS* and *HRAS*) that encode guanosine-5'-triphosphate (GTP)-binding proteins, which acts as a molecular switch, linking receptor and non-receptor tyrosine kinase activation to downstream cytoplasmic or nuclear events. Activating mutations in *RAS* result in stimulating cell proliferation and inhibiting apoptosis. Around 32%-40% of CRC harbor a *KRAS* mutation^[7,8] which is a predictor of response to anti-EGFR treatment^[9,10]. *BRAF* is a *KRAS* downstream abnormally activated kinase that has been shown to have a similar adverse effect on treatment response^[7,8].

The backbone of mCRC chemotherapy are fluoropyrimidines (5-FU and capecitabine) that cause inhibition of *de novo* thymidine creation from uracil by the *TYMS* enzyme. Potential resistance mechanisms to fluoropyrimidines include *TYMS* gene amplification^[11], loss of heterozygosity (LOH)^[12] and a negative feedback mechanism^[13]. The *TYMS* gene (GeneID 7298^[14]) is located on the short arm of chromosome 18 (18p11.32) and several polymorphisms of the *TYMS* gene have been connected to variable *TYMS* protein levels and therapeutic outcome in relation to 5-FU.

The first polymorphism has been identified in the 5' untranslated region (UTR) and includes an insertion of a 28 base-pair (bp) repeat (rs34743033^[14]), that adds an extra binding site for the upstream stimulatory factor-1 (USF-1) transcription factor (E-box CACTTG^[15]). This USF-1 extra binding site acts as an enhancer to the *TYMS* promoter which leads to increased *TYMS* expression and thus to increased *TYMS* enzyme activity^[16]. This results in alleles with two or three 28 bp tandem repeats (2R or 3R respectively). The second polymorphism (rs2853542^[14]) is a G>C single nucleotide polymorphism (SNP) in the second 28 bp repeat of 3R alleles that abolishes the extra USF-1 binding site^[17] and leads to conversion of the transcriptional activity from a 3R to a 2R. The third polymorphism is located on the 3'UTR (rs34489327^[14]) and is a 6 bp insertion linked to stabilization of the mRNA transcript^[18,19]. The above polymorphisms produce three genotypes: ins/ins (homozygous for insertion of 6bp), del/del (homozygous for deletion of the 6bp) and ins/del (heterozygous).

This study aims to investigate the associations of *TYMS* polymorphisms, LOH, *KRAS/BRAF* mutations and clinicopathologic characteristics with the survival outcomes of patients with mCRC treated with 1st line fluoropyrimidine-based chemotherapy.

MATERIALS AND METHODS

Patients and clinical data

This was a retrospective study carried out by a single

institution (University General Hospital "ATTIKON", Athens, Greece). Clinical data were collected from records of consecutive patients with mCRC treated with fluoropyrimidine-based chemotherapy from 1/2005 to 1/2007. Formalin-fixed paraffin-embedded tissues (FFPE) from consecutive patients with mCRC were retrieved for analysis.

The study protocol was approved by the Institutional Review Board and Ethical Committee (University General Hospital "ATTIKON").

DNA extraction

Five 5-μm thick FFPE sections from a site containing at least 30% malignant cells were used for DNA extraction by means of a commercially available kit (Purelink Genomic DNA Kit; Thermo Fisher Scientific, Schwerte, Germany). DNA was quantified using qPCR (Quant-iT[™] PicoGreen[®] dsDNA Assay Kit; Thermo Fisher Scientific) and was diluted accordingly to achieve a concentration of 10 ng/μL for *TYMS* polymorphisms and 4 ng/μL for *KRAS* mutation detection.

TYMS polymorphisms

Analysis was performed as previously described with minor modifications^[20]. PCR was performed using 1 U of Platinum[®] Taq DNA Polymerase (Thermo Fisher Scientific), 1.5 mmol/L of Mg, 200 nmol/L of dNTPs, and primers. The same primers were used, but 5' UTR amplification was performed using a GC-rich amplification kit (PCR Enhancer System; Thermo Fisher Scientific) adding 1 × of PRCx Enhancer. Genotyping for the 2R/3R polymorphism was performed by running 10 μL of the PCR product on a 1.5% agarose gel and staining with EtBr (Figure 1). For the 12 G>C substitution, 10 μL of PCR product was digested with 1 U of *Hae*III (Takara, Shiga, Japan) for 1 h at 37 °C and run on an 8% 19:1 polyacrylamide gel (Figure 2).

The 3'UTR was also analyzed on polyacrylamide gels (Figure 3). LOH analysis was performed *via* analyzing the intensity of the 5'UTR and 3'UTR bands on the pictures acquired using GeneTools software (Syngene, Cambridge, United Kingdom) (Figures 1 and 3). When either of the bands had an intensity of < 50% of the other, the sample was categorized as having a LOH. Samples showing LOH were defined as 2R/3RGLOH, 2RLOH/3RG, 2R/3RCLOH and 2RLOH/3RC to indicate the allele that was partially lost. Selected products were sequenced to verify the sequence amplified. Blast of the sequenced products and alignment with the latest human assemblies revealed that the amplified product was 242 bp for 3R and 214 bp for 2R genotypes.

Mutation detection

Detection of *KRAS* mutations of codons 12 and 13 was performed with a commercially available Real-Time (RT) PCR kit (Therascreen *KRAS*; DxS Diagnostics, Manchester, United Kingdom) detecting six mutations of codon 12 (G12D, G12A, G12V, G12S, G12R, G12C)

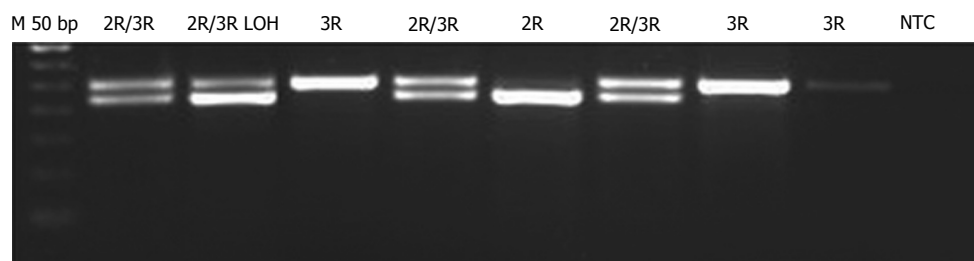


Figure 1 Agarose gel with PCR products for the thymidylate synthase 5'untranslated region 28 bp insertion. A DNA ladder of repeated 50 bp fragments was used (M 50 bp). All potential genotypes (2R 241 bp and 3R 242 bp) are depicted as well, as a sample with LOH for 3R (Lane 3). bp: Base pair; LOH: Loss of heterozygosity; UTR: Untranslated region.

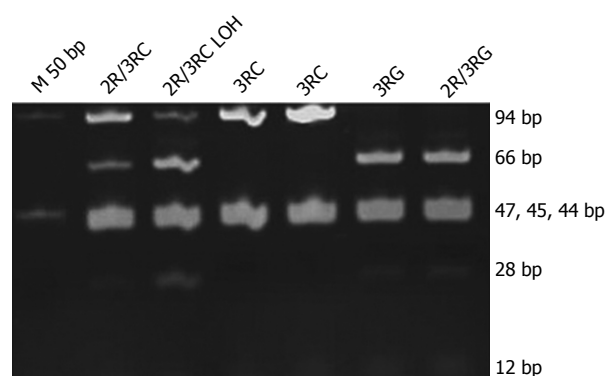


Figure 2 Polyacrylamide gel resolution showing the 12G>C substitution in the 5'untranslated region after digestion. Expected bands are 12, 44, 45 and 47 bp for all genotypes. Digestion of a sample with 2R or 3R12G genotype results in production of two bands of 66 and 28 bp, while in 3R12C genotypes those two fragments are left undigested in a single 94 bp fragment. bp: Base pair; LOH: Loss of heterozygosity; UTR: Untranslated region.

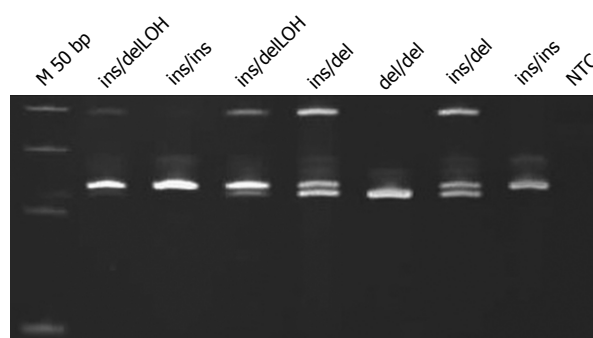


Figure 3 Polyacrylamide gel resolution showing the 3'untranslated region products. Expected bands are 104 and 110 bp. In heterozygotes, a second band of approximately 200 bp was observed due to heteroduplex mismatches. bp: Base pair; UTR: Untranslated region.

and one mutation of codon 13 (G13D)^[21]. A positive reaction mix for all mutations was included. A second exogenous reaction was simultaneously taking place, to avoid false negative results caused by PCR inhibitors. Samples were characterized as bearing a mutation only if ΔC_t (Ct of control reaction - Ct mutation reaction) was lower than the value set by the manufacturer.

The activating mutation V600E of *BRAF* was identified using molecular beacons, as previously described^[21]. One beacon for the wild-type and one for the mutant allele were added at a final concentration of 100 nmol/L in a 25 μ L PCR reaction containing 1 \times PCR Buffer, 6 mmol/L MgCl₂, 200 nmol/L dNTPs, 300 nmol/L of each primer and 1U of Platinum[®] Taq. The PCR thermocycling profile used was 95 $^{\circ}$ C for 2 min, followed by 40 cycles of 95 $^{\circ}$ C for 10 s, 62 $^{\circ}$ C for 60 s and 72 $^{\circ}$ C for 20 s. SKMEL2 and SKMEL20 DNA extracts were used as positive controls for both the wild-type and mutant allele (CLS, Germany). All RT-PCR experiments were performed on an ABI 7500 Fast instrument (Thermo Fisher Scientific).

***TYMS* gene polymorphism stratification model**

Polymorphisms of the *TYMS* gene in each the 3'UTR and 5'UTR were classified into three groups according to the probability they have for high, medium and low *TYMS* expression (and similar levels of risk^[22]), taking

into account the following evidence from previous studies: (1) 3R polymorphism has higher translation efficiency than that of the 2R, leading to higher *TYMS* protein expression associated with resistance to 5FU-based chemotherapy^[18,23], while the 2R/3R has an intermediate *TYMS* protein expression profile^[24]; (2) the SNP G>C results in the 3RC genotype, reported to display a similar transcriptional activity as the 2R genotype (since 3RC and 2R have the same number of binding sites for the USF-1)^[17,25]; (3) the 6 bp insertion, located in the 3'UTR of the *TYMS* primary transcript, favors the *TYMS* mRNA stability, increasing *TYMS* protein expression^[26] and the possibility of resistance to 5FU^[18]; (4) Lower *TYMS* protein expression leads to higher sensitivity to fluoropyrimidine-based therapy^[27,28]; and (5) LOH is associated with a higher risk of resistance to 5FU chemotherapy^[12,29]. Genotypes categorized into expression groups are shown in Table 1.

Statistical analyses

OS was defined as the time from the initiation of 1st line chemotherapy to the date of death by any cause. Progression-free survival (PFS) was calculated as the time from 1st line chemotherapy initiation to the date of verified progression of the disease or the date of death by any cause. Surviving patients were censored at the date of last contact.

The relationship of *TYMS* polymorphisms groups with OS and PFS was assessed by univariate Cox regression analysis. Time-to-event distributions were

Table 1 Thymidylate synthase polymorphisms' groups per untranslated region

	Low expression	Medium expression	High expression
TYMS	del/del	ins/del	ins/ins
3'UTR		del/LOH	ins/LOH
TYMS	2RG	2RG/3RG	3RG
5'UTR	2RG/3RC	2RG/3RG	3RG/3RC
	3RC	2RG/3RCLOH	2RGLOH/3RG
		2RG/3RGLOH	
		2RGLOH/3RC	

LOH: Loss of heterozygosity; UTR: Untranslated region.

Table 2 Clinicopathologic data for patients with metastatic colorectal cancer *n* (%)

Clinicopathologic data	Relapses	De novo metastatic	Total
Age	48 (53.9)	41 (46.1)	89 (100)
Male	65 (40-84.1)	64 (27-86)	65 (27-86)
Primary site	34 (70.8)	23 (56.1)	57 (64.8)
Colon	20 (41.7)	26 (63.4)	46 (51.7)
Rectum	28 (58.3)	15 (36.6)	43 (48.3)
Histological grade			
I + II	27 (56.3)	28 (68.3)	55 (61.8)
III + IV	21 (43.7)	13 (31.7)	34 (38.2)
KRAS mutation	22 (45.8)	18 (43.9)	40 (44.9)
BRAF V600E mut	2 (4.2)	3 (7.3)	5 (5.6)
TYMS LOH	15 (31.3)	11 (26.8)	26 (29.2)
Fluoropyrimidine-based CT			
Monotherapy or with	5 (10.4)	5 (12.2)	10 (11.2)
Irinotecan	18 (37.5)	10 (24.4)	28 (31.4)
Oxaliplatin	22 (45.8)	22 (53.7)	44 (49.4)
Oxaliplatin and irinotecan	3 (6.3)	3 (7.3)	6 (6.7)
Bevacizumab	31 (64.6)	30 (73.2)	61 (68.5)
No chemotherapy	0 (0.0)	1 (2.4)	1 (1.1)
Overall survival			
Deaths	30 (62.5)	33 (80.5)	63 (70.8)
Time ¹ in mo	21.4 (12.2-30.6)	18.2 (14.3-22.0)	19.8 (15.8-23.9)
Progression-free survival			
Events	44 (91.7)	41 (100.0)	85 (95.5)
Time ¹ in mo	10.8 (9.0-12.5)	9.9 (7.0-12.8)	10.6 (8.8-12.5)
Follow-up in mo	14.2 (0-72.5)	17.0 (0.8-119.8)	14.8 (0-119.8)

Data are presented as *n* (%) or median (range) or ¹(95%CI). CT: Chemotherapy; LOH: Loss of heterozygosity.

estimated using Kaplan-Meier curves. Correlation of *TYMS* polymorphisms among them and with selected clinicopathological characteristics were performed using the χ^2 test. For all correlations, the level of statistical significance was set at $P = 0.05$.

The Cox proportional hazards model was used to assess the relationship of clinicopathological parameters and the examined polymorphisms with OS and PFS. In the multivariate Cox regression analysis, a backward selection procedure with a removal criterion of $P > 0.10$ based on likelihood ratio test was performed to identify significant variables among the

following: age, sex (female vs male), histological grade (III-IV vs I - II), primary site (rectal vs colon), *KRAS* and *BRAF* status, groups of *TYMS* polymorphisms, existence of LOH, history of relapse or *de novo* metastatic disease and treatment.

Statistical analysis was conducted using SPSS software for Windows (version 24; SPSS Inc, Chicago, IL, United States).

RESULTS

Patient and tumor characteristics

Patients' information including age, sex, primary tumor site, histological grade, treatment and survival are presented in Table 2. The median age was 65 years (range: 27-86), and the primary site was colon or rectum in 46 and 43 patients respectively. *De novo* metastatic disease was present in 41 patients (46.1%). First-line fluoropyrimidine-based chemotherapy was administered to 88 patients, with a median number of 6 cycles (range: 1-12). In total, 5FU-based chemotherapy was given to 13 patients (14.6%), while 75 patients (84.3%) received capecitabine-based chemotherapy. Fluoropyrimidine-based regimens were combined with irinotecan (31.4%), oxaliplatin (49.4%) or both drugs (6.7%). Bevacizumab was included in the 1st line treatment of 61 patients (68.5%). With a median follow-up of 14.8 mo (range: 0-119.8), 85 patients (95.5%) experienced disease progression and 63 deaths (70.8%) were recorded. The 3-year and 5-year OS rate was 25.4% and 7.7% respectively, while the 3-year PFS rate was 7.1%.

TYMS genotypes

The detected genotypes of *TYMS* according to *de novo* metastatic or relapsed patients are shown in Supplemental Table 1. The wide variations deriving from *TYMS* polymorphism combinations and the presence of LOH according to *de novo* metastatic and relapsed patients are shown in Supplemental Table 2. The 3'UTR polymorphisms had no association with the 5'UTR polymorphism or the SNP G>C. The ins alleles correlated almost statistically significantly with LOH, as shown in Supplemental Table 3.

Analysis of significant association of *TYMS* polymorphisms with patient and tumor characteristics is shown in Table 3. Younger patients (< 65 years old) were more frequently found to carry 2R, but not in a statistically significant way. Also, low grade tumors (I, II) associated with 2RG/3RG ($P < 0.05$). The absence of mutations in *KRAS* correlated with 3RG/3RC ($P < 0.04$).

Correlations of clinicopathological features and genotype with survival outcomes

Analysis of patients according to *TYMS* expression groups and genotypes are shown in Table 4. Univariate Cox regression analysis of clinicopathological parameters in relation to PFS and OS showed no significant

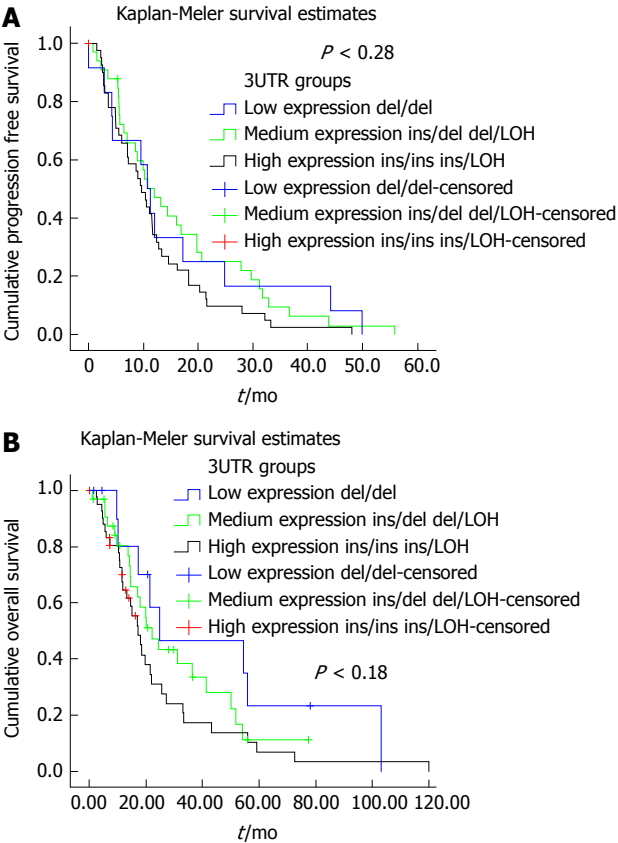


Figure 4 Kaplan-Meier survival curve. A: Kaplan-Meier curve for PFS according to *TYMS* 3'UTR groups; B: Kaplan-Meier curve for OS according to *TYMS* 3'UTR groups. Comparisons were made using long-rank tests. OS: Overall survival; PFS: Progression-free survival; UTR: Untranslated region.

association in our set of data. Univariate Cox regression analyses of *TYMS* polymorphisms and groups, *KRAS* and *BRAF* mutations and LOH are shown in Table 5. The univariate analysis of *TYMS* 3'UTR polymorphisms and LOH demonstrated a trend of lower risk for disease progression and death for the genotypes del/del, ins/del and even ins/LOH compared with ins/ins. There is a trend for increased risk of death for patients with *KRAS* mutation. The analysis of *TYMS* 5'UTR polymorphisms, whether taking into consideration the SNP G>C and LOH or not, also showed no significant effect.

Multivariate analysis of *TYMS* polymorphism groups and selected clinicopathological parameters is shown in Table 6. *KRAS* mutation, existence of LOH and the group of *TYMS* polymorphisms ins/LOH - ins/ins were associated with increased risk for disease progression, while the addition of irinotecan in the 1st line chemotherapy was associated with lower risk. In terms of OS, the group of *TYMS* polymorphisms ins/LOH - ins/ins was associated with increased statistical risk both of disease progression and death. Kaplan-Meier curves for PFS and OS according to *TYMS* 3'UTR polymorphisms groups are shown in Figure 4A and B respectively. Furthermore, the addition of irinotecan or oxaliplatin to fluoropyrimidine-based chemotherapy was associated with lower risk of death. Also, a

Table 3 Association between thymidylate synthase polymorphisms and patient characteristics

Polymorphism	Characteristic	RR (95%CI)	P value
2R	Age < 65-years-old	1.708 (1.158-2.520)	0.090
2RG/3RG	Grade 1-2	1.449 (1.077-1.948)	0.044
2RG/3RC	Female	1.943 (1.152-3.275)	0.036
ins/ins	<i>KRAS</i> G12D	3.563 (1.163-10.912)	0.045
3RG/3RC	<i>KRAS</i> wild-type	1.753 (1.156-2.657)	0.031

Table 4 Risk groups of thymidylate synthase polymorphisms

Group	Relapsed	De novo metastatic	Total
<i>TYMS</i> 5'UTR			
Low expression			
2RG	2 (4.2)	6 (14.6)	8 (9.0)
2RG/3RC	8 (16.7)	3 (7.3)	11 (12.4)
3RC	6 (12.5)	5 (12.2)	11 (12.4)
Medium expression			
2RG/3RG	4 (8.3)	4 (9.8)	8 (9)
2RG/3RCLOH	7 (14.6)	3 (7.3)	10 (11.2)
2RG/3RGLOH	3 (6.3)	2 (4.9)	5 (5.6)
2RGLOH/3RC	1 (2.1)	2 (4.9)	3 (3.4)
High expression			
3RG	4 (8.3)	4 (9.8)	8 (9.0)
3RG/3RC	9 (18.8)	8 (19.5)	17 (19.1)
2RGLOH/3RG	4 (8.3)	4 (9.8)	8 (9.0)
<i>TYMS</i> 3'UTR			
Low expression			
del/del	6 (12.5)	7 (17.1)	13 (14.6)
Medium expression			
ins/del	19 (39.6)	10 (24.4)	29 (32.6)
del/LOH	1 (2.1)	3 (7.3)	4 (4.5)
High expression			
ins/ins	8 (16.7)	13 (31.7)	21 (23.6)
ins/LOH	14 (29.2)	8 (19.5)	22 (24.7)

LOH: Loss of heterozygosity; UTR: Untranslated region.

statistical trend for a higher risk of death was shown in male patients. These findings were consistent in multivariate Cox regression analysis when the history of relapse or *de novo* metastatic disease was considered.

DISCUSSION

This is a retrospective study of 89 patients with mCRC treated with fluoropyrimidine-based chemotherapy, interrogating the association of *TYMS* polymorphisms, LOH, *KRAS*/*BRAF* status with survival outcome. To the best of our knowledge, this is the first time that *TYMS* genotype, LOH and mutations in *KRAS* and *BRAF* have been analyzed in relation to the chemotherapy treatment and survival outcome of patients with mCRC. We report that the polymorphisms of the *TYMS* 3'UTR represent an independent factor, increasing the risk for both disease progression and death of mCRC patients under fluoropyrimidines-based treatment as monotherapy or in combination with oxaliplatin or/and irinotecan, or/and targeted therapy. Also, an independent factor decreasing the risk of both

Table 5 Univariate Cox regression analysis for clinicopathological features and genotype

Variable	PFS			OS		
	HR	95%CI	P value	HR	95%CI	P value
KRAS mutated	1.390	0.895-2.164	0.142	1.669	0.996-2.797	0.052
BRAF V600E	0.884	0.356-2.196	0.791	1.514	0.545-4.207	0.426
LOH	1.013	0.632-1.624	0.957	1.020	0.592-1.758	0.944
TYMS 5'UTR			0.561			0.845
2R	1.000			1.000		
2R/3R	1.243	0.616-2.508	0.543	1.276	0.556-2.928	0.565
3R	0.974	0.474-2.003	0.944	1.239	0.535-2.870	0.616
TYMS 5'UTR			0.887			0.486
2RG	1.000			1.000		
2RG/3RC	1.151	0.535-2.475	0.720	0.978	0.388-2.468	0.963
2RG/3RG	1.351	0.625-2.921	0.444	1.688	0.689-4.132	0.252
3RC	1.038	0.428-2.517	0.935	0.876	0.293-2.620	0.813
3RG/3RC	0.883	0.391-1.995	0.764	1.648	0.660-4.113	0.284
3RG	1.107	0.433-2.832	0.832	1.054	0.348-3.189	0.926
TYMS 5'UTR			0.726			0.562
2R	1.000			1.000		
2RG/3RC	1.864	0.738-4.713	0.188	1.678	0.546-5.160	0.366
2RG/3RCLOH	0.783	0.300-2.044	0.617	1.044	0.328-3.323	0.942
2RG/3RG	1.058	0.372-3.014	0.916	1.869	0.537-6.504	0.325
2RG/3RGLOH	1.936	0.630-5.948	0.248	3.875	1.019-14.740	0.047
2RGLOH/3RC	1.155	0.301-4.441	0.834	1.091	0.126-9.412	0.937
2RGLOH/3RG	1.656	0.617-4.442	0.317	1.745	0.546-5.576	0.348
3RC	1.096	0.428-2.806	0.848	1.070	0.325-3.521	0.912
3RG	1.163	0.432-3.134	0.765	1.281	0.385-4.270	0.687
3RG/3RC	1.001	0.413-2.426	0.998	2.144	0.758-6.064	0.151
TYMS 5'UTR groups			0.812			0.489
Low expression	1.063	0.633-1.784	0.818	0.696	0.384-1.261	0.232
Medium expression	0.888	0.518-1.523	0.667	0.851	0.460-1.572	0.606
High expression	1.000			1.000		
TYMS 3'UTR			0.295			0.340
del/del	0.602	0.305-1.190	0.144	0.563	0.259-1.224	0.147
ins/del	0.764	0.475-1.228	0.267	0.910	0.522-1.587	0.739
ins/ins	1.000			1.000		
TYMS 3'UTR			0.067		1	0.095
del/del	0.421	0.194-0.912	0.028	0.311	0.125-0.772	0.012
del/LOH	0.784	0.263-2.334	0.662	0.773	0.175-3.417	0.734
ins/del	0.438	0.240-0.802	0.007	0.459	0.230-0.918	0.028
ins/LOH	0.516	0.274-0.973	0.041	0.488	0.233-1.020	0.057
ins/ins	1.000			1.000		
TYMS 3'UTR groups			0.225			0.187
Low expression	0.639	0.323-1.263	0.198	0.503	0.230-1.102	0.086
Medium expression	0.435	0.435-1.118	0.135	0.738	0.426-1.279	0.279
High expression	1.000			1.000		

CT: Chemotherapy; LOH: Loss of heterozygosity; OS: Overall survival; PFS: Progression-free survival; UTR: Untranslated region.

disease progression and death was the administration of fluoropyrimidine-based chemotherapy in combination with irinotecan, while the combination of fluoropyrimidines with oxaliplatin was associated with lower risk of death.

In search of prognostic markers towards personalized therapy, studies have investigated *TYMS* gene polymorphisms^[30,31], *TYMS* mRNA expression^[32,33] and *TYMS* protein expression^[34-38]/activity^[39]. Such studies have conflicting results for the way *TYMS* polymorphisms seem to affect the therapeutic result in CRC patients^[30,36,38,40-46]. The numerous *TYMS* polymorphisms and their combination could explain the inconclusive results. For example, the SNP G>C was not considered for many years until its discovery^[15,17,19]. Thus, the homozygous 3R group was considered to be

related with high expression^[15] and could include three subgroups with a different impact in *TYMS* expression (low expression subgroup 3RC/3RC and high expression subgroups 3RG/3RG and 3RG/3RC^[24,28]).

Our results indicate that only 8 (21.6%) out of 37 tumors with the 3R polymorphism are 3RG, without the presence either of LOH or SNP G>C. Similarly, 21 (50%) out of 42 heterozygous 2R/3R tumors are 2RG/3RG. The different distribution of these subgroups in various studies could explain the differential effect on survival. Moreover, another factor held responsible for generating inconclusive results is the addition to fluoropyrimidines of newer chemotherapeutics and targeted agents^[28] which incommode the interpretation of how *TYMS* polymorphisms influence survival outcome across different treatment populations. Moreover, there

Table 6 Multivariate Cox regression analysis

	PFS			OS		
	HR	95%CI	P value	HR	95%CI	P value
KRAS mutated	1.600	1.011-2.531	0.045			
LOH	1.674	0.912-3.071	0.096			
Fluoropyrimidine-based CT						
With irinotecan	0.600	0.372-0.969	0.037	0.352	0.164-0.757	0.007
Without irinotecan	1.000			1.000		
With oxaliplatin				1.000		
Without oxaliplatin				2.702	1.273-5.738	0.010
TYMS 3'UTR groups			0.043			0.027
Low expression	0.432	0.198-0.946	0.036	0.366	0.162-0.827	0.016
Medium expression	0.513	0.287-0.919	0.025	0.559	0.309-1.013	0.055
High expression	1.000			1.000		
Sex						
Males				1.580	0.916-2.724	0.100

CT: Chemotherapy; LOH: Loss of heterozygosity; OS: Overall survival; PFS: Progression-free survival; UTR: Untranslated region.

are other genes, such as *p53*^[47], astrocyte elevated gene-1^[48], and enolase superfamily member 1^[49] that have been proven to participate in the final level of *TYMS* expression^[18,47-50]. Thus, the rather small size samples used in most studies could not examine thoroughly the plethora of all these factors and possible interactions among them, without conflicting results.

Another reason responsible for conflicting results across studies is the categorization of *TYMS* polymorphisms into only two groups^[18,51], which leads to misclassifying polymorphisms with uncertain effect. Although such a classification model is preferred because it facilitates statistical processing (e.g., by increasing the size of each group) and the interpretation of statistical processing, it also entails the risk of increasing the probability of classification error.

Different to previous studies^[30,31,50-53], ours took into consideration the extensive number of *TYMS* polymorphisms, as well as their combinations with LOH and *KRAS* / *BRAF* mutations. Additionally, for the first time, we classified the polymorphisms of each UTR region into three groups according to the level of *TYMS* expression.

The low expression group of 5'UTR polymorphism includes tumors with two alleles, each with one being an active USF-1 binding site. Members of the high expression group have no 2RG allele and they include heterozygous tumors in which, due to LOH, the allele 2RG was deleted. Medium expression group includes the heterozygous tumors with three USF-1 binding sites (one in the 2RG and two in the 3RG), resulting in one more than the low expression and one less than the high expression group. Also in this group, we included tumors with only one 2R allele, as LOH eliminates the 3R allele. Although they have less than three USF-1 binding sites, the LOH situation bears a loss of genetic material from chromosome 18q that, in ways not fully understood, adversely affects survival^[54].

The low expression group of 3'UTR polymorphism contains the homozygous deletion of the 6 bp insertion that leads to destabilization of *TYMS* mRNA, resulting in reduced translation and eventually reduced *TYMS* activity. The high expression group has only ins alleles, homozygous or in combination with LOH, which impart stability to *TYMS* mRNA and thus, by increasing *TYMS* production/activity, increases the risk of poor response or development of resistance^[18,19]. Tumors in the medium expression group have an allele with deletion, which coexists with either ins allele or LOH, that have been associated with increased risk of relapse^[54].

On the basis of previous studies, *TYMS* 5'UTR may be linked to survival outcomes^[41,55]. Contrary to these, in the multivariate Cox regression analysis of our data, the groups of 5'UTR polymorphisms did not emerge as factors of survival outcome. However, the 3'UTR polymorphisms' groups, were identified as independent factors of disease progression and death.

More specifically, the high expression group was identified as an independent risk factor of disease progression and death compared to the medium/low-risk groups (Table 5). Similar to our findings, a previous study showed that mCRC patients with del/del genotype treated with 5FU/oxaliplatin had significantly longer OS^[31]. The ins allele, present in high-risk genotypes (ins/ins and ins/LOH) has been associated with higher *TYMS* mRNA stability and *TYMS* protein expression^[18]. It is logical to assume that the mRNA stability has a more significant role in *TYMS* protein production than the number of transcripts. Hence, even if *TYMS* 5'UTR has a 3RG polymorphism leading to higher mRNA production, the complete absence of ins allele in *TYMS* 3'UTR could cause *TYMS* mRNA instability and therefore decreased *TYMS* translation. On the contrary, in theory the final outcome of decreased mRNA production of 2R cases combined with ins/ins genotype could be an increase of protein production due to the stability of transcribed mRNA and translational efficacy.

Tumors with 2R/3RLOH genotype have been shown

to be expressing significantly lower levels of TYMS protein than those with 2RLOH/3R^[56]. Also, patients with mCRC bearing 2R/3RLOH genotype have been shown to have better survival than those with 2RLOH/3R^[12]; although in the later study the SNP G>C was not taken into consideration. LOH is as likely to lead to altered genotypes, either with high or low TYMS protein expression (2RLOH/3RG and 2R/3RGLOH respectively). But the loss of chromosomal material from 18q, the cause of LOH, has been shown to act as a molecular marker of adverse prognosis^[29], even if combined with the low-risk 2R allele. This is in agreement with our results as LOH remained in the Cox proportional hazards model as a factor that associates with disease progression with marginal statistical significance (HR = 1.674, 95%CI: 0.912-3.071, $P < 0.1$). This association was not observed for risk of death, probably due to the numerous factors that affect this outcome, such as the additional chemotherapy lines.

It has been previously shown that patients with *KRAS* mutant tumors had significantly lower *TYMS* mRNA levels, especially in proximal colon tumors^[57]. In our study, we were able to identify an association of *KRAS* wild-type only with polymorphism 3RG/3RC (RR = 1.753, 95%CI: 1.156-2.657, $P < 0.04$), a member of the high TYMS protein expression group.

The addition of bevacizumab in the fluoropyrimidine-based 1st line chemotherapy for mCRC did not emerge, in the Cox model, as a factor affecting survival outcome in our study. To date, no prospectively validated biomarkers have emerged to include or exclude patients from anti-VEGF therapy^[58]. Pander *et al.*^[59] have shown that there is a genetic interaction between the polymorphisms in the *TYMS* enhancer region (5'UTR) and VEGF +405g>c polymorphisms as a predictor of the efficacy of capecitabine/oxaliplatin/bevacizumab in mCRC patients, but only for PFS. Also, Watanabe *et al.*^[60] have found that higher TYMS levels are associated with an adverse response to bevacizumab therapy. In this context, it could be proposed that in studies applying anti-VEGF and targeted therapy, *TYMS* polymorphisms should be considered. Overall, there is great need for a prognostication model that would include all these polymorphisms with *RAS* mutations for treatment tailoring.

In our study, we did not examine TYMS protein expression, as this could be affected by a plentitude of factors^[47,48,50] and altered in the course of the disease. For example, discordance in *TYMS* mRNA expression and TYMS protein levels has been found between primary and secondary tumors^[33,61,62]. Also, in an autoregulatory manner, the binding of TYMS protein to its own mRNA, as well as the binding of TYMS to *p53* mRNA, causes translational repression^[13,63,64].

Some limitations of this study should be addressed. The plethora of genotypes resulting from the polymorphisms occurring in the UTRs of *TYMS* is difficult to be analyzed with a small patient group. Moreover,

previous exposure to adjuvant therapy with fluoropyrimidines, that could associate with resistance to fluoropyrimidines, was not taken into consideration. The allocation of *TYMS* polymorphisms into groups was based on published research but the conflicting results observed in these studies and ours highlight the need for further analysis on larger scale datasets. Also, we did not examine the TYMS protein expression and activity. Finally, due to the retrospective nature of this analysis we could not correlate these findings to the treatment toxicity.

After taking into account the SNP G>C and LOH, only the polymorphisms in the *TYMS* 3'UTR, affecting the stability of mRNA, independently influenced survival outcome for patients with mCRC treated with fluoropyrimidines-based chemotherapy. Genotypes that include del alleles, linked to *TYMS* mRNA instability, had better survival outcome. *KRAS* mutation was associated with high risk of disease progression. Combinations that included irinotecan were associated with lower risk of disease progression and death. Future studies should focus on gathering large samples and carefully selecting batteries of biomarkers to be examined in multivariate analysis. For the more complete assessment of the effect of *TYMS* gene polymorphisms, LOH should be considered. Further prospective studies are needed to elucidate the role of *TYMS* polymorphisms in tailoring treatment of patients with mCRC.

COMMENTS

Background

Metastatic colorectal cancer (mCRC) remains a significant cause of cancer-related death worldwide, although important improvements have been achieved in the last decades. It has been found that large variation exists in individual patient prognosis and response to chemotherapy, caused by molecular heterogeneity. Around 32%-40% of CRC harbor a *KRAS* mutation, which is a predictor of response to anti-EGFR treatment, while *BRAF* is a *KRAS* downstream abnormally activated kinase that has been shown to have similar adverse effects on treatment response. Several polymorphisms of the thymidylate synthase (*TYMS*) gene have been connected to variable TYMS protein levels and therapeutic outcome in relation to 5-FU, while loss of heterozygosity (LOH) is included in potential resistance mechanisms to fluoropyrimidines. This study aimed to investigate the associations of *TYMS* polymorphisms, LOH, *KRAS/BRAF* mutations and clinicopathologic characteristics with the survival outcome of patients with mCRC treated with 1st line fluoropyrimidine-based chemotherapy.

Research frontiers

To the best of the authors' knowledge, this is the first study that analyzes the extensive number of *TYMS* polymorphisms, particularly their combination with LOH and *KRAS* and *BRAF* mutations in relation to the chemotherapy treatment and the survival outcome of patients with mCRC. Additionally, for the first time, the authors classified the polymorphisms of each untranslated region (UTR) into three groups according to the level of *TYMS* expression. The results of this study contribute to clarifying the significance of *TYMS* polymorphisms for patients with mCRC.

Innovations and breakthroughs

In this study, the groups of *TYMS* 5'UTR polymorphisms did not emerge as factors of survival outcome. However, the 3'UTR polymorphisms' groups were identified as independent factors of disease progression and death. Genotypes

that included del alleles, linked to *TYMS* mRNA instability, had better survival outcome.

Applications

This study suggests that *TYMS* 3'UTR polymorphisms independently influence survival outcome for patients with mCRC treated with fluoropyrimidines-based chemotherapy. Genotypes that include del alleles may benefit from fluoropyrimidine-based chemotherapy. Future studies should gather large sample sets and carefully select the biomarkers to be examined in multivariate analysis, taking into consideration LOH.

Terminology

UTR: Regions of the mRNA that are not translated into protein but, among other things, affect the post-transcriptional regulation of gene expression. Upstream stimulatory factor: Factors that enhance the gene promoter and lead to increased gene expression.

Peer-review

Good overview of the role of *TYMS* in the treatment protocol. Will be of interest to the readership.

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Clinical Trials Study

Characterizing gastrointestinal stromal tumors and evaluating neoadjuvant imatinib by sequencing of endoscopic ultrasound-biopsies

Per Hedenström, Bengt Nilsson, Akif Demir, Carola Andersson, Fredrik Enlund, Ola Nilsson, Riadh Sadik

Per Hedenström, Riadh Sadik, Division of Medical Gastroenterology, Department of Internal Medicine, Sahlgrenska University Hospital, S-413 45 Gothenburg, Sweden

Bengt Nilsson, Department of Surgery, Sahlgrenska University Hospital, S-413 45 Gothenburg, Sweden

Akif Demir, Carola Andersson, Fredrik Enlund, Ola Nilsson, Department of Clinical Pathology and Genetics, Sahlgrenska University Hospital, S-413 45 Gothenburg, Sweden

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Correspondence to: Riadh Sadik, Associate Professor, Division of Medical Gastroenterology, Department of Internal Medicine, Sahlgrenska University Hospital, Blå Stråket 3, S-413 45 Gothenburg, Sweden. riadh.sadik@vgregion.se

Telephone: +46-31-3421000

Fax: +46-31-827458

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Abstract

AIM

To evaluate endoscopic ultrasound (EUS)-guided biopsies for the pretreatment characterization of gastrointestinal stromal tumors (GIST) to personalize the management of patients.

METHODS

All patients with lesions suspected to be GIST who were referred for EUS-sampling at a tertiary Swedish center were eligible for inclusion 2006-2015. During the observational study phase (2006-2011), routine fine-needle-aspiration (EUS-FNA) was performed.

In 2012-2015, we converted to an interventional, randomized protocol with dual sampling EUS-FNA and fine-needle-biopsy-sampling (EUS-FNB) for all lesions. c-KIT- and DOG-1-immunostaining was attempted in all samples and a manual count of the Ki-67-index was performed. FNB-sampled tissue and the resected specimens were subjected to Sanger sequencing of the *KIT* and platelet-derived growth factor alpha (*PDGFRA*) genes.

RESULTS

In all, 64 unique patients with GIST were included, and of these, 38 were subjected to pretreatment dual sampling. EUS-FNB had a higher diagnostic sensitivity when compared head-to-head with EUS-FNA (98% *vs* 58%, $P < 0.001$) and was more adequate for Ki-67-indexing (Ki-67_{EUS}) (92% *vs* 40%, $P < 0.001$). Sequencing of EUS-biopsies was successful in 43/44 (98%) patients, and the mutation profiles (*KIT*-mutation 73%, *PDGFRA*-mutation 18%, wild-type 7%) were fully congruent with those detected in the corresponding resected specimens. In imatinib-naïve patients, the Ki-67_{EUS} was comparable with the Ki-67-index in the corresponding surgical specimens (Ki-67_{SURG}) (2.7% *vs* 2.9%, $P = 0.68$). In patients treated with neoadjuvant imatinib who also carried mutations indicating sensitivity, the Ki-67_{EUS} was higher than the Ki-67_{SURG} (2.5% *vs* 0.2%, $P = 0.005$), with a significant reduction in the Ki-67-index of -91.5% (95%CI: -82.4 to -96.0, $P = 0.005$).

CONCLUSION

EUS-guided biopsy sampling is accurate for the pre-treatment diagnosis and characterization of GISTs and allows the prediction and evaluation of tumor response to neoadjuvant imatinib therapy.

Key words: Endosonography; Fine-needle biopsy; Gastrointestinal stromal tumor; *KIT*; Platelet-derived growth factor alpha; Tumor proliferation rate; Ki-67 index; Neoadjuvant treatment; Imatinib

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Core tip: Personalization of the management and treatment of gastrointestinal stromal tumors (GIST) requires an extensive characterization of individual tumors. Information on the tumor proliferation rate and the *KIT*- and platelet-derived growth factor alpha (*PDGFRA*)-mutation profile is essential. While endoscopic ultrasound (EUS)-FNA is reported to be suboptimal for the diagnosis of GIST, EUS-guided biopsy sampling (EUS-FNB) has not been evaluated for the characterization of GISTs. This prospective, long-term study showed that EUS-FNB was safe and highly accurate for the pretreatment diagnosis of GISTs, for the sequencing of *KIT* and *PDGFRA*, and for the assessment of the tumor proliferation rate (Ki-67-index). By obtaining this information, we managed to guide and evaluate neoadjuvant imatinib therapy in patients with GIST.

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INTRODUCTION

In personalized medicine, a detailed characterization of tumors is essential for accurate patient management. A gastrointestinal stromal tumor (GIST) is an example of a tumor entity that illustrates the potential for genotype-driven targeted therapy^[1]. However, to turn this potential into clinical reality, an extensive characterization of the tumor is needed.

First, GISTs are difficult to diagnose preoperatively. A sufficient quantity of tumor material is required for a conclusive diagnosis, which is reached by immunostaining for c-KIT (CD117), anoctamin 1 (DOG-1), or CD34^[2].

Second, the tumor response to the tyrosine kinase inhibitor imatinib depends upon the mutation profile of the individual tumor. The genes commonly mutated in GIST are *KIT* proto-oncogene receptor tyrosine kinase (*KIT*) (exon 9, 11, 13 or 17), and less frequently, platelet-derived growth factor alpha (*PDGFRA*) (exon 12, 14 or 18). Primary resistance, or reduced sensitivity to imatinib, is related to mutations in exon 9, 13, or 17 of *KIT*, exon 18 of *PDGFRA*, or to the wild type profile (WT)^[3]. Secondary resistance may evolve during imatinib treatment due to additional mutations^[4]. Imatinib treatment of GISTs has led to a significant improvement in survival^[5,6], and neoadjuvant imatinib is valuable, especially in advanced tumors^[7,8]. The mutation profile is also a predictor of overall survival^[9].

Third, the prognostic risk of GIST varies from excellent to poor^[10]. In resected GIST specimens, the National Institutes of Health prognostic risk classification is used to assess the prognosis based on the tumor size and the tumor proliferation rate (the mitotic index^[11]). The Ki-67-index is an alternative indicator of the tumor proliferation rate in GIST as well as in many other tumor entities. The level of the Ki-67-index in GIST strongly correlates with the prognosis^[12-16].

Endoscopic ultrasound (EUS) enables the visualization of tumors such as GIST and the sampling with fine-needle aspiration (FNA) for cytology. The analysis of EUS-FNA-samples by mass spectrometry has been shown to facilitate the challenging assessment of cystic pancreatic lesions, a potential precursor of pancreatic adenocarcinoma^[17]. In GISTs however, EUS-FNA-samples are often non-diagnostic^[18-20], which also leads to an evident lack of prognostic information based on the tumor mutation profile and the tumor proliferation rate. This drawback of EUS-FNA is a major obstacle for the early personalized management of

patients with GIST. Confronted with the difficulties in the characterization of GISTs, clinicians have to decide on surgical resection based on the mere suspicion of malignancy and without knowledge of the tumor proliferation rate. Finally, the decision on expensive neoadjuvant imatinib treatment can only be based on probability and not on the actual mutation profile of *KIT* and *PDGFRA*.

The primary aim of the study was to evaluate EUS-guided sampling for the diagnosis and the pretreatment characterization of GIST with respect to the tumor proliferation rate and the mutation profile of *KIT* and *PDGFRA*. The secondary aim was to evaluate the Ki-67-index in EUS-biopsies and in resected specimens as a marker for individual tumor response to neoadjuvant imatinib therapy.

MATERIALS AND METHODS

Study patients

Sahlgrenska University Hospital is a tertiary center for advanced endoscopy and for the management of GIST in the region of west Sweden (population: 1.6 million). All patients who were referred to the unit for a diagnostic EUS-guided sampling of a suspicious GIST were eligible for inclusion in this prospective study as consecutive subjects. Findings suspected to be GIST were defined as lesions previously detected at gastroscopy or cross-sectional imaging with a probable origin from within the gastric or duodenal wall and with a hypoechoic appearance on ultrasound. Ongoing treatment with imatinib was a criterion for non-eligibility. Subjects were later excluded if the follow-up was consistent with an alternative diagnosis of the suspected lesion or if the GIST diagnosis could never be firmly established by conclusive histopathology including positive immunostaining for c-KIT or DOG-1.

The time frame for this study was February 2006 to December 2015. The medical records and the data from a parallel, prospective study on the long-term outcome of all patients with GIST in the region^[15] were used to assess the results of EUS-guided sampling with respect to the clinical follow-up and the surgical outcome.

This study was reviewed and approved by the Regional Ethical Review Board of Gothenburg. Written informed consent was obtained from all patients.

This study is registered at ClinicalTrials.gov. The registration identification number is NCT02360839.

EUS - examination and sampling

All study subjects were examined by EUS under conscious sedation. Linear echoendoscopes [2006-2012: Pentax EG3830UT (Tokyo, Japan), 2012-: Pentax EG3870UTK] and an ultrasound processor (HI VISON Ascendus, Hitachi, Tokyo, Japan) were used for this purpose. The examinations were performed by the study endosonographer (RS). The tumor location, size,

echogenicity, and vascularization were assessed before the optimal sampling route was chosen.

The Baseline Period (2006-2011): During the baseline period (BP), the study design was observational. All patients were prospectively included except for the first nine patients of the study start-up phase.

The suspicious GISTs were sampled at the discretion of the endosonographer with no specific interventional procedure. EUS-FNA was performed with either a 22 G or a 25 G needle (Olympus, Aomori, Japan/Boston Scientific, Spencer, United States/Wilson-Cook Medical, Limerick, Ireland), while a 19 G trucut-needle (TCB) was used (Wilson-Cook Medical) for biopsies.

The Study Period (2012-2015): We designed an interventional study protocol in 2011 and modified the sampling procedure in 2012. From 2012 to 2015 (Study Period, SP), dual sampling was performed on *each* individual subject using both EUS-FNA for cytology (needles as described above) and EUS-guided core biopsy sampling (EUS-FNB) for histology (22 G Procore or 19 G Procore, Wilson-Cook Medical)^[21]. In blocks of four and by using sealed envelopes, the patients were randomized to a first pass with FNA or FNB. This was performed to eliminate the introduction of a bias related to the sampling sequence. Further passes were performed by alternating the needles. A non-necrotic area of the tumors was targeted and sampling was performed by fanning. If the yield was poor, the sampling time and the suction were increased.

The first six subjects of the *Study Period* underwent EUS-FNB only to accustom the endosonographer to the new sampling technique. With some limitations, a cytotechnician was present for rapid on-site cytology evaluation.

Cytopathology and histopathology

FNA-samples and FNB-biopsies were processed and analyzed as described in the Supplementary Methods.

Ki-67-indexing

Representative samples were subjected to immunostaining for Ki-67 as described in the Supplementary Methods. The quality and the adequacy of the FNA-samples and the FNB-biopsies for the assessment of the Ki-67-index were categorized as adequate or non-adequate by the study cytopathologist (AD) and pathologist (ON).

Given the superior quality of the FNB-biopsies compared with FNA-samples, only the Ki-67-index of FNB-biopsies (Ki-67_{EUS}) was calculated in detail on printouts of digital images captured *via* an x40-magnification objective (Eclipse E1000, Nikon, Japan) with a ProgResC7-camera (Jenoptik, Germany). Manual counting of positive nuclei including 2000 tumor cells was performed. Counting by eyeballing and digital

counting are considered less accurate and were not used^[22]. The result was recorded as the fraction of positive tumor cells (%). Similarly, the Ki-67-index of the corresponding surgical specimens (Ki-67_{SURG}) was analyzed in subjects who underwent resection.

In each case sampled by EUS-FNB and subjected to surgical resection, we calculated the following parameters: (1) The pairwise difference in the Ki-67-index (%-units): $Ki-67_{DIFF} = Ki-67_{EUS} - Ki-67_{SURG}$; and (2) The pairwise reduction in the Ki-67-index (%): $Ki-67_{RED} = -100 \times [(Ki-67_{SURG}) / (Ki-67_{EUS})]$

Sequencing and mutational analysis

No sequencing of FNA-samples was performed since the sample quantity and quality were poor compared with that of FNB-biopsies. All FNB-biopsies were subjected to mutational analysis by Sanger sequencing as were the corresponding resected specimens (in subjects who underwent resection). In the early part of the SP, the sequencing of FNB-biopsies was performed for research purposes after EUS. In the latter part of the SP, the procedure was implemented into clinical practice and was performed directly after EUS to supply the genetic information to the clinician (BN).

The preparation of FNB-biopsies for DNA-extraction followed by sequencing is described in the Supplementary Methods.

Follow-up, reference standard, and definitions

Subjects were followed-up by the clinician (BN) for 5 year or until death. Neoadjuvant imatinib therapy was considered and initiated by the clinician (BN). Patients having small tumors (size < 20 mm) were not evaluated for neoadjuvant imatinib. The cases subjected to surgical resection, either treated or not treated with neoadjuvant imatinib, were designated as: (1) Neo- (no neoadjuvant imatinib therapy); (2) Neo + s (neoadjuvant imatinib and imatinib-sensitive mutation profile); or (3) Neo + r (neoadjuvant imatinib therapy and imatinib-resistant mutation profile) according to the table in the Supplementary Methods. The tumor response was evaluated on a clinical basis in some cases *via* the comparison of the fluorodeoxyglucose positron emission tomography (¹⁸FDG-PET) signal at baseline and at 3-8 wk after the start of imatinib treatment.

Resected specimens were used to validate the diagnosis of GIST. In patients not subjected to surgery, the GIST-diagnosis was considered established if cytopathology or histopathology of tumor sampling was conclusive for GIST including positive immunostaining for KIT or DOG-1.

The FNA-samples and FNB-biopsies were classified as diagnostic only if they contained adequate GIST material for accurate diagnostic KIT or DOG-1 immunostaining. Samples with adequate tumor yield but with failed or inconclusive immunostaining were classified as suggestive of GIST. Samples without

adequate tumor yield were considered non-diagnostic.

Outcome

The primary outcome of this study was the diagnostic sensitivity of EUS-guided sampling for GIST. The secondary outcome was the EUS-sample adequacy (1) for the assessment of the Ki-67-index; (2) for the sequencing of *KIT* and *PDGFRA* (FNB-biopsies only); and (3) for the evaluation of response to neoadjuvant imatinib therapy (FNB-biopsies only), which was measured as the difference in the Ki-67-index of FNB-biopsies compared with that of resected specimens.

Statistical analysis

Demographics, tumor characteristics, and procedures were compared using Fisher's exact test and the Mann-Whitney *U*-test. Prior to the interventional phase (the SP), a sample size calculation was performed for paired, dichotomous variables (statistical power = 80%, alpha error = 0.05), which aimed to detect a difference in sensitivity of 35% in order to compare EUS-FNA and EUS-FNB at dual sampling. A sample size of 33 cases was returned.

The diagnostic sensitivity for GIST as a binary outcome was compared between sampling groups using Fisher's exact test (unpaired data) and McNemar's test (paired data) in an intention-to-treat analysis. The Ki-67-index of FNB-biopsies and resected specimens was compared using the Wilcoxon signed-rank test. The mutation profile of FNB-biopsies and resected specimens was compared on a case-by-case basis. The (95%CI) was calculated when possible. The statistical significance level was set at $P < 0.05$. All authors had access to the study data and approved the manuscript. The STARD protocol was applied throughout the study.

RESULTS

Patient characteristics

In total, 64 patients [34 women/30 men, median age 70 (range: 23-89)] were included (Figure 1). Validation specimens were available in 43/64 (67%) cases (resected specimen: 42 cases, endoscopy forceps: one case). The baseline characteristics are shown in Table 1.

Primary outcome

The diagnostic sensitivity of EUS-FNB (dual procedures FNB+FNA: $n = 38$, single FNB-procedures: $n = 6$) was superior both compared with routine EUS-FNA performed during the *Baseline Period* [43/44 (98%) vs 8/16 (50%), ^a $P < 0.001$] and compared with EUS-FNA in a head-to-head comparison of dual sampling procedures during the SP [37/38 (97%) vs 22/38 (58%), ^b $P < 0.001$], as shown in Figure 2.

Supposing that the two cases in Figure 1, which had an unclear final diagnosis during the *Study Period*, were actually true GISTs, the worst scenario of the

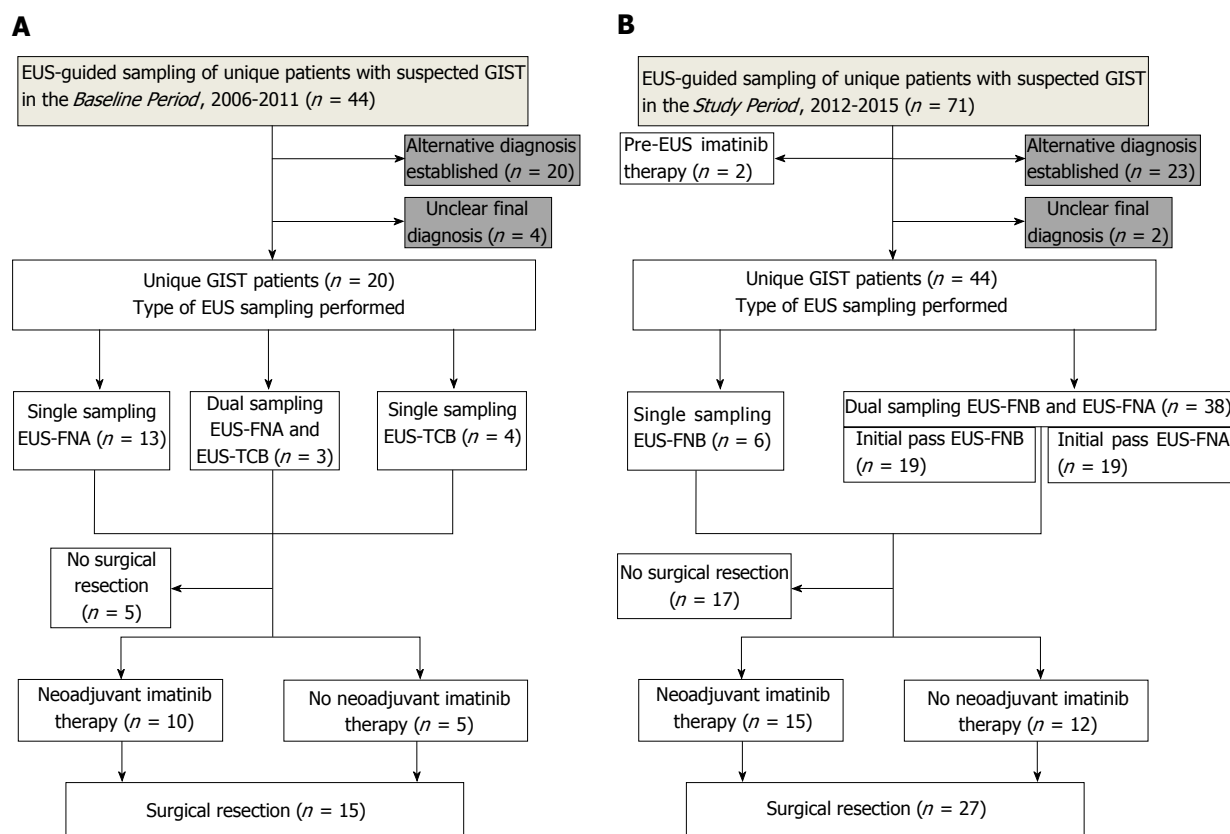


Figure 1 Flow charts of the study inclusion process, February 2006-December 2015. A: The *Baseline Period*, observational study design (2006-2011); B: The *Study Period*, interventional study design (2012-2015). GIST: Gastrointestinal stromal tumor; EUS: Endoscopic ultrasound; FNA: Fine-needle aspiration; FNB: Fine-needle biopsy.

Table 1 Baseline characteristics, follow-up, and clinical outcome n (%)

Parameter	Baseline period	Study period	P value
Age, median (range)	75 (23-89)	68 (49-89)	0.07
Gender (M/F)	11/9	19/25	0.43
Study patients (n)	20	44	
Tumor location (n)			
Stomach	18	40	
Duodenum	2	4	
Tumor size (mm), median (range)	60 (12-200)	38 (13-220)	0.29
Tumor endosonographic appearance			
Homogenous (solid)	8	17	
Heterogeneous (necrotic)	12	27	
EUS-FNA (n)	16	38	
Needle (22 G/25 G)	12/4	26/12	0.75
Passes (n), median (range)	2 (1-3)	3 (1-4)	0.10
EUS-FNB (n)	7	44	
Needle (TCB 19 G/FNB 19 G/FNB 22 G)	7/0/0	0/5/39	< 0.001
Passes (n), median (range)	1 (1-4)	2 (1-4)	0.15
ROSE ¹	9 (56)	26 (68)	0.53
Study cytologist	5 (31)	32 (84)	< 0.001
Study pathologist	2 (29)	38 (86)	0.003
Resected cases	15 (75)	27 (61)	0.40
Resection margin (R0/R1/R2)	13/1/1	24/3/0	
Follow-up time ² , mo (range)	72 (16-105)	19 (1-45)	
Overall survival (OS) ³ , 12 mo	20/20 (100)	31/31 (100)	1.00
OS, 24 mo	19/20 (95)	17/18 (94)	1.00
OS, 36 mo	18/20 (90)	10/11 (91)	1.00
Patients deceased	9/20 (45)	2/44 (5)	

¹Rapid on-site cytology evaluation by a cytotechnician; ²From the date of EUS until death or until end of follow-up; ³From the date of EUS until 12, 24 and 36 mo post-EUS. EUS: Endoscopic ultrasound; FNA: Fine needle aspiration; FNB: Fine needle biopsy.

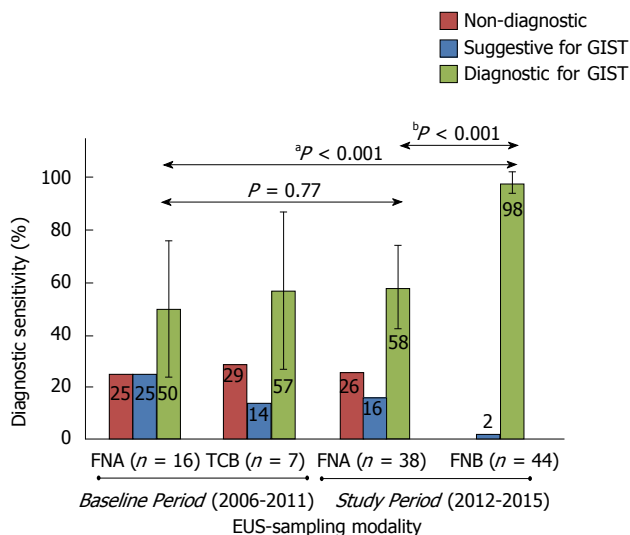


Figure 2 The diagnostic sensitivity of endoscopic ultrasound-guided sampling in unique Gastrointestinal stromal tumor-cases ($n_{\text{tot}} = 64$) examined at the Sahlgrenska University Hospital from 2006 to 2015. The error bars represent the 95%CI. FNA: Fine-needle aspiration; FNB: Fine-needle biopsy; TCB: Trucut-biopsy; GIST: Gastrointestinal stromal tumor; EUS: Endoscopic ultrasound.

sensitivity of EUS-FNB would still be superior to that of EUS-FNA in a head-to-head comparison (37/40, 93% vs 22/40, 55%, $P < 0.001$). The sensitivity of FNA-samples was not affected by the recorded variables, as shown in Table 2.

One minor adverse event was recorded (1/64, complication rate 1.6%). Patient #33, who had a 3-cm GIST in the stomach, experienced local bleeding post-EUS, which was stopped by adrenalin injection. No technical failure was observed for any needle. No tumor seeding was observed in any of the patients during follow-up.

Secondary outcomes

Ki-67-indexing: During the BP, the FNA-samples were of adequate quality for the assessment of the Ki-67-index in 3/16 (19%) cases. In the dual sampling procedures during the SP, FNB-biopsies were more often of adequate quality (37/38, 92%) compared with FNA-samples (15/38, 40%, $P < 0.001$), and the FNB-biopsies were adequate for the assessment of the Ki-67-index in all cases subjected to surgical resection 27/27 (100%).

In non-resected cases with adequate FNB-biopsies ($n = 14$), the mean Ki-67_{EUS} was 6.1% (95%CI: 2.5 to 9.7).

In resected cases not treated with neoadjuvant imatinib ($n = 12$, Neo- Group), the median Ki-67_{EUS} was not significantly different from the median Ki-67_{SURG} [2.7% vs 2.9%, $^aP = 0.68$, median Ki-67_{DIFF} = -0.30 (95CI: -0.62 to 0.57, $P = 0.64$)] (Figures 3A, 4A and B). No significant reduction was observed in the Ki-67-index [median Ki-67_{RED} = 10.7% (95%CI: -22.3

Table 2 Parameters with potential influence on the sensitivity of Endoscopic ultrasound-fine needle aspiration n (%)

Parameter			P value
Tumor echogenicity	Homogenous (solid)	Heterogeneous (necrotic)	
EUS-FNA-sensitivity	11/20 (55)	18/32 (56)	1.0
Tumor size	< 30 mm	≥ 30 mm	
EUS-FNA-sensitivity	10/18 (56)	19/34 (56)	1.0
ROSE	ROSE	non-ROSE	
EUS-FNA-sensitivity	21/34 (62)	8/18 (44)	0.26
FNA-needle	22 gauge	25 gauge	
EUS-FNA-sensitivity	20/37 (54)	9/15 (60)	0.77
FNA-passes	< 3 passes	≥ 3 passes	
EUS-FNA-sensitivity	9/17 (53)	20/35 (57)	1.0
Sampling order ¹	EUS-FNA first	EUS-FNB first	
EUS-FNA-sensitivity	12/19 (61)	10/19 (53)	0.63

¹Only the GISTs ($n = 38$) sampled during the Study Period (2012-2015). All study GISTs examined by EUS-FNA ($n = 52$) from 2006 to 2015. GIST: Gastrointestinal stromal tumor; EUS: Endoscopic ultrasound; FNA: Fine needle aspiration; FNB: Fine needle biopsy.

to 26.5, $P = 0.70$)).

Sequencing of KIT and PDGFRA: The FNB-biopsies were adequate for successful Sanger sequencing of KIT and PDGFRA in 43/44 (98%) cases (Table 3). Among resected cases, full congruence (100%) was found in the comparison of the mutations detected in the FNB-biopsies and the mutations detected in the corresponding resected specimens ($n = 27$). Additional mutations in KIT or PDGFRA were not observed in any of the resected specimens. The sole FNB-biopsy (case #2) with inadequate material for diagnostic immunohistochemistry still contained sufficient material for successful sequencing.

Evaluation of neoadjuvant imatinib therapy: (1) Neoadjuvant imatinib + imatinib-sensitive mutation detected (Neo + s Group): In resected patients who were treated with neoadjuvant imatinib and who carried a mutation suggestive of primary sensitivity to imatinib [$n = 10$: KIT exon 11 ($n = 9$); PDGFRA exon 12 ($n = 1$)], the median Ki-67_{EUS} was significantly higher than the median Ki-67_{SURG} [2.5% vs 0.2%, $P = 0.005$, median Ki-67_{DIFF} = 2.3 (95%CI: 0.67 to 5.37, $P = 0.005$)] (Figures 3B, 4C and D). Consequently, a significant reduction was observed in the Ki-67-index [median Ki-67_{RED} = -91.5% (95%CI: -82.4 to -96.0, $P = 0.005$)).

In the five patients with a positive baseline ¹⁸FDG-PET, a signal reduction was recorded in the post-treatment ¹⁸FDG-PET signal.

(2) Neoadjuvant imatinib + imatinib-resistant mutation detected (Neo + r Group): Five resected patients who were treated with neoadjuvant imatinib carried a mutation suggestive of primary resistance to imatinib [$n = 5$: PDGFRA exon 18 D842V ($n = 2$); WT ($n = 2$); KIT exon 13 p. K642E ($n = 1$)]. The median Ki-67_{EUS}

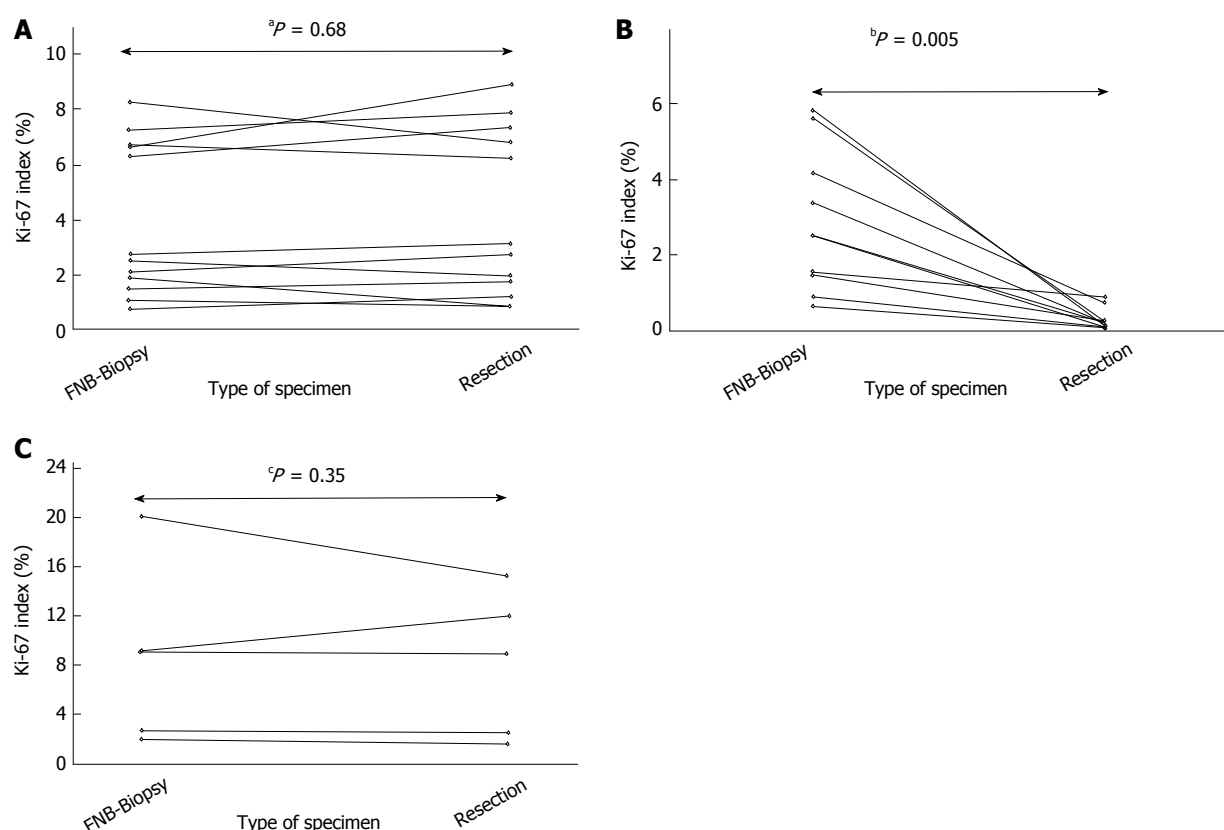


Figure 3 The Ki-67-index (%) of the fine-needle biopsy and of the corresponding resected specimen in each patient who underwent resection. A: Patients not treated with neoadjuvant imatinib; B: Patients treated with neoadjuvant imatinib who carried an imatinib-sensitizing *KIT*- or *PDGFRA*-mutation; C: Patients treated with neoadjuvant imatinib who carried a *KIT*- or *PDGFRA*-mutation (or wild type profile), which indicates resistance to imatinib.

was not significantly different from the median Ki-67_{SURG} (9.1% vs 9.0%, $P = 0.35$) (Figures 3C, 4E and F). In addition, no significant reduction was observed in the Ki-67-index (median Ki-67_{RED} = -10.2%, $P = 0.50$). The baseline ¹⁸FDG-PET signal was measured and was positive in two patients. In one patient, no reduction was observed in the post-treatment ¹⁸FDG PET-signal, while a weak reduction was recorded in the other (case #20).

DISCUSSION

This study provides new knowledge on the ability to perform extensive preoperative and pretreatment characterization of gastrointestinal stromal tumors. This knowledge enables the introduction of an early personalized management and treatment of patients with GIST.

According to the results of this work, endoscopic ultrasound-guided biopsy sampling is a safe and accurate method for the purpose of diagnosis and for further analyses of the tumor material in GIST.

A correct and reliable diagnosis of GIST is important to avoid unnecessary resections of benign lesions that are merely suspected GISTs. A non-diagnostic sample will result in uncertain management and a resection based on suspicion alone. Prospective studies that evaluate the accuracy of EUS-guided sampling of

GIST are scarce. Studies have reported a diagnostic sensitivity of approximately 50%^[18], which is in agreement with the sensitivity of EUS-FNA in our work. A sensitivity of 80% was reported in a recent study that excluded small tumors (< 20 mm)^[23].

In the present study, a new method of dual sampling with both EUS-FNA and EUS-FNB was used on all tumors during a 4-year period, and these modalities were compared head-to-head. As a result, we have now shown that EUS-FNB can be used for the reliable and safe diagnosis of GIST in up to 98% of cases including small tumors.

The treatment decision for GIST requires a balance between the benefits and drawbacks of both surgical and pharmacological therapies. The initiation of adjuvant and neoadjuvant treatment can vary in between institutions and the prognostic risk needs to be addressed since there are potential side-effects of imatinib. Nevertheless, the treatment with tyrosine kinase inhibitors should be prescribed only to patients who carry sensitive mutations. Without the mutational status of *KIT* and *PDGFRA*, the clinician randomly selects the therapy. The mutation profile is also valuable for the prediction of survival^[9].

This study shows that a mutational analysis of pretreatment FNB-biopsies by Sanger sequencing provides the genetic information needed. In the early part of the SP the clinician had to decide on

Table 3 Individual case data on the mutation profile, treatment, and Ki-67-index

Case	Mutation gene and exon	Mutation	Treatment neoadj ¹	Surgery	Group	EUS-surgery (mo)	Ki67EUS	Ki67SURG	Ki67RED
1	<i>KIT</i> exon 11	p.V560del	No	Yes	Neo-	2	2.2	2.8	26
2	<i>PDGFRA</i> exon 18	p.D842V ²	No	No	NA	-	NC	-	-
3	Wild Type	Wild Type	Yes	Yes	Neo + r	2	2.1	1.7	-19
4	<i>KIT</i> exon 11	p.V559D	Yes	Yes	Neo + s	6	4.2	0.7	-82
5	<i>KIT</i> exon 11	p.Y553-Q556del	Yes	Yes	Neo + s	13	1.5	0.2	-84
6	Unknown	Unknown	No	No	NA	-	NC	-	-
7	<i>KIT</i> exon 11	V559D	No	No	NA	-	2.4	-	-
8	<i>KIT</i> exon 11	p.P577-R586dupl	No	Yes	Neo-	2	6.3	7.4	17
9	<i>KIT</i> exon 11	V559del	Yes	Yes	Neo + s	9	2.5	0.1	-96
10	<i>KIT</i> exon 11	p.V560D	No	Yes	Neo-	1	1.5	1.8	17
11	<i>KIT</i> exon 11	p.V560D	No	Yes	Neo-	2	0.8	1.2	47
12	<i>KIT</i> exon 11	p.V560del	Yes	No	NA	-	19.3	-	-
13	<i>KIT</i> exon 11	p.V559D	No	Yes	Neo-	3	1.9	0.9	-52
14	<i>KIT</i> exon 11	V559G	Yes	Yes	Neo + s	16	1.6	0.9	-43
15	<i>PDGFRA</i> exon 18	p.D842V	Yes	Yes	Neo + r	2	9.1	9.0	-1
16	<i>KIT</i> exon 11	p.W557G	Yes	Yes	Neo + s	12	0.6	0.1	-93
17	<i>KIT</i> exon 11	p551-W557delinsR	Yes	Yes	Neo + s	12	3.4	0.2	-94
18	<i>KIT</i> exon 11	D579del	No	Yes	Neo-	2	6.7	6.3	-6
19	<i>PDGFRA</i> exon 12	E556-I565dupl	Yes	Yes	Neo + s	2	0.9	0.1	-89
20	<i>KIT</i> exon 13	p K642E	Yes	Yes	Neo + r	4	20.1	15.3	-24
21	<i>KIT</i> exon 11	p.W557R	No	Yes	Neo-	2	7.2	7.8	9
22	<i>KIT</i> exon 11	V559D	Yes	Yes	Neo + s	2	5.6	0.3	-95
23	<i>KIT</i> exon 11	P551-E554delinsQ	Yes	Yes	Neo + s	12	5.8	0.1	-98
24	<i>KIT</i> exon 11	K558-G565delinsR	Yes	No	NA	-	21.5	-	-
25	<i>PDGFRA</i> exon 18	p.D842V	No	No	NA	-	1.5	-	-
26	<i>PDGFRA</i> exon 18	p.D842V	Yes	Yes	Neo + r	1	2.7	2.5	-10
27	<i>KIT</i> exon 11	V559D	No	Yes	Neo-	3	2.8	3.1	13
28	<i>KIT</i> exon 11	p.V559D	No	Yes	Neo-	2	1.1	0.8	-25
29	<i>KIT</i> exon 11	p.V559D	Yes	Yes	Neo + s	17	2.5	0.2	-90
30	<i>KIT</i> exon 11	pQ575-L576dupl	No	No	NA	-	2.7	-	-
	<i>KIT</i> exon 13	V654A							
31	<i>KIT</i> exon 11	V559D	No	No	NA	-	1.4	-	-
32	<i>KIT</i> exon 11	p.L576P	No	No	NA	-	1.8	-	-
33	<i>PDGFRA</i> exon 18	p.D842V	No	Yes	Neo-	2	6.6	8.9	35
34	<i>PDGFRA</i> exon 18	p.D846Y	No	Yes	Neo-	2	2.6	2.0	-22
35	<i>KIT</i> exon 11	p P551-W560del	No	No	NA	-	3.0	-	-
36	<i>KIT</i> exon 11	V560E	Yes	No	NA	-	NC	-	-
37	Wild type	Wild type	Yes	Yes	Neo + r	3	9.2	12.0	31
38	<i>KIT</i> exon 11	pL567del	Yes	No	NA	-	10.1	-	-
39	<i>PDGFRA</i> exon 12	M578-S584del	Yes	No	NA	-	11.0	-	-
40	<i>KIT</i> exon 11	p.P551-Q556del	Yes	No	NA	-	4.6	-	-
41	<i>KIT</i> exon 11	p.N567-T574del	No	Yes	Neo-	1	8.3	6.8	-18
42	<i>KIT</i> exon 11	57-E561del	Yes	No	NA	-	4.6	-	-
43	Wild type	Wild type	No	No	NA	-	0.1	-	-
44	<i>KIT</i> exon 9	A502-Y503dupl	Yes	No	NA	-	0.7	-	-

¹Neoadjuvant treatment with imatinib; ²For comparison, the sequencing was performed on an endoscopy biopsy. The 44 GIST study cases sampled with EUS-FNB and ordered by the date of study enrollment. Ki-67_{EUS}: The Ki-67_{index} (%) of the EUS-biopsies; Ki-67_{SURG}: The Ki-67_{index} (%) of the resected specimens; Ki-67_{RED}: The percentage of the reduction in the Ki-67_{index}, comparison of the Ki-67_{EUS} with the Ki-67_{SURG} (see Methods); NA: Not annotated; NC: Not countable; *KIT*: KIT proto-oncogene receptor tyrosine kinase; *PDGFRA*: Platelet-derived growth factor receptor alpha.

neoadjuvant imatinib therapy without information on the mutational status. Consequently, in five patients who were treated with neoadjuvant imatinib, the sequencing of FNB-biopsies later showed a genetic profile consistent with primary resistance to imatinib, which led to a modification in the treatment regimen. To assist clinicians during the preoperative management of patients with GIST, we implemented the immediate sequencing of FNB-biopsies during the latter part of the SP. One recent retrospective study revealed that it is possible to obtain the mutation profile of GISTs in a selected pool of EUS-FNA-samples using next-

generation sequencing^[24]. No comparison was made between the sequencing of EUS-FNA-samples and the sequencing of any corresponding resected specimens.

The prognosis of an individual patient with GIST is dependent on the tumor proliferation rate and the size of the tumor^[15,25]. In our study, the FNB-biopsies were highly accurate for the precise assessment of the Ki-67-index by manual counting. The Ki-67-index measured in the FNB-biopsies seems reliable since it was in agreement with the Ki-67-index of the resected specimens of the study patients who were not treated with neoadjuvant imatinib. More

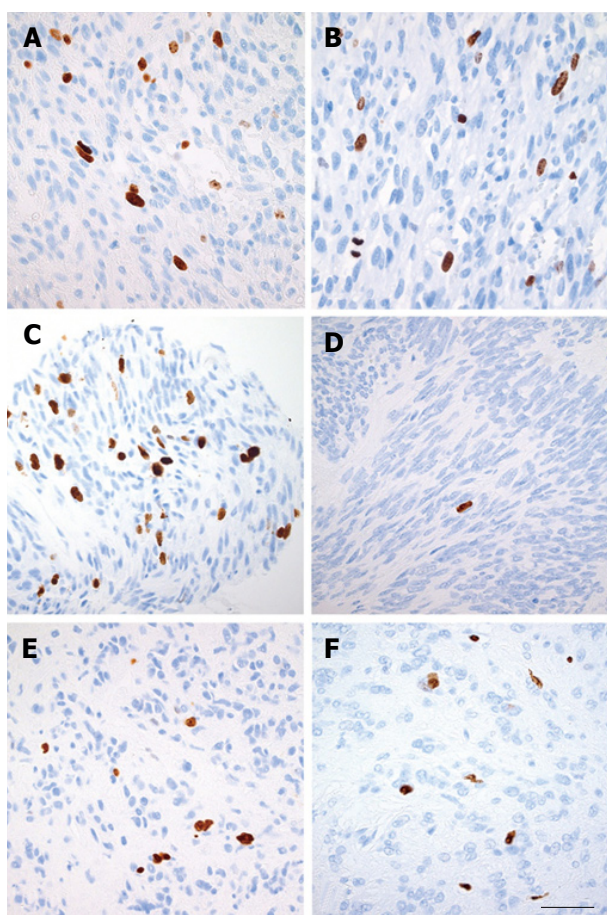


Figure 4 Ki-67-immunostaining of gastrointestinal stromal tumors-tumor tissue in three endoscopic ultrasound-biopsies and in the three corresponding resected specimens. Digital photos (magnification $\times 40$): EUS-biopsy-tissue (left) and resected specimen tissue (right). Cell nuclei (brown color) are positive for Ki-67 while other cell nuclei (blue color) are negative for Ki-67. Scale bar equals 50 μm . A and B: Case #18, Neo- group (*KIT* exon 11 D579del, Ki-67_{EUS}: 6.6%, Ki-67_{SURG}: 6.3%); C and D: Case #22, Neo + s group (*KIT* exon 11 V559D, Ki-67_{EUS}: 5.6%, Ki-67_{SURG} 0.3%); E and F: Case #26, Neo + r group (*PDGFRA* exon 18 p.D842V, Ki-67_{EUS}: 2.7%, Ki-67_{SURG} 2.5%). EUS: Endoscopic ultrasound.

importantly, in patients who are sensitive to imatinib and who are treated with neoadjuvant imatinib, the Ki-67-index of FNB-biopsies probably better reflects the accurate proliferation rate of tumors compared with the Ki-67-index of resected specimens, which may erroneously be found to be low. A substantial danger of the overestimation of survival and the under-prescription of adjuvant therapy can emerge in these groups of patients. An assessment of the mitotic rate of specimens obtained by FNB-biopsy is probably challenging, and it was not an aim of this study. The maximum quantity of FNB-material obtained in this study reached 40 high-power fields.

The pretreatment assessment of the Ki-67-index has a range of clinical applications. This assessment provides clinicians with prognostic information for a discussion of therapeutic options with their patients. The tumor response to neoadjuvant treatment by measurement of the reduction in the Ki-67-index, as

described in the current study, may guide adjuvant treatment in patients who undergo resection. ^{18}F FDG-PET is expensive and some tumors may have a negative baseline signal; the demonstration of the Ki-67-indexing of repeated EUS-biopsies is an attractive method by which the therapeutic response may be evaluated.

This prospective study was conducted in a large Swedish region over several years and involved dedicated experts and the use of advanced techniques. The centralized management of GIST facilitated good control of patients and reliable follow-up data. We used pretreatment tumor tissue not only to diagnose GIST but also to clarify the sensitivity to imatinib, to assess the tumor proliferation rate, and finally, to evaluate the treatment response to imatinib. To the best of our knowledge, the presented results are more detailed and accurate than those of any comparable publications in the literature.

A limitation of EUS is that GISTs in the jejunum or ileum can be punctured only if they are visible from the stomach or the duodenum. However, the majority of GISTs are located in the stomach. Some study patients were treated with neoadjuvant imatinib even if they carried mutations with primary resistance to imatinib, which highlights the importance of sequencing prior to therapy. Sampling errors may result in an erroneously low Ki-67-index. However, such a phenomenon was probable only in two patients in this study (case #33 and #37).

The described pretreatment characterization of tumors should be incorporated in future management guidelines of GIST to facilitate personalized treatment. Moreover, the work-up of complex tumors such as GISTs should be centralized to high-volume centers in order to enable a rational and effective treatment.

We conclude that this study provides clear support for endoscopic ultrasound as the front-line diagnostic procedure in GIST, as it enables an early diagnosis and a personalized, genotype-driven targeted therapy of patients. The presented approach with the extensive characterization of GISTs based on the analysis of EUS-guided biopsies may also serve as a model for other tumor entities.

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COMMENTS

Background

The early personalized management and treatment of gastrointestinal stromal tumors (GISTs) require an extensive characterization of individual tumors. Information on the tumor proliferation rate and the *KIT*- and platelet-derived

growth factor alpha-mutation profile is essential.

Research frontiers

Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) has been reported to be suboptimal for the diagnosis of GIST, but endoscopic ultrasound (EUS)-guided biopsy sampling (EUS-FNB) has not been evaluated for the characterization of GISTs. Neither the Ki-67 index nor KIT/PDGFRA-sequencing has been evaluated in EUS-FNB-tissue.

Innovations and breakthroughs

This prospective, long-term study showed that EUS-FNB was safe and highly accurate for the pretreatment diagnosis of GISTs, for the sequencing of *KIT* and *PDGFRA*, and for the assessment of the tumor proliferation rate (Ki-67-index). To the best of our knowledge, other relevant publications in this field demonstrate a diagnostic accuracy of EUS-FNA of approximately 50%. The sequencing of EUS-FNA-smears of GIST has not been evaluated in a prospective cohort but only in a single, retrospective study that included 20 patients.

Applications

By obtaining the extensive, preoperative diagnostic and prognostic information described in the present study, it will be possible to personalize the clinical and surgical management of patients with GIST especially with respect to the guidance and evaluation of neoadjuvant imatinib therapy.

Peer-review

This manuscript is about endoscopic ultrasound-guided biopsy in the diagnosis of gastrointestinal stromal tumors and evaluating neoadjuvant imatinib by sequencing of EUS-biopsies. It's an interesting and valuable manuscript.

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Observational Study

Novel predictors for lymph node metastasis in submucosal invasive colorectal carcinoma

Kwangil Yim, Daeyoun David Won, In Kyu Lee, Seong-Taek Oh, Eun Sun Jung, Sung Hak Lee

Kwangil Yim, Eun Sun Jung, Sung Hak Lee, Department of Hospital Pathology, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul 137-701, South Korea

Daeyoun David Won, In Kyu Lee, Department of Surgery, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul 137-701, South Korea

Seong-Taek Oh, Department of Surgery, Uijeongbu St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul 137-701, South Korea

ORCID number: Kwangil Yim (0000-0001-8767-9033); Daeyoun David Won (0000-0001-5227-8141); In Kyu Lee (0000-0001-9074-5214); Seong-Taek Oh (0000-0002-5962-581X); Eun Sun Jung (0000-0002-8451-939X); Sung Hak Lee (0000-0003-1020-5838).

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Correspondence to: Sung Hak Lee, MD, PhD, Assistant Professor, Department of Hospital Pathology, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, 222, Banpo-daero, Seocho-gu, Seoul 137-701, South Korea. hakjjang@catholic.ac.kr
Telephone: +82-2-22581617
Fax: +82-2-22581627

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Abstract

AIM

To evaluate a novel grading system to predict lymph node metastasis (LNM) in patients with submucosal invasive colorectal carcinoma (SICRC).

METHODS

We analyzed the associations between LNM and various clinicopathological features in 252 patients with SICRC who had undergone radical surgery at the Seoul Saint Mary's hospital between 2000 and 2015.

RESULTS

LNM was observed in 31 patients (12.3%). The depth and width of the submucosal invasion, lymphatic invasion, tumor budding, and the presence of poorly differentiated clusters (PDCs) were significantly

associated with the incidence of LNM. Using multivariate analysis, the receiver operating characteristic curves were calculated and the area under curve (AUC) was used to compare the ability of the different parameters to identify the risk of LNM. The most powerful clinicopathological parameter for predicting LNM was lymphatic invasion (difference AUC = 0.204), followed by the presence or absence of tumor budding (difference AUC = 0.190), presence of PDCs (difference AUC = 0.172) and tumor budding graded by the Ueno method (difference AUC = 0.128).

CONCLUSION

Our results indicate that the tumor budding and the depth multiplied by the width measurements of submucosal invasion can provide important information for patients with SICRC.

Key words: Colorectal cancer; Neoplasm invasion; Lymph node; Metastasis

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Core tip: The appropriacy of endoscopic resection for patients with submucosal invasive colorectal carcinoma (SICRC) is still questionable. Therefore, highly precise predictors of lymph node metastasis (LNM) are needed to optimize the outcome of treatments for SICRC. We determined the value of a novel grading system based on histopathological parameters to predict LNM in patients with SICRC. Our results indicate that the presence or absence of tumor budding and the depth multiplied by the width measurements of the submucosal invasion can provide important information regarding the treatment options for patients with SICRC.

Yim K, Won DD, Lee IK, Oh ST, Jung ES, Lee SH. Novel predictors for lymph node metastasis in submucosal invasive colorectal carcinoma. *World J Gastroenterol* 2017; 23(32): 5936-5944 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i32/5936.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i32.5936>

INTRODUCTION

Endoscopic resection of intramucosal carcinomas is considered to be a curative therapy since the risk of lymph node metastasis (LNM) is low^[1]. However, up to 12% of patients with submucosal invasive colorectal carcinoma (SICRC) have LNM^[2,3], and it is unclear whether endoscopic resection is the most appropriate treatment option for these patients. Prior to endoscopic resection, patients with SICRC require careful pathological assessment to determine whether there is a significant risk of LNM which may require additional surgical treatment^[4].

Various histopathological parameters such as angiolymphatic invasion, poor tumor differentiation, and the depth and/or width of submucosal invasion are reported to be associated with LNM in patients with SICRC^[3-6]. The 2014 guidelines of the Japanese Society for Cancer of the Colon and Rectum (JSCCR) for the treatment of colorectal cancer (CRC) by endoscopic resection, suggest that cases with tumor-positive vertical resection margin, submucosal invasion of $\geq 1000 \mu\text{m}$, and unfavorable histology such as poorly differentiated adenocarcinoma, signet-ring cell carcinoma, or mucinous carcinoma should be considered for additional surgery with LN dissection^[7]. The presence of vascular invasion and/or grade 2/3 budding at the site of the deepest invasion are also included as surgical candidates.

However, not all surgical cases of SICRC present LNM, resulting in overtreatment. Conversely, some patients without surgery eventually present LNM^[2,6,8,9]. In order to provide the appropriate treatment to patients with SICRC, and reduce the number of unnecessary additional surgical resections, there needs to be an improvement in discriminating between patients with a high risk of developing LNM and those with a low risk of developing LNM.

In this study, we analyzed the incidence of LNM in relation to several histopathological findings in a large cohort of patients with SICRC, with an aim to help pathologists and clinicians in identifying the best treatment strategy for such patients.

MATERIALS AND METHODS

Patients and clinicopathological data

A total of 252 patients with SICRC who had undergone surgery for systematic lymph node dissection in Seoul Saint Mary's hospital between 2000 and 2015 were enrolled in this study. Clinicopathological parameters including age, sex, tumor location, size, and LNM status were reviewed retrospectively from the medical records. Approval for this study was acquired from the Institutional Review Board of the College of Medicine at the Catholic University of Korea (KC16RISI0817).

Histopathological analysis

Hematoxylin and eosin stained tumor sections were evaluated for the following: depth and width of the submucosal invasion, tumor budding, poorly differentiated clusters (PDCs), histological grade, lymphatic invasion, venous invasion, perineural invasion, peritumoral inflammation, and desmoplasia. Two pathologists (Lee SH and Yim KI) independently examined each tumor section.

Depth of submucosal invasion

The depth of submucosal invasion was measured by three methods. The first method followed the JSCCR guidelines^[10]. In brief, when the muscularis mucosa (MM) was clearly visible, the depth of the submucosal

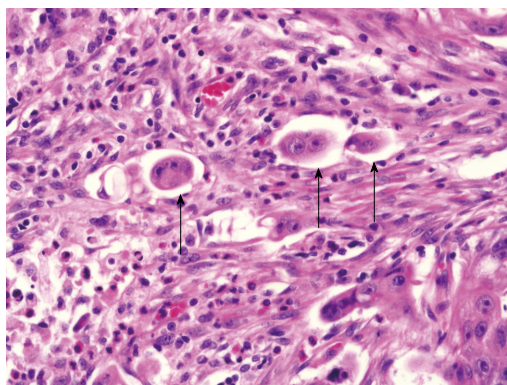


Figure 1 Representative histopathological micrograph of tumor budding (magnification $\times 400$). Hematoxylin and eosin staining of a tumor section showing tumor budding (black arrows) at the invasive front.

invasion was measured from the lowest border of the MM to the deepest invasion front. When the MM was deformed or not easily identified, the depth of the submucosal invasion was measured from the surface of the tumor to the invasion front, for both pedunculated and sessile forms. For pedunculated lesions with tangled MM, the depth of the submucosal invasion was measured as the distance between the boundary line of the head and stalk (level 2 by Haggitt classification) and the deepest point of the tumor. The second method followed the protocol by Kitajima *et al.*^[5] The tumor was classified as a pedunculated or sessile type with an identifiable MM or a sessile type without an identifiable MM. For pedunculated lesions, the depth of the submucosal invasion was measured as the distance between Haggitt's level 2 and the deepest invasion point. For sessile tumor types with an identifiable MM, the depth of invasion was measured from the lowest border of the MM to the deepest invasion front. For sessile tumor types without an identifiable MM, the depth of the submucosal invasion was measured from the surface of the tumor to the invasion front. Lastly, we followed the method by Ueno *et al.*^[6] The depth of the submucosal invasion was simply measured as the distance between the tumor surface and the deepest invasion point.

Width of submucosal invasion

The width of the submucosal invasion was defined as the largest (longest) horizontal measurement of the submucosal invasive area.

Depth multiplied by width

Assuming the shape of the submucosal invasion was an ellipse, the area is $\pi \times \text{depth} \times \text{width}$. Therefore, we postulated that the depth multiplied by width could represent the area of submucosal invasion. The results of depth multiplied by width were obtained by the three different methods described previously for measuring the depth of invasion.

Lymphatic and vascular invasion

The diagnosis of lymphatic invasion was made based on the presence of at least one tumor cell cluster within vascular space lined by a single layer of endothelial cells with no supporting smooth muscle, elastic lamina and/or red blood cells, whose lumens are sometimes filled with lymphocytes. Similarly, we defined vascular invasion as tumor cell nests in spaces that were lined by endothelium and filled with red blood cells, located in the vicinity of an artery and distant from the main lesion. Tumor cell nests in spaces that were not lined by endothelial cells were considered as stroma-invasive tumor cell nests, that is, retraction artifacts due to tissue shrinkage during fixation. In this study, only tumor cell nests in spaces lined by endothelial cells were counted as lymphovascular invasion.

Additional immunohistochemical staining with Podoplanin (clone D2-40, 1:50, Cell Marque, Hot Springs, AR, the United States of America), to detect lymphatic invasion, and with CD34 (clone QBEnd 10, 1:100, DAKO, Glostrup, Denmark) or CD31 (clone JC70A, 1:200, Dako, Glostrup, Denmark), to detect venous invasion were performed in those sections in which it was difficult to judge the presence or absence of lymphovascular invasion.

Tumor budding

An isolated tumor cell or small clusters of < 5 cancer cells in the invasive front was defined as tumor budding (Figure 1). In the present study, this was assessed by two different methods. The method described by Ueno *et al.*^[6] identified a microscopic field with intense budding and counted the number of budding foci using the $\times 20$ objective lens and classified the number of foci by grade (grade 0; 0 focus, grade 1; 1-4 foci, grade 2; 5-9 foci, grade 3; ≥ 10 foci per field). The second method assessed the presence or absence of the tumor budding.

PDCs

PDCs were defined as cancer cell clusters of ≥ 5 carcinoma cells that are lacking a glandular formation at the invasive front (Figure 2). However, when evaluating the mucinous adenocarcinoma, cancer cell clusters within a large mucin pool were not classified as PDCs; whereas, cancer cell clusters infiltrating the stroma with minimal extracellular mucin formation were classified as PDCs^[11]. In our study, the assessment of PDCs was based on the presence or absence of PDCs.

Statistical analysis

χ^2 test, Fisher's exact test, or Wilcoxon rank sum test were used to analyze the differences between the absence and presence of LNM. When predicting LNM, the sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV) were evaluated for each factor. Using multivariate logistic

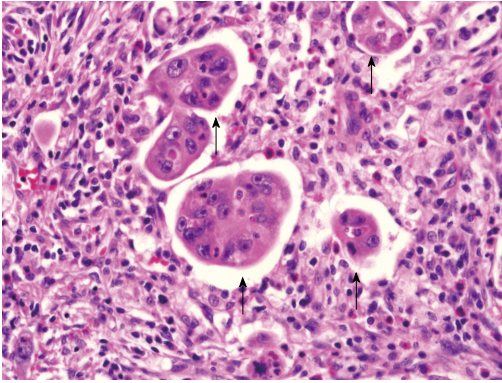


Figure 2 Representative histopathological micrograph of poorly differentiated clusters (magnification $\times 400$). Hematoxylin and eosin stain of tumor section showing cancer cell clusters of ≥ 5 carcinoma cells lacking a glandular formation (poorly differentiated clusters, black arrows).

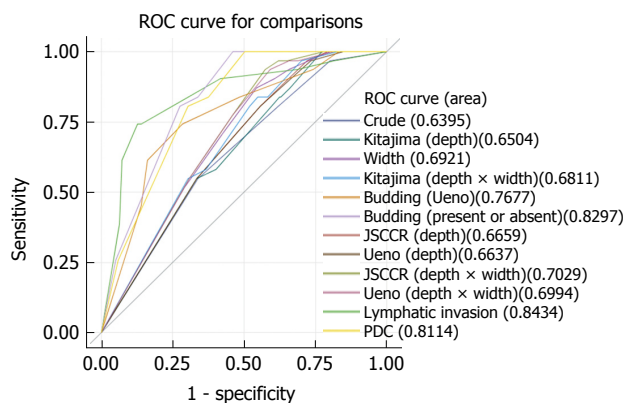


Figure 3 Comparison of receiver operating characteristic curves by multivariate logistic regression tests. Desmoplasia and tumor type were used as compounding factors.

regression analysis, ROC was calculated and the AUC was used to compare how effective the parameters were at identifying the risk of LNM; tumor type and desmoplasia were used as compounding factors. Two-sided P values of < 0.05 were considered statistically significant. JMP software (version 9.0.2; SAS Institute, Cary, NV, the United States of America) was used for all statistical calculations.

RESULTS

Patients and clinicopathological data

Of the 252 patients, 130 were male and 122 were female; the mean age was 61.8 years. One hundred and fifty eight patients had tumors in the colon, including 1 in the vermiform appendix, and 94 in the rectum. The mean tumor size was 20.6 mm (range 2.0 mm–65.0 mm). LNM were observed in 31 cases (12.3%). Tumor budding was identified in 133 cases (52.8%) and 86 cases (34.1%) were grade 2 or 3 using the Ueno method [grade 0; 119 cases (47.2%), grade 1; 47 cases (18.7%), grade 2; 46 cases (18.2%), grade 3; 40 cases (15.9%)]. The baseline

clinicopathological characteristics are summarized in Table 1.

LNM in relation to histopathological parameters

Table 1 summarizes the correlations between the histopathological parameters and LNM. The depth and width of the submucosal invasion, depth multiplied by width measurement, and the presence of lymphatic invasion, tumor budding, and PDCs were all significantly associated with the incidence of LNM. The depth multiplied by width values were significantly higher in tumors with LNM than in tumors without LNM, irrespective of the method used ($P < 0.001$). The incidence of LNM was 23.3% in tumors with tumor budding and 0% in cases without tumor budding ($P < 0.0001$).

Comparison of risk factors for LNM

The sensitivity, specificity, accuracy, PPVs, and NPVs were calculated for the parameters that were significantly associated with LNM using univariate analysis (Table 2). In addition, ROC curves were obtained for each parameter and the AUC was used to compare how well the various risk factors could identify the risk of LNM (Table 3 and Figure 3).

We found that the JSCCR method for measuring the depth of invasion was the most predictive for LNM (difference AUC = 0.026). In addition, the width of the submucosal invasion was more accurate for predicting LNM than the depth of invasion (difference AUC = 0.053). Furthermore, when using the JSCCR method for the depth of invasion, we found that the depth multiplied by width was even more powerful than the depth of invasion or width of invasion alone (difference AUC = 0.063). Meanwhile, the most powerful clinicopathological parameter for predicting LNM was lymphatic invasion (difference AUC = 0.204), followed by the presence or absence of tumor budding (difference AUC = 0.190), PDCs (difference AUC = 0.172) and tumor budding assessed using the Ueno method (difference AUC = 0.128). Simply classifying tumor budding by the presence or absence was more predictive than the method proposed by Ueno.

DISCUSSION

The presence of LNM is known to be one of the most significant and independent predictor for 5 years cancer specific survival and 5 years disease-free survival in patients with SICRC^[12]. It is essential to select the most appropriate treatment options considering the risk of LNM. However, each treatment modality needs to balance a potential cure with the mortality and morbidity risks that accompany such treatment options. Therefore, it is crucial to identify those patients with a potential risk for LNM before proceeding with additional surgical treatments.

To date, various histopathological parameters,

Table 1 Univariate analysis of clinicopathological and histological parameters for lymph node metastasis

	LNM		P value
	No (n = 221)	Yes (n = 31)	
Age (yr)	61.81 ± 11.40 63.0 (31.0, 86.0)	61.35 ± 10.63 66.0 (38.0, 78.0)	0.8334
Sex			
Male	115 (88.46)	15 (11.54)	0.7205
Female	106 (86.89)	16 (13.11)	
Tumor location			
Ascending colon	34 (89.48)	4 (10.52)	0.6018
Transverse colon	12 (85.71)	2 (14.29)	
Descending colon	6 (100.00)	0 (0.00)	
Sigmoid colon	73 (91.25)	7 (8.75)	
Rectosigmoid colon	14 (93.33)	1 (6.67)	
Rectum	78 (82.98)	16 (17.02)	
Cecum	3 (75.00)	1 (25.00)	
Vermiform appendix	1 (100.00)	0 (0.00)	
Tumor size (cm)	2.05 ± 1.67 1.8 (0.2, 6.5)	2.07 ± 1.27 2.0 (0.4, 6.5)	0.6902
Tumor type			
Pedunculated type	84 (93.33)	6 (6.67)	0.0424
Sessile type	137 (84.57)	25 (15.43)	
Depth of submucosal invasion by JSCCR (μm)	2473.94 ± 2003.78 2200.0 (0.0, 13500.0)	3777.42 ± 2167.10 3500.0 (1050.0, 12000.0)	0.0002
< 1000	46 (100.00)	0 (0.00)	0.0021
≥ 1000	175 (84.95)	31 (15.05)	
by Kitajima (μm)	1944.3 ± 1885.18 1900.0 (0.0, 13500.0)	3380.65 ± 2390.28 3000.0 (500.0, 12000.0)	0.0002
< 1000	76 (95.00)	4 (5.00)	0.0218
≥ 1000	145 (94.30)	27 (15.70)	
by Ueno (μm)	2671 ± 1898.39 2300.0 (50.0, 13500.0)	3908.06 ± 2147.17 3500.0 (1050.0, 12000.0)	0.0002
< 1000	34 (100.00)	0 (0.00)	0.0113
≥ 1000	187 (85.78)	31 (14.22)	
Width of submucosal invasion (μm)	5831.45 ± 4724.02 5000.0 (50.0, 31000.0)	9261.29 ± 4692.03 8000.0 (1900.0, 21000.0)	< 0.0001
< 4000 μm	87 (96.67)	3 (3.33)	0.0010
≥ 4000 μm	134 (82.72)	28 (17.28)	
Depth multiplied by width by JSCCR (mm ²)	20.22 ± 35.07 11.6 (0.0, 303.8)	38.91 ± 36.50 26.6 (2.0, 163.8)	< 0.0001
< 6.5	84 (98.82)	1 (1.18)	< 0.0001
≥ 6.5	137 (82.04)	30 (17.96)	
by Kitajima (mm ²)	16.79 ± 34.52 8.8 (0.0, 303.8)	36.42 ± 37.97 23.1 (2.0, 163.8)	< 0.0001
< 6.5	100 (95.24)	5 (4.76)	0.0017
≥ 6.5	121 (82.31)	26 (17.69)	
by Ueno (mm ²)	20.91 ± 34.82 12.8 (0.0, 303.8)	40.12 ± 36.52 26.6 (2.0, 163.8)	< 0.0001
< 6.5	76 (98.70)	1 (1.30)	0.0001
≥ 6.5	145 (82.86)	30 (17.14)	
Tumor differentiation			
Well and moderately	211 (87.92)	29 (12.08)	0.6470
Poorly	10 (83.33)	2 (16.67)	
Lymphatic invasion			
No	190 (95.96)	8 (4.04)	< 0.0001
Yes	31 (57.41)	23 (42.59)	
Venous invasion			
No	215 (87.76)	30 (12.24)	1.0000
Yes	6 (85.71)	1 (14.29)	
Perineural invasion			
No	219 (87.60)	31 (12.40)	1.0000
Yes	2 (100)	0 (0.00)	
Inflammation			
No	100 (87.72)	14 (12.28)	1.0000
Yes	121 (87.68)	17 (12.32)	
Desmoplasia			
No	106 (92.17)	9 (7.83)	0.0550

Yes	115 (83.94)	22 (16.06)	
Tumor budding			
Ueno			
Grade 0, 1	158 (95.18)	8 (4.82)	< 0.0001
Grade 2, 3	63 (73.26)	23 (26.74)	
Present or absent			
No	119 (100)	0 (0.00)	< 0.0001
Yes	102 (76.69)	31 (23.31)	
PDCs			
No	110 (100.00)	0 (0.00)	< 0.0001
Yes	111 (78.17)	31 (21.83)	

Data are presented as *n* (%) and as mean \pm SD, median (range). *P* value of significant difference between present/absent of LMN, by χ^2 , Fisher's exact, Wilcoxon rank sum test. LNM: Lymph node metastasis; JSCCR: Japanese Society for Cancer of the Colon and Rectum; PDCs: Poorly differentiated clusters.

Table 2 Predictive powers of histopathological factors for lymph node metastasis

	Sensitivity	Specificity	Accuracy	Positive <i>P</i> value	Negative <i>P</i> value
Depth of submucosal invasion					
by JSCCR	100.00%	20.81%	30.56%	15.05%	100.00%
by Kitajima	87.10%	34.39%	40.87%	15.70%	95.00%
by Ueno	100.00%	15.38%	25.79%	14.22%	100.00%
Width of submucosal invasion	90.32%	39.37%	45.63%	17.28%	96.67%
Depth multiplied by width					
by JSCCR	96.77%	38.01%	45.24%	17.96%	98.82%
by Kitajima	83.87%	45.25%	50.00%	17.69%	95.24%
by Ueno	96.77%	34.39%	42.06%	17.14%	98.70%
Lymphatic invasion	74.19%	85.97%	84.52%	42.59%	95.96%
Tumor budding					
by Ueno	74.19%	71.49%	71.83%	26.74%	95.18%
Present or absent	100.00%	53.84%	59.52%	23.31%	100.00%
PDCs	100.00%	49.77%	55.95%	21.83%	100.00%

JSCCR: Japanese Society for Cancer of the Colon and Rectum; PDCs: Poorly differentiated clusters.

Table 3 Comparison of histopathological factors for predicting of lymph node metastasis by multivariate analysis

Model	AUC	SE	95%CI	Difference AUC	95%CI	<i>P</i> value
Crude (unadjusted)	0.640	0.046	(0.549, 0.730)			
JSCCR (depth)	0.666	0.041	(0.585, 0.747)	0.026	(0.009, 0.043)	0.0022
Kitajima (depth)	0.650	0.044	(0.565, 0.736)	0.011	(-0.022, 0.043)	0.5103
Ueno (depth)	0.664	0.042	(0.582, 0.746)	0.024	(0.009, 0.040)	0.0024
Width	0.692	0.040	(0.613, 0.771)	0.053	(-0.007, 0.112)	0.0829
JSCCR (depth \times width)	0.703	0.038	(0.629, 0.776)	0.063	(0.024, 0.103)	0.0017
Kitajima (depth \times width)	0.681	0.042	(0.599, 0.763)	0.042	(-0.002, 0.085)	0.0612
Ueno (depth \times width)	0.699	0.038	(0.624, 0.775)	0.060	(0.020, 0.099)	0.0030
Budding (ueno)	0.768	0.044	(0.682, 0.853)	0.128	(0.043, 0.214)	0.0033
Lymphatic invasion	0.843	0.041	(0.763, 0.924)	0.204	(0.114, 0.294)	< 0.0001
Budding present itself	0.830	0.0277	(0.776, 0.884)	0.190	(0.106, 0.274)	< 0.0001
PDCs	0.811	0.0296	(0.753, 0.870)	0.172	(0.087, 0.256)	< 0.0001

Crude: Tumor type, desmoplasia each model was adjusted to the tumor type and desmoplasia. AUC: Area under curve; JSCCR: Japanese Society for Cancer of the Colon and Rectum; PDCs: Poorly differentiated clusters.

including those related to the degree of submucosal invasion (measured as the depth or width of the submucosal invasion) and the status of the MM, have been proposed to predict the risk of LNM in patients with SICRC. The depth of submucosal invasion has long been identified as a predictor of LNM in SICRC, but the level of submucosal invasion associated with LNM and how the depth of invasion should be measured remain undefined. Several measurement

systems have been proposed to evaluate the depth of the submucosal invasion in SICRC, although there are still controversies regarding their ability to accurately predict LNM. Haggitt *et al*^[8] revealed that level 4 invasion is an adverse prognostic factor associated with LNM in cases of the pedunculated type of SICRC^[8]. Similarly, Kikuchi *et al*^[12] showed that the submucosal invasion level in sessile type tumors is an important risk factor for the development of LNM and local

recurrence. However, the Haggitt system is of little use in sessile type tumors without an identifiable stalk, which are always classified as level 4. The Kikuchi system is difficult to apply to specimens obtained by endoscopic resection as they do not usually include the muscularis propria and therefore submucosal invasion levels cannot easily be evaluated. For both the Haggitt and Kikuchi systems to be successfully applied, the tumors should be resected en bloc.

Owing to the variability in the shapes of SICRCs, the usefulness of quantitatively measuring the actual submucosal depth from the MM as an invasion value is being widely accepted^[5,7]. In the JSCCR guidelines, a depth of submucosal invasion of 1000 μm or more has been adopted as the criteria for additional intestinal resection^[7]. Similarly, in the present study, we found that a submucosal invasion depth of 1000 μm or more was significantly correlated with the incidence of LNM.

In the evaluation of SICRC, there have been difficulties in identifying the depth of the submucosal invasion, because the MM is sometimes poorly defined and disrupted, leading to variability in the measurements. Moreover, the greater the length of the submucosal invasive fronts, the higher the chances of contact with and subsequent invasion into the lymphovascular structures. Thus, Ueno *et al.*^[6] addressed the importance of the width of submucosal invasion in predicting LNM. They suggested that a submucosal invasion width of $\geq 4000 \mu\text{m}$ together with an invasion depth of $\geq 2000 \mu\text{m}$ could increase the probability of LNM^[6]. The chances of the lymphovascular invasion would be higher with a greater area of submucosal invasion. Toh *et al.*^[13] introduced the area of tumor involvement within the submucosa as a predictor for LNM, suggesting that the assessment of the submucosal invasion area would provide more valuable information.

Our study revealed that lymph node-positive SICRCs had a significantly greater width of invasion ($P = 0.001$) and multiplication of depth and width of invasion ($P < 0.001$) compared with those of lymph node-negative SICRCs. Additionally, the ROC analysis demonstrated that the width of invasion and multiplication of depth and width of invasion had increased sensitivity and specificity compared with the submucosal invasion depth in predicting LNM. Toh *et al.*^[13] measured the area of submucosal tumor involvement very accurately using digital pathology, which is impractical and laborious for routine clinical use. On the other hand, in the present study, we assessed the depth and width of the submucosa invasion, and then simply multiplied these two parameters. This can easily be applied in routine practice. Moreover, we have found that the multiplication of depth and width of submucosal invasion, roughly reflects the area of submucosal invasion. It showed to be a good predictor for LNM.

Tumor budding means individual malignant cells and/or small clusters of undifferentiated malignant cells

seen in the tumor stroma, which are located near the invasive front of the tumor^[14]. It has been associated with the process of epithelial-mesenchymal transition (EMT), which gives tumor cells a more mesenchymal phenotype with increased migratory capacity and invasiveness^[15]. Tumor budding is postulated to be akin to EMT, and is now considered a predictor of LNM, lymphovascular invasion, tumor relapse and poor prognosis among CRC patients of all stages^[14]. In the same context, it is proposed as an adverse prognostic factor in early CRC by the European Society for Medical Oncology consensus guidelines^[16]. Nevertheless, tumor budding has not yet been used in routine clinical practice, because there is no consensus criteria concerning the exact definition, and methodology of assessment. Therefore, standardization of methods for defining and quantifying tumor budding is needed to unequivocally confirm its prognostic value.

The present study defined tumor budding by its presence or absence and demonstrated that tumor budding was the second highest impact factor for predicting LNM in patients with SICRC, after lymphatic invasion. Moreover, in the evaluation of tumor budding, we revealed that our classification is superior to that of Ueno *et al.*^[6] suggesting that presence of tumor budding itself can significantly affect patient outcomes. In addition, our defining system is more practical since it does not require any additional grading procedures^[17].

Recent studies have emphasized the potential role of PDC as a prognostic marker for LNM in SICRC, and the present study confirmed the significant impact of PDC in identifying the risk of LNM^[11,18]. Furthermore, both ROC curves of tumor budding and PDC showed a relatively large AUC, suggesting that these two parameters had high sensitivity and specificity in predicting LNM compared with submucosal invasion depth, width and multiplication of depth and width.

Many researchers have shown that there is a directly proportional relationship between the number of examined lymph nodes and survival, particularly in patients with advanced CRC^[19-22]. Similarly, Wang *et al.*^[23] revealed that the total number of lymph nodes sampled significantly correlated with the prognosis of SICRC. Therefore, retrieving a sufficient number of nodes is crucial for investigating the relationship between clinicopathological parameters and lymph node status in CRC. However, most previous studies with early CRC have reported upon a relatively small number of lymph nodes: mean number of 9.4 ± 7.8 retrieved lymph nodes in the study by Wang *et al.*^[23] 10 in the study by Okabe *et al.*^[24] and 14 in the study by Tateishi *et al.*^[25]. A median of 18 lymph nodes were retrieved in our study, which is the largest number collected to date.

This study has several limitations. First, it is a retrospective cohort study that has a relatively low statistical power due to the small number of SICRC with LNM. In addition, SICRC case selection in this study might have been biased toward those with high

risk for LNM. Therefore, a prospective trial with large scaled cohort is necessary to verify the findings of this study. Despite these limitations, our study revealed relatively simple and novel predictors for LNM in SICRC.

In conclusion, we have revealed that the presence of tumor budding might be a powerful predictor for LNM in patients with SICRC. In addition, we have found that submucosal invasion depth and width were significantly correlated with the incidence of LNM. In particular, the multiplication of depth and width measurements of the submucosal invasion proposed in our study may provide important information regarding treatment options for patients with SICRC.

COMMENTS

Background

Endoscopic resection of intramucosal carcinomas is considered to be a curative therapy since the risk of lymph node metastasis (LNM) is low. However, up to 12% of patients with submucosal invasive colorectal carcinoma (SICRC) have LNM. Thus, it is unclear whether endoscopic resection is appropriate after carcinomatous cells have penetrated the muscularis mucosa. Prior to endoscopic resection, patients with SICRC require careful pathological assessment to determine whether there is a significant risk of LNM which may require additional surgical treatment.

Research frontiers

The depth of submucosal invasion has long been identified as a predictor of LNM in SICRC, but the level of submucosal invasion associated with LNM and how the depth of invasion should be measured remain undefined. Several measurement systems have been proposed to evaluate the depth of the submucosal invasion in SICRC, although there are still controversies regarding their ability to accurately predict LNM. There needs to be an improvement in discriminating between patients with a high risk of developing LNM and those with a low risk of developing LNM.

Innovations and breakthroughs

It has revealed that the width of the submucosal invasion was more accurate for predicting LNM than the depth of invasion and the depth multiplied by width was even more powerful than the depth of invasion or width of invasion alone in the present study. In addition, it has revealed that the presence of tumor budding might be a powerful predictor for LNM in patients with SICRC.

Applications

In the present study, it assessed the depth and width of the submucosa invasion, and then simply multiplied these two parameters. This can easily be applied in routine practice. In the evaluation of tumor budding, it revealed that presence of tumor budding itself can significantly affect patient outcomes. This defining system is more practical since it does not require any additional grading procedures.

Peer-review

This is a well-written manuscript showing predictive factors for LNM of SICRC.

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Observational Study

Changes with aging in gastric biomarkers levels and in biochemical factors associated with *Helicobacter pylori* infection in asymptomatic Chinese population

Jin-Hua Shan, Xiao-Juan Bai, Lu-Lu Han, Yuan Yuan, Xue-Feng Sun

Jin-Hua Shan, Department of Gerontology and Geriatrics, the First Affiliated Hospital of China Medical University, Shenyang 110001, Liaoning Province, China

Xiao-Juan Bai, Lu-Lu Han, Department of Gerontology and Geriatrics, Shengjing Hospital of China Medical University, Shenyang 110004, Liaoning Province, China

Yuan Yuan, Department of Tumor Research, the First Affiliated Hospital of China Medical University, Shenyang 110001, Liaoning Province, China

Xue-Feng Sun, Department of Kidney, General Hospital of Chinese People's Liberation Army, Beijing 100853, China

ORCID number: Jin-Hua Shan (0000-0002-1130-0784); Xiao-Juan Bai (0000-0003-3890-8391); Lu-Lu Han (0000-0002-8306-4506); Yuan Yuan (0000-0002-9314-3371); Xue-Feng Sun (0000-0002-6569-6737).

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Correspondence to: Dr. Xiao-Juan Bai, Department of Gerontology and Geriatrics, Shengjing Hospital of China Medical University, Shenyang 110004, Liaoning Province, China. baixj@sj-hospital.org
Telephone: +86-24-23516346

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Abstract

AIM

To observe changes in gastric biomarker levels with age and effects of *Helicobacter pylori* (*H. pylori*) infection in a healthy population, and explore factors associated with gastric biomarkers.

METHODS

Three hundred and ninety-five subjects were selected and underwent physical examinations, biochemical tests, and measurement of serum pepsinogen (PG)

I and II, gastrin-17 (G-17) and *H. pylori* antibody levels. Analyses were made by Student's *t*-test, ANOVA, Pearson's correlation and multiple linear regressions.

RESULTS

PGII levels were higher in the ≥ 65 -years-old age group ($P < 0.05$) and PGI/PGII were lower in the ≥ 75 -years-old age group ($P = 0.035$) compared to the 35-44-years-old age group. Levels of low-density lipoprotein cholesterol (LDL-C) were higher ($P = 0.009$) in *H. pylori*-infected subjects that were male. LDL-C levels were higher in 55-74-years-old age group ($P < 0.05$) for *H. pylori*-infected subjects and 45-64-years-old age group ($P < 0.05$) for non-infected subjects compared to 35-44-years-old age group. Hp-IgG level positively correlated with PG I, PG II and G-17 ($P < 0.001$, $P < 0.001$, $P = 0.006$), and negatively correlated with PGI/PGII ($P < 0.001$). Creatinine positively correlated with PG I, PG II and G-17 ($P < 0.001$, $P < 0.001$, $P < 0.001$). Fasting blood glucose (FBG) positively correlated with PG I /PG II and G-17 ($P < 0.001$, $P = 0.037$). Age positively correlated with PGII and G-17 ($P = 0.005$, $P = 0.026$).

CONCLUSION

PGII levels increased while PGI/PGII declined with age in a healthy population. *H. pylori* infection had an effect on raising LDL-C levels to increase the risk of atherosclerosis in males, especially those of elderly age. Age, *H. pylori* infection, levels of renal function and FBG were associated with levels of pepsinogens and gastrin.

Key words: *Helicobacter pylori* antibody; Pepsinogen; Gastrin; Gastric ageing

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Core tip: Our study showed that in an entire healthy population, levels of serum pepsinogen (PG) II increased while PG I /PG II declined with age. We discovered that *Helicobacter pylori* (*H. pylori*) infection had an effect on raising levels of low-density lipoprotein cholesterol to increase the risk of atherosclerosis in males, especially those who are elderly. We also found that age, *H. pylori* infection, serum levels of renal function indicators and fasting blood glucose (FBG) were associated with levels of serum PGs and gastrin; it was assumed that they may influence the secretory function of gastric mucosa and that abnormal serum levels of FBG and renal function might participate in the occurrence and development of gastric diseases.

Shan JH, Bai XJ, Han LL, Yuan Y, Sun XF. Changes with aging in gastric biomarkers levels and in biochemical factors associated with *Helicobacter pylori* infection in asymptomatic Chinese population. *World J Gastroenterol* 2017; 23(32): 5945-5953 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i32/5945.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23>.

INTRODUCTION

Ageing of the gastric tract is an early manifestation of overall ageing, and mainly presents as a decline in the secretory function of the gastric mucosa. Histomorphological studies have demonstrated that atrophy of gastric mucosa increases with age^[1-3]. In addition, studies have also shown that *Helicobacter pylori* (*H. pylori*) infection plays an important role in the progression of gastric mucosa lesions^[4]. It has been demonstrated that the prevalence of *H. pylori* infection increases with age, and *H. pylori* is closely related with the occurrence and development of peptic ulcers, chronic atrophic gastritis and gastric cancer^[5,6].

Serum pepsinogen (PG) levels reflect the number of glands and cells in gastric corpus mucosa. Therefore, they can reflect the secretory function of the gastric mucosa^[7-10]. It has been reported that the levels of serum PGs are influenced by age, sex, pathophysiologic status of gastric mucosa and *H. pylori* infection^[11]. Thus, serum PGs are indicators of the functional and morphological status of gastric corpus mucosa, and lower serum levels of PG I or PG I /PG II represent existence and degree of atrophy in gastric corpus mucosa^[12].

Serum level of gastrin-17 (G-17) can act as a biomarker that reflects the function and structure of gastric antral mucosa. Combining serum PG and G-17 levels has been shown to provide diagnostic information on gastric mucosa^[13-16], and may also reflect the degree of gastric aging. Non-invasive biomarker tests may, therefore, evaluate the secretory function of gastric mucosa and differentiate pathological conditions, such as *H. pylori*-associated gastritis and atrophic gastritis, from the healthy condition by combining tests for PGs, G-17 and *H. pylori*-immunoglobulin G (Hp-IgG)^[17].

Previous studies have investigated patients with peptic ulcer, chronic atrophic gastritis and gastric cancer. To date, few studies have observed levels of the aforementioned biomarkers and effects of *H. pylori* infection in a healthy ageing population nor explored the associated factors. In our study, we selected PGs and G-17 as gastric biomarkers and measured their serum levels along with Hp-IgG. The aim of the current study was to observe changes in gastric biomarker levels with age in a healthy Chinese population and effects of *H. pylori* infection on biochemical tests, as well as to explore associated factors which influence the levels of gastric biomarkers.

MATERIALS AND METHODS

Study subjects

This was a cross-sectional study of a healthy population, defined as having no respiratory, cardiovascular,

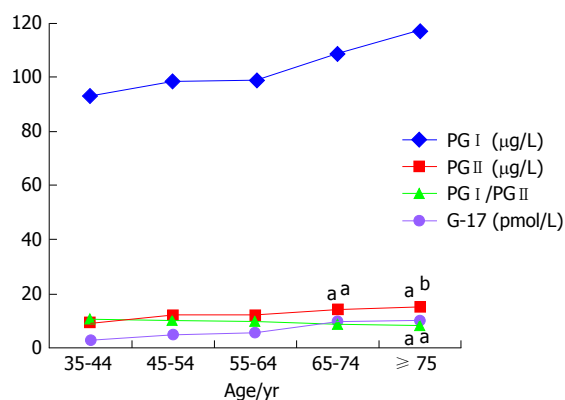


Figure 1 Comparison of serum gastric biomarker levels in various age groups. There was no significant difference in serum levels of PGI and G-17 between each age group with increasing age. In contrast, serum levels of PGII increased with age, and were significantly higher in subjects ≥ 65 -years-old compared to 35-44-years-old group. The ratio of PGI/PGII decreased with age, and was significantly lower in subjects ≥ 75 -years-old compared to 35-44-years-old group. The "a" denotes comparison with 35-44-years-old age group, ^a $P < 0.05$, ^b $P < 0.01$. G-17: Gastrin-17; PG I: Pepsinogen I; PG II: Pepsinogen II.

digestive, neurological, endocrine or urinary system diseases, as well as having absence of neoplastic and chronic infectious diseases and no history of psychiatric disorders. We screened 505 healthy persons out of 1500 volunteers in Shenyang, China between September 2007 and June 2008. The screening included inquiries on medical history, symptoms, smoking, alcohol intake, diet and family history obtained by a questionnaire that was completed by each participant. Physical examinations (*i.e.*, electrocardiogram, chest radiograph, etc.) were carried out along with biochemical tests, including assessments of fasting blood glucose (FBG), blood lipids, liver function, renal function and uric acid levels.

A total of 395 subjects (168 males and 227 females) out of the 505 persons, having a mean age of 59.4 years (range: 37-87 years), were enrolled from November 2010 to May 2011 by the same screening method. Patients with circulatory, respiratory, endocrine, neurological, digestive, urinary diseases and chronic infections or neoplastic diseases, or abnormal physical examinations and test results, as well as those with psychiatric disorders or who were unable to complete instructions and self-evaluations were excluded. Blood samples were obtained and sera were stored (within 2 h of collection) at -75°C until use for measurement of gastric biomarker levels (within 6 mo).

Informed consent was obtained from each participant. This study was reviewed and approved by the Medical Ethics Committees of General Hospital of Chinese People's Liberation Army and China Medical University.

Serological assays

Serum PGI and PGII, G-17 and *H. pylori* antibody levels were measured with enzyme-linked immunosorbent assay (ELISA)^[18] (Biohit Oyj, Laipatie 1, FIN-00880

Helsinki, Finland). All procedures were carried out according to the manufacturer's instructions.

Study groups

Subjects were divided into five age groups (35-44, 45-54, 55-64, 65-74 and ≥ 75 years). Hp-IgG-positive or -negative groups (Hp-IgG-positive defined as serum Hp-IgG ≥ 35 EIU)^[19] were also established.

Statistical analysis

Serum biomarker levels and serum biochemical tests were analyzed in *H. pylori*-positive and *H. pylori*-negative patients, separately in male and female subjects, by Student's *t*-test. Levels of serum gastric biomarkers among age groups and levels of serum gastric biomarkers and biochemical tests among age groups divided by *H. pylori* infection status were compared by ANOVA, and multiple comparisons were carried out by the Bonferroni method (homogeneity of variance) or Tamhane method (heterogeneity of variance). Relationships among serum gastric biomarker levels, age and biochemical tests were analyzed by Pearson's correlation coefficient matrix. Serum gastric biomarkers as dependent variables and other related factors as independent variables were analyzed by multiple linear regression analysis with stepwise method and multiple-collinearity. For all statistical analyses, we used SPSS V.17.0, and a two-sided *P* value of < 0.05 was considered statistically significant.

RESULTS

Comparison of serum gastric biomarker levels in various age groups

There was no significant difference in serum levels of PG I and G-17 between each age group with increasing age. In contrast, serum levels of PGII increased with age, and were significantly higher in subjects ≥ 65 -years-old compared to the 35-44-years-old group ($P = 0.024$, $P = 0.004$). The ratio of PG I / PG II decreased with age and was significantly lower in subjects ≥ 75 -years-old compared to those in the 35-44-years-old group ($P = 0.035$) (Table 1 and Figure 1).

Comparison of serum gastric biomarker levels by *H. pylori* infection status

Compared to non-infected subjects, serum levels of PG I, PG II and G-17 were significantly higher ($P < 0.001$, $P < 0.001$, $P = 0.025$), while the ratio of PG I / PG II was significantly lower ($P < 0.001$), in the *H. pylori*-infected subjects (Figure 2).

Comparison of serum biochemical tests between *H. pylori* infection statuses by sex

There was no significant difference in serum levels of biochemical tests between *H. pylori*-infected and non-infected female subjects. In males, levels of low-density lipoprotein cholesterol (LDL-C) were higher (P

Table 1 Comparison of serum gastric biomarker levels in various age groups

	35-44 yr, n = 58	45-54 yr, n = 84	55-64 yr, n = 117	65-74 yr, n = 76	≥ 75 yr, n = 60	F	P value
PG I, µg/L	92.98 ± 5.16	98.47 ± 4.15	98.65 ± 3.66	108.56 ± 8.01	117.04 ± 8.30	2.326	0.056
PG II, µg/L	9.65 ± 0.73	12.26 ± 0.91	12.43 ± 0.76	14.23 ± 1.17 ^{1a}	15.33 ± 1.25 ^{1b}	3.915	0.004
PG I /PG II	10.94 ± 0.44	9.89 ± 0.40	9.67 ± 0.36	9.29 ± 0.61	8.71 ± 0.52 ^{1a}	2.407	0.049
G-17, pmol/L	2.93 ± 0.55	5.10 ± 1.29	5.75 ± 1.49	9.93 ± 3.00	10.03 ± 3.18	1.950	0.101

Data are presented as mean ± SD. ¹Comparison with the 35-44-years-old group, ^a $P < 0.05$, ^b $P < 0.01$. G-17: Gastrin-17; PG I: Pepsinogen I; PG II: Pepsinogen II.

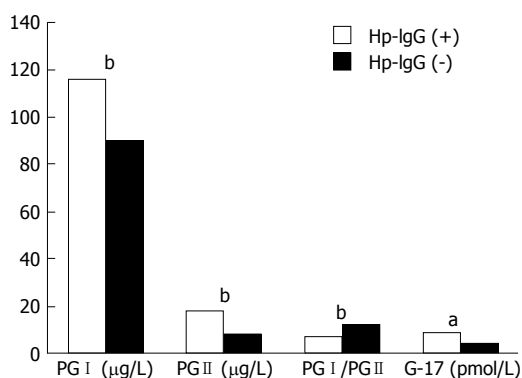


Figure 2 Comparison of serum gastric biomarker levels by *Helicobacter pylori* infection status. Compared to non-infected subjects, serum levels of PG I, PG II and G-17 were significantly higher, while the ratio of PG I /PG II was significantly lower in *Helicobacter pylori*-infected subjects. ^a $P < 0.05$, ^b $P < 0.01$. G-17: Gastrin-17; PG I: Pepsinogen I; PG II: Pepsinogen II.

= 0.009) in *H. pylori*-infected subjects compared to non-infected subjects (Table 2).

Comparison of serum gastric biomarker levels and biochemical tests in various age groups by *H. pylori* infection status

There was no significant difference in serum levels of gastric biomarkers between each age group with increasing age in *H. pylori*-infected subjects. In non-infected subjects, levels of serum PG II increased with age and were significantly higher in subjects ≥ 75-years-old compared to subjects between 35- and 54-years-old ($P = 0.007$, $P = 0.004$).

In *H. pylori*-infected subjects, serum levels of total cholesterol ($P = 0.002$, $P = 0.001$) and LDL-C ($P = 0.016$, $P = 0.002$) were significantly higher in subjects between 55- and 74-years-old compared to those in the 35-44-years-old age group. In non-infected subjects, serum levels of total cholesterol ($P = 0.023$, $P = 0.035$) and LDL-C ($P = 0.015$, $P = 0.006$) were significantly higher in subjects between 45- and 64-years-old compared to those in the 35-44-years-old group (Table 3 and Figure 3).

Correlation analysis among serum gastric biomarker levels, age and biochemical tests

Age positively correlated with serum levels of Hp-IgG, PG I, PG II and G-17 ($P = 0.038$, $P = 0.001$, $P < 0.001$, $P = 0.005$) and negatively correlated with ratio of PG I

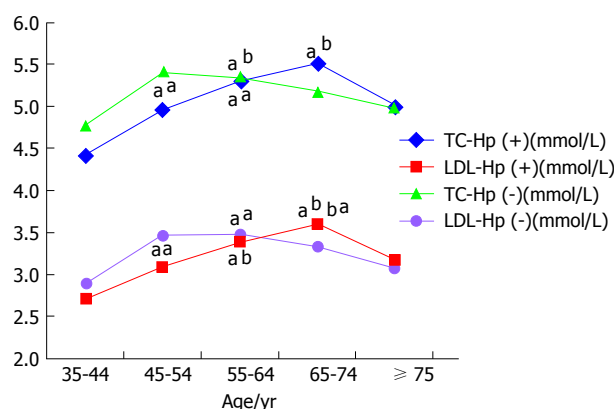


Figure 3 Comparison of serum cholesterol levels in various age groups by *Helicobacter pylori* infection status. In *H. pylori*-infected subjects, serum levels of TC and LDL were significantly higher in subjects between 55- and 74-years-old compared to those in the 35-44-years-old age group. In non-infected subjects, serum levels of TC and LDL were significantly higher in subjects between 45- and 64-years-old compared to those in the 35-44-years-old age group. The "a" denotes comparison with the 35-44-years-old age group and the "b" denotes comparison with the 45-54-years-old age group, ^a $P < 0.05$, ^b $P < 0.01$. *H. pylori*: *Helicobacter pylori*; LDL-C: Low-density lipoprotein cholesterol; TC: Total cholesterol.

/PG II ($P = 0.002$). Levels of serum Hp-IgG positively correlated with serum levels of PG I, PG II and G-17 ($P < 0.001$, $P < 0.001$, $P = 0.038$) and negatively correlated with ratio of PG I /PG II ($P < 0.001$).

Levels of serum PG I positively correlated with serum levels of uric acid, creatinine and cystatin-C ($P < 0.001$, $P < 0.001$, $P < 0.001$). Levels of serum PG II positively correlated with serum levels of creatinine and cystatin-C ($P < 0.001$, $P < 0.001$). Levels of serum G-17 positively correlated with serum levels of FBG, creatinine and cystatin-C ($P = 0.018$, $P = 0.011$, $P = 0.037$).

Levels of serum Hp-IgG were strongly associated with serum levels of PG II and PG I /PG II ($r = 0.592$, $P < 0.001$; $r = -0.587$, $P < 0.001$), and levels of serum PG II were strongly associated with serum levels of PGI and PG I /PG II ($r = 0.682$, $P < 0.001$; $r = -0.588$, $P < 0.001$)(Table 4).

Analysis of factors associated with serum levels of gastric biomarkers

With serum PGI as a dependent variable, serum levels of creatinine, Hp-IgG and FBG positively correlated with levels of serum PG I ($P < 0.001$, $P < 0.001$,

Table 2 Comparison of serum biochemical tests between *Helicobacter pylori* infection statuses by sex

	Male			Female		
	Hp-IgG (+), <i>n</i> = 81	Hp-IgG (-), <i>n</i> = 87	<i>P</i> value	Hp-IgG (+), <i>n</i> = 104	Hp-IgG (-), <i>n</i> = 123	<i>P</i> value
TG, mmol/L	1.33 ± 0.11	1.30 ± 0.17	0.875	1.24 ± 0.06	1.26 ± 0.06	0.767
TC, mmol/L	5.07 ± 0.11	4.80 ± 0.09	0.052	5.16 ± 0.09	5.42 ± 0.09	0.050
HDL-C, mmol/L	1.31 ± 0.04	1.35 ± 0.03	0.381	1.52 ± 0.03	1.54 ± 0.03	0.575
LDL-C, mmol/L	3.30 ± 0.10	2.99 ± 0.07	0.009	3.26 ± 0.09	3.48 ± 0.08	0.071
FBG, mmol/L	5.45 ± 0.09	5.26 ± 0.06	0.073	5.29 ± 0.06	5.27 ± 0.09	0.857
Cr, μmol/L	72.58 ± 1.76	73.26 ± 1.40	0.760	60.66 ± 2.59	55.34 ± 0.89	0.054
Cys-C, mg/L	0.93 ± 0.02	0.91 ± 0.02	0.520	0.88 ± 0.03	0.81 ± 0.02	0.059
UA, μmol/L	339.05 ± 8.19	337.48 ± 9.04	0.898	265.13 ± 6.19	273.22 ± 5.30	0.319

Data are presented as mean ± SD. *H. pylori*-IgG (+) is defined as *H. pylori*-IgG ≥ 35 EIU. Cr: Creatinine; Cys-C: Cystatin-C; FBG: Fasting blood glucose; HDL-C: High-density lipoprotein cholesterol; Hp-IgG: *Helicobacter pylori*-immunoglobulin G; LDL-C: Low-density lipoprotein cholesterol; TC: Total cholesterol; TG: Triglycerides; UA: Uric acid; *H. pylori*: *Helicobacter pylori*.

Table 3 Comparison of serum gastric biomarker levels and biochemical tests in various age groups by *Helicobacter pylori* infection status

	35-44 yr <i>n</i> 1 = 21 <i>n</i> 2 = 37	45-54 yr <i>n</i> 1 = 39 <i>n</i> 2 = 45	55-64 yr <i>n</i> 1 = 57 <i>n</i> 2 = 60	65-74 yr <i>n</i> 1 = 36 <i>n</i> 2 = 40	≥ 75 yr <i>n</i> 1 = 33 <i>n</i> 2 = 27	F	<i>P</i> value
PGI, μg/L							
Hp-IgG (+)	89.43 ± 7.46	100.03 ± 6.45	90.25 ± 5.10	92.61 ± 12.23	94.95 ± 13.47	0.824	0.920
Hp-IgG (-)	83.6 ± 6.47	81.28 ± 3.89	83.96 ± 5.04	96.67 ± 10.31	105.49 ± 8.08	1.730	0.143
PGII, μg/L							
Hp-IgG (+)	14.68 ± 1.08	18.45 ± 1.34	17.47 ± 1.16	19.52 ± 1.46	19.51 ± 1.91	1.393	0.260
Hp-IgG (-)	6.80 ± 0.58	6.89 ± 0.41	7.64 ± 0.46	9.46 ± 1.42	10.23 ± 0.76 ^{1b2b}	1.115	0.011
PGI/PGII							
Hp-IgG (+)	7.92 ± 0.53	7.17 ± 0.39	7.03 ± 0.38	6.65 ± 0.58	6.97 ± 0.52	0.662	0.616
Hp-IgG (-)	12.66 ± 0.41	12.25 ± 0.42	12.18 ± 0.39	11.68 ± 0.89	10.85 ± 0.80	1.163	0.353
G-17, pmol/L							
Hp-IgG (+)	4.11 ± 0.74	9.81 ± 2.58	8.18 ± 2.17	7.16 ± 0.95	13.7 ± 4.79	1.258	0.285
Hp-IgG (-)	2.26 ± 0.73	1.02 ± 0.26	3.44 ± 2.03	12.43 ± 5.65	5.54 ± 3.90	2.254	0.066
TC, mmol/L							
Hp-IgG (+)	4.42 ± 0.14	4.96 ± 0.14	5.30 ± 0.11 ^{1b}	5.51 ± 0.19 ^{1b}	5.00 ± 0.15	6.604	< 0.001
Hp-IgG (-)	4.77 ± 0.10	5.41 ± 0.17 ^{1a}	5.35 ± 0.13 ^{1a}	5.18 ± 0.16	4.98 ± 0.14	2.709	0.031
LDL-C, mmol/L							
Hp-IgG (+)	2.71 ± 0.14	3.10 ± 0.12	3.40 ± 0.11 ^{1a}	3.60 ± 0.17 ^{1b2a}	3.17 ± 0.14	7.291	< 0.001
Hp-IgG (-)	2.89 ± 0.09	3.47 ± 0.15 ^{1a}	3.48 ± 0.11 ^{1b}	3.33 ± 0.13	3.08 ± 0.13	3.544	0.008
FBG, mmol/L							
Hp-IgG (+)	5.25 ± 0.08	5.22 ± 0.13	5.38 ± 0.12	5.46 ± 0.12	5.44 ± 0.08	0.791	0.532
Hp-IgG (-)	5.01 ± 0.06	5.21 ± 0.08	5.29 ± 0.08	5.34 ± 0.08	5.39 ± 0.34	1.186	0.318
Cr, μmol/L							
Hp-IgG (+)	56.95 ± 2.57	60.38 ± 2.35	63.79 ± 1.71	63.61 ± 3.04	81.4 ± 7.24 ^{1a}	4.974	0.001
Hp-IgG (-)	59.19 ± 2.05	62.78 ± 1.95	61.20 ± 1.67	63.53 ± 2.29	73.19 ± 3.40 ^{1b2a3b}	4.174	0.003
Cys-C, mg/dL							
Hp-IgG (+)	0.69 ± 0.02	0.78 ± 0.02 ^{1a}	0.84 ± 0.02 ^{1b}	0.97 ± 0.03 ^{1b2b3a}	1.19 ± 0.07 ^{1b2b3b}	19.952	< 0.001
Hp-IgG (-)	0.71 ± 0.02	0.78 ± 0.02	0.83 ± 0.02 ^{1b}	0.93 ± 0.03 ^{1b2b}	1.14 ± 0.04 ^{1b2b3b4b}	28.435	< 0.001

Data are presented as mean ± SD. Hp-IgG (+) is defined as Hp-IgG ≥ 35 EIU. *n*1: number in the Hp-IgG (+) group; *n*2: number in the Hp-IgG (-) group. ¹35-44-years-old group, ²45-54-years-old group, ³55-64-years-old group, ⁴65-74-years-old group, ^a*P* < 0.05, ^b*P* < 0.01. Cr: Creatinine; Cys-C: Cystatin-C; FBG: Fasting blood glucose; G-17: Gastrin-17; Hp-IgG: *Helicobacter pylori*-immunoglobulin G; LDL-C: Low-density lipoprotein cholesterol; PG I: Pepsinogen I; PG II: Pepsinogen II; TC: Total cholesterol.

P = 0.037), while serum levels of G-17 negatively correlated with levels of serum PG I (*P* < 0.001). With serum PGII as a dependent variable, serum levels of creatinine, Hp-IgG and age positively correlated with levels of serum PGII (*P* = 0.006, *P* < 0.001, *P* = 0.007). With PG I /PGII as a dependent variable, serum levels of FBG positively correlated with PGI/PGII (*P* < 0.001), while serum levels of Hp-IgG, G-17 and age negatively correlated with PG I /PGII (*P* < 0.001, *P* < 0.001, *P* = 0.024). With serum G-17 as a dependent variable,

age and serum levels of creatinine, Hp-IgG and FBG positively correlated with levels of serum G-17 (*P* = 0.032, *P* < 0.001, *P* = 0.037, *P* = 0.045), while serum levels of PGI and uric acid negatively correlated with levels of serum G-17 (*P* < 0.001, *P* = 0.009)(Table 5).

DISCUSSION

A European gastric biomarkers test^[17] has been developed to measure serum PG and G-17 levels, and

Table 4 Correlation matrix among serum gastric biomarker levels, age and biochemical tests

	Age	PG I	PG II	PG I / II	G-17	Hp-IgG	TG	TC	HDL-C	LDL-C	FBG	UA	Cr	Cys-C	BMI
Age	1	0.161 ^b	0.215 ^b	-0.155 ^b	0.140 ^b	0.104 ^a	-0.009	0.108 ^a	0.010	0.136 ^b	0.145 ^b	0.148 ^b	0.265 ^b	0.548 ^b	-0.016
PG I		0.001	0.000	0.002	0.005	0.038	0.854	0.033	0.844	0.007	0.004	0.003	0.000	0.000	0.781
	1.000	0.682 ^b	0.000	0.047	-0.140 ^b	0.260 ^b	-0.014	-0.066	-0.051	-0.023	0.056	0.188 ^b	0.301 ^b	0.355 ^b	0.011
PG II			0.000	0.357	0.005	0.000	0.788	0.188	0.308	0.654	0.266	0.000	0.000	0.000	0.843
			1.000	-0.588 ^b	0.149 ^b	0.592 ^b	-0.029	-0.062	-0.075	-0.019	-0.005	0.077	0.209 ^b	0.278 ^b	0.006
PG I / II				0.000	0.003	0.000	0.571	0.216	0.135	0.705	0.915	0.124	0.000	0.000	0.913
				1.000	-0.384 ^b	-0.587 ^b	0.035	0.032	0.040	0.027	0.074	0.080	-0.019	-0.060	0.003
G-17					0.000	0.000	0.488	0.532	0.427	0.590	0.140	0.112	0.712	0.234	0.961
					1.000	0.105 ^a	0.055	-0.020	-0.068	-0.016	0.119 ^a	-0.062	0.129 ^a	0.105 ^a	-0.023
Hp-IgG						0.038	0.279	0.695	0.177	0.749	0.018	0.219	0.011	0.037	0.690
						1.000	0.000	-0.025	-0.062	-0.004	0.057	0.009	0.058	0.095	0.006
TG							0.985	0.618	0.220	0.938	0.257	0.865	0.250	0.059	0.919
							1.000	0.278 ^b	-0.327 ^b	0.145 ^b	0.166 ^b	0.156 ^b	0.059	0.068	0.048
TC								0.000	0.000	0.004	0.001	0.002	0.243	0.176	0.402
								1.000	0.295 ^b	0.896 ^b	0.123 ^a	0.023	-0.032	-0.039	0.111
HDL-C									0.000	0.000	0.015	0.647	0.530	0.442	0.053
									1.000	0.032	-0.093	-0.282 ^b	-0.176 ^b	-0.164 ^b	0.017
LDL-C										0.528	0.064	0.000	0.000	0.001	0.762
										1.000	0.149 ^b	0.074	10.000	0.022	0.085
FBG											0.003	0.142	0.000	0.664	0.138
											1.000	0.119 ^a	0.111 ^a	0.086	0.034
UA												0.018	0.027	0.086	0.558
												1.000	0.465 ^b	0.403 ^b	-0.003
Cr													0.000	0.000	0.961
													1.000	0.706 ^b	-0.010
Cys-C														0.000	0.858
														1.000	-0.026
BMI															0.648
															1.000

^a *p* < 0.05, ^b *p* < 0.01. BMI: Body mass index; Cr: Creatinine; Cys-C: Cystatin-C; FBG: Fasting blood glucose; G-17: Gastrin-17; Hp-IgG: *Helicobacter pylori*-immunoglobulin G; LDL-C: Low-density lipoprotein cholesterol; PG I: Pepsinogen I; PG II: Pepsinogen II; TC: Total cholesterol; HDL-C: High-density lipoprotein cholesterol; TG: Triglyceride; UA: Uric acid.

Hp-IgG antibodies by ELISA technique. Compared to endoscopic biopsy findings, the test classified the subjects into groups with “healthy” or “diseased” gastric mucosa with 94% accuracy, 95% sensitivity and 93% specificity. Compared to endoscopic histological findings, the accuracy of the biomarkers test in diagnosing atrophic gastritis was 87%, with a sensitivity of 40% and a specificity of 94%. Combined testing of Hp-IgG, PG and G-17 levels is of great clinical significance for general assessment of gastric mucosa secretion.

It has been previously shown that levels of serum PG I decreased with age. Levels of serum PGII increased with age, but declined in participants aged over 60. Ratio of PG I / PGII decreased with age, but it increased after age 60^[20]. It has also been observed that levels of PGI and PGII increased with age. In a healthy population, levels of PG I and PGII varied amongst age groups, and the average PG level was highest in the senile group^[21].

Our study showed that in the entire healthy study population, levels of serum PG II increased with age, while the ratio of PG I / PGII decreased with age. The correlation between age and PG II is stronger and more significant than that of PG I ; possibly, the distribution of PG II-secreting cells is more extensive, and this could

Table 5 Factors associated with serum levels of gastric biomarkers

Dependent variable	Associated factors	Non-standard coefficient		Standard coefficient	P value
		B	SE	β	
PGI	Constant	50.347	200.845		0.798
	Cr	0.712	0.138	0.273	0.000
	Hp-IgG	0.334	0.066	0.263	0.000
	G-17	-0.647	0.138	-0.247	0.000
	FBG	70.859	30.744	0.110	0.037
PGII	Constant	-10.657	20.078		0.426
	Hp-IgG	0.120	0.010	0.556	0.000
	Cr	0.058	0.021	0.131	0.006
	Age	0.089	0.033	0.129	0.007
PGI /PGII	Constant	90.251	10.461		0.000
	Hp-IgG	-0.054	0.004	-0.520	0.000
	G-17	-0.075	0.009	-0.349	0.000
	FBG	10.037	0.255	0.177	0.000
	Age	-0.033	0.015	-0.101	0.024
G-17	Constant	-140.817	80.992		0.100
	Age	0.192	0.089	0.1240	0.032
	PGI	-0.103	0.022	-0.269	0.000
	Cr	0.228	0.063	0.228	0.000
	Hp-IgG	0.058	0.027	0.119	0.037
	UA	-0.042	0.016	-0.160	0.009
	FBG	30.054	10.520	0.112	0.045

Cr: Creatinine; FBG: Fasting blood glucose; G-17: Gastrin-17; Hp-IgG: *Helicobacter pylori*-immunoglobulin G; PG I : Pepsinogen I ; PG II : Pepsinogen II ; UA: Uric acid.

be one of the reasons to explain this finding. Since the ratio of PG I /PG II reflects the degree of atrophy in gastric mucosa, the current study indicated that atrophy of gastric mucosa occurred and developed with increasing age in a non-invasive serological method.

It has been suggested that serum levels of PGI and PGII significantly correlated with age in *H. pylori*-positive subjects. Increased PG I and PG II levels associated with age in a healthy population were caused by increased rates of *H. pylori* infection. Levels of PG I and PG II were dependent on the presence of *H. pylori* infection^[22]. It was suggested that serum levels of G-17, PGI and PG II increased in subjects with *H. pylori* infection, especially PG II, while the ratio of PG I /PG II decreased.

Hypergastrinemia and hyperpepsinogenemia may be secondary to *H. pylori* infection^[23,24]. The results of the current study on the effects of *H. pylori* infection on serum gastric biomarker levels were consistent with those of previous studies, and it was suggested that *H. pylori* infection had a closer correlation with PG II than with PG I and may influence the levels of PG II more.

It has been shown that *H. pylori* infection was independently associated with elevated LDL-C levels and contributed to the atherosclerotic process^[25]. The current study showed the difference on levels of serum LDL-C between *H. pylori*-infected and non-infected male subjects, which suggested an effect of *H. pylori* infection on raising levels of LDL-C in males. Meanwhile, the highest level of LDL-C was found in the middle-aged group (45-64 years) in non-infected subjects, while in *H. pylori*-infected subjects it was found in the elderly group (55-74 years). Increased

LDL-C level is a risk factor for the development of atherosclerosis, and the current study indicated that Hp infection may increase the risks of atherosclerosis in males, especially those of elderly age.

It has been reported that renal function status may influence levels of serum PG and gastrin. Levels of serum PG and gastrin were found to be increased in patients with renal function insufficiency. This may have been due to reduced renal clearance of PG and gastrin^[26,27]. There have been few studies investigating the relationship between renal function and serum PG and gastrin in a healthy population. The current study showed that age and serum levels of Hp-IgG, creatinine and FBG were the main factors associated with levels of serum PG and G-17. Since different levels of PG and G-17 represent different pathophysiological status of gastric mucosa, it was assumed that age, *H. pylori* infection, and serum levels of FBG and markers of renal function may influence the secretory function of gastric mucosa, and that abnormal serum levels of FBG and renal function might participate in the occurrence and development of gastric diseases.

In summary, the current study observed changes in gastric biomarker levels with age and effects of *H. pylori* infection in a healthy Chinese population, and explored factors associated with gastric biomarkers. Our data provide a theoretical basis for the recognition of gastric aging and its related diseases, which is of important clinical significance. However, there are some limitations in the study. Firstly, the sample size was relatively small and may, therefore, not represent the whole healthy population. Secondly, we found the effects of *H. pylori* infection and the correlation between gastric biomarkers and other associated factors, but the

mechanisms are not clear. More studies are needed to illustrate the mechanisms in the future.

COMMENTS

Background

Combined testing of *Helicobacter pylori* (*H. pylori*)-immunoglobulin G (Hp-IgG), pepsinogen (PG) and G-17 levels is of great clinical significance for general assessment of gastric mucosa secretion, and may also reflect the degree of gastric aging. Previous studies have investigated patients with peptic ulcer, chronic atrophic gastritis and gastric cancer. To date, few studies have observed levels of the gastric biomarkers and effects of *H. pylori* infection in a healthy ageing population nor explored the associated factors.

Research frontiers

Non-invasive biomarker tests may evaluate the secretory function of gastric mucosa, and distinguish pathological conditions, such as atrophic gastritis, from the healthy condition by combining tests for PGs, G-17 and Hp-IgG. Lower serum levels of PGI or PG I /PG II represent existence and degree of atrophy in gastric corpus mucosa. Studies have indicated that serum levels of PGs and G-17 are related to *H. pylori* infection and age, and could be significantly influenced by *H. pylori* infection. Furthermore, it has been shown that *H. pylori* infection was independently associated with elevated low-density lipoprotein cholesterol (LDL-C) levels and contributed to the atherosclerotic process.

Innovations and breakthroughs

The current study observed changes in gastric biomarker levels with age and effects of *H. pylori* infection in a healthy Chinese population, and explored factors associated with gastric biomarkers. This study showed that in the entire healthy study population, levels of serum PGI increased while PGI/PGII declined with age, which indicated that atrophy of gastric mucosa occurred and developed with increasing age, observed via a non-invasive serological method. Meanwhile, we discovered that *H. pylori* infection had an effect on raising levels of LDL-C to increase the risk of atherosclerosis in males, especially those of elderly age. Moreover, it is suggested that age, *H. pylori* infection, serum levels of renal function and fasting blood glucose (FBG) were associated with levels of serum PGs and gastrin.

Applications

In this study, the authors' discovered that *H. pylori* infection had an effect on raising levels of LDL-C to increase the risk of atherosclerosis in males, especially those who were elderly, which indicated that *H. pylori* infection should be afforded a more important status and given active treatment in elderly males to prevent atherosclerotic diseases. This study suggested that age, *H. pylori* infection, serum levels of renal function and FBG were associated with levels of serum PGs and gastrin. It was assumed that serum levels of renal function and FBG may influence the secretory function of gastric mucosa, and abnormal serum levels of FBG and renal function might participate in the occurrence and development of gastric diseases.

Terminology

H. pylori: A curved Gram-negative bacillus which is found in gastric mucosa; *H. pylori* is closely related with multiple gastric diseases, such as peptic ulcers, chronic atrophic gastritis and gastric cancer. PG: A precursor of pepsin which is mainly secreted by cells in the gastric corpus and can be divided into two groups, PGI and PGII; serum PG levels reflect the number of glands and cells, as well as the secretory function in gastric corpus mucosa. G-17: A hormone which is mainly secreted by G cells in gastric antrum and plays multiple physiological roles; serum level of G-17 reflects the number of cells and the secretory function in gastric antral mucosa.

Peer-review

The authors have carried out a detailed study of biomarkers and *H. pylori* infection in a large cohort of patients. The manuscript is detailed, the study well carried out and the data is comprehensive and complex.

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Prospective Study

Modified *Helicobacter* test using a new test meal and a ¹³C-urea breath test in *Helicobacter pylori* positive and negative dyspepsia patients on proton pump inhibitors

Bojan Tepeš, Peter Malfertheiner, Joachim Labenz, Sitke Aygen

Bojan Tepeš, AM DC Rogaška, Prvomajska 29 A, 3250 Rogaška Slatina, Slovenia

Peter Malfertheiner, Universitätsklinikum Magdeburg A. ö. R Klinik für Gastroenterologie, Hepatologie und Infektiologie, 39120 Magdeburg, Germany

Joachim Labenz, Department of Internal Medicine and Gastroenterology, Diakonie Klinikum, Jung-Stilling Hospital, 57074 Siegen, Germany

Sitke Aygen, Institut für Biomedizinische Analytik und NMR-Imaging GmbH (INFAI), Gottfried-Hagen-Str. 60-62, 51105 Köln, Germany

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Correspondence to: Bojan Tepeš, MD, PhD, FEBGH, Professor, AM DC Rogaška, Prvomajska 29A, 3250 Rogaška Slatina, Slovenia. bojan.tepes@siol.net
Telephone: +386-3-8191411
Fax: +386-3-8191412

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Abstract

AIM

To determine the sensitivity and specificity of the ¹³C-urea breath test (UBT) in patients taking proton pump inhibitors (PPIs), using a new test meal Refex.

METHODS

One hundred and fourteen consecutive patients with dyspepsia, 53 *Helicobacter pylori* (*H. pylori*) positive, 49 *H. pylori* negative, were included in the study. The patients were then given esomeprazole 40 mg for 29 consecutive days, and the ¹³C-UBT with the new test meal was performed the next morning.

RESULTS

The sensitivity of the ¹³C-UBT with a cut off 2.5‰ was

92.45% (95%CI: 81.79%-97.91%) by per-protocol (PP) analysis and 78.13% (95%CI: 66.03%-87.49%) by intention-to-treat (ITT) analysis. The specificity of the ¹³C-UBT test was 96.00% in the ITT population (95%CI: 86.29%-99.51%) and 97.96% in the PP population (95%CI: 89.15%-99.95%).

CONCLUSION

The new test meal based ¹³C-UBT is highly accurate in patients on PPIs and can be used in those unable to stop their PPI treatment.

Key words: Urea breath test; New test meal; Proton pump inhibitors; Prospective randomized clinical trial; *Helicobacter pylori*

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Core tip: The urea breath test (UBT) with new test meal Reflex (5.5 g powder mixture of citric, malic and tartaric acid) was tested in one hundred and fourteen consecutive patients with dyspepsia, 53 *Helicobacter pylori* (*H. pylori*) positive, 49 *H. pylori* negative. After being on esomeprazole 40 mg for 29 consecutive days, the ¹³C-UBT was performed the next morning. The sensitivity of the ¹³C-UBT (cut off 2.5‰) was 92.45% by per-protocol (PP) analysis and 78.13% by intention-to-treat (ITT) analysis. The specificity of the ¹³C-UBT test was 96.00% in the ITT population and 97.96% in the PP population.

Tepeš B, Malfertheiner P, Labenz J, Aygen S. Modified *Helicobacter* test using a new test meal and a ¹³C-urea breath test in *Helicobacter pylori* positive and negative dyspepsia patients on proton pump inhibitors. *World J Gastroenterol* 2017; 23(32): 5954-5961 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i32/5954.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i32.5954>

INTRODUCTION

The urea breath test (UBT) is recommended as the test of choice for determining the success of eradication treatment^[1]. In the management of dyspeptic patients in primary care settings, non-invasive *Helicobacter pylori* (*H. pylori*) testing is the initial step in the management of dyspeptic patients (*i.e.*, test and treat strategy)^[2,3]. The UBT is highly sensitive and specific, except in patients taking proton pump inhibitors (PPIs)^[4]. In studies with patients on PPI therapy, the UBT resulted in 10%-40% false negatives^[5-7]. Current guidelines recommend stopping these medications for 14 d before the UBT or stool test^[2].

PPIs are widely available and are over-the-counter agents in some countries^[8]. Clinicians are frequently confronted with making a diagnosis of *H. pylori* infection in patients who may knowingly or unknowingly

be taking a PPI. Patients who self-administer certain medications that can cause dyspepsia (*e.g.*, low dose aspirin to prevent myocardial infarction or nonsteroidal anti-inflammatory drugs) often take PPIs to treat dyspepsia symptoms, and the majority of these patients cannot stop PPI therapy for two weeks without suffering dyspeptic symptoms. Therefore, the UBT might not be properly performed in a substantial number of these patients. If *H. pylori* is diagnosed late or remains undiagnosed, the risk of stomach cancer is increased^[9,10].

The breath tests that are currently available are reliable 12-14 d after discontinuing PPI therapy^[4,11]. Acid inhibition with PPIs can reduce the number of *H. pylori* colonies, especially in the antrum, which may be one possible explanation for a false negative UBT^[12]. Some studies have suggested that acidification of the stomach may partially reverse a false negative UBT^[11,13]. However, the results have been inconsistent, and the correct procedure for acidifying the stomach has not been established.

Reflex is a new acidified test meal for the ¹³C-UBT that contains a mixture of three organic acids - citric acid, malic acid and tartaric acid - and has been developed to increase the sensitivity of the test in patients taking PPIs.

The aim of this study was to determine the sensitivity, specificity and accuracy of a specially formulated UBT test meal, Reflex, in patients taking proton pump inhibitors.

MATERIALS AND METHODS

Study objectives

Primary objective: To determine the sensitivity of the ¹³C-UBT test using the new test meal for *H. pylori* in patients with dyspepsia taking PPIs with a one day break in medication.

Secondary objectives: To determine the specificity of the ¹³C-UBT using the new test meal for *H. pylori* in patients with dyspepsia taking PPIs with a one day break in medication and to determine the safety and tolerance of the new test meal.

Inclusion criteria and study protocol

This was an observer-blind, multicentre study (one in Slovenia and two in Germany) in which consecutive dyspeptic *H. pylori* positive or negative patients were included. The inclusion criteria were as follows: male and female patients of at least 18 year of age; all acid-related disorders requiring long-term PPI treatment, including functional dyspepsia, according to the Rome II classification; and positive or negative standard ¹³C-UBT at screening. Diagnosis of *H. pylori* infection was confirmed or excluded by a combination of culture, histology and the rapid urease test (RUT; PyloriTek®, Serim Research Corp., Elkhart, United

States) on samples obtained by endoscopy. "True positive patients" were patients with a positive culture or when at least two of the following tests were positive: UBT, histology, or rapid urease test (RUT). "True negative patients" were patients with at least two negative tests and a negative culture. True negative patients were also those with non-evaluable cultures and negative histology and urease test. Patients with negative UBT underwent upper endoscopy only if this was deemed necessary by the investigator for medical reasons. This study was conducted in outpatients.

Two biopsy samples were obtained from the antrum and corpus for histology. One biopsy sample for RUT was taken from the angular fold, and two samples from the antrum were taken for culture.

The biopsies for histology were stained with haematoxylin and eosin and Giemsa stains, and gastritis was scored using the Updated Sydney System. All biopsy samples were analysed at each respective medical centre.

Gastric biopsies for culture were collected and transported in Portagerm pylori (bioMerieux, France) transport medium. After homogenization in 1 mL PBS, 0.1 mL aliquots were inoculated for gram stain and culture. Two selective and one non-selective media were used. Plates were incubated at 37 °C in a microaerophilic atmosphere for 9 d and inspected for growth every 72 h. An enriched atmosphere was created using Anoxomat (Mart Microbiology). Typical colonies were identified with a typical gram stain and positive urease, catalase and oxidase reactions.

Starting on Day 1, *H. pylori* positive and negative patients in both study arms took Nexium capsules (40 mg) orally once daily 30 min before breakfast. They were instructed not to take antibiotics, bismuth compounds, H₂ receptor antagonists or other acid-suppressive agents during the treatment period. All other concomitant medications were recorded in the case report form with the name of the drug, active ingredient(s), strength of active ingredient(s), indication, single dose, daily dose, dosage interval, route of administration, and the times of initiation and discontinuation.

Patients returned to the hospital/medical practice for breath tests on day 30. Nexium capsules were discontinued after day 29. The patients were requested to return unused PPI medication on day 30. Treatment compliance was assessed by calculating the difference in the number of tablets issued and returned.

The ¹³C-UBT was performed in *H. pylori* positive and negative patients using the new test meal Reflex on day 30. A delta value ≥ 2.5‰ was set as a positive result. The test started with a breath sample taken at baseline. Thereafter, the patient had to ingest the new test meal Reflex dissolved in 200 mL tap water and 75 mg ¹³C-urea dissolved in 30 mL tap water. The new test meal had to be ingested and was followed by the ¹³C-urea solution. A second breath sample was taken

30 min after ingestion of the test meal. Breath samples were collected in pre-labelled test-tubes. The breath samples were sent in the original outer packaging to the laboratory in Germany.

We could not compare the new test meal Reflex with the classic meal with 2.0 g of citric acid, because according the UBT protocol the second test should be performed earliest one day later in order to avoid a false positive result. This means second UBT meal can be performed earliest after two days break of PPI treatment instead one day what will implement great bias in the study.

Patients were followed-up for 14 d after discontinuation of PPI treatment. At the end of the study, positive patients were offered eradication therapy according to the current European guidelines. *H. pylori* negative patients were treated according to national dyspepsia guidelines.

Exclusion criteria

Patients were not included in the study if they have previously been treated for their *H. pylori* infection, if they have used PPI, H₂ receptor antagonists, NSAIDs, antibiotics, antisecretory drugs, bismuth compounds, or sucralfate in the 4 wk prior to enrolment, if they had manifest coagulopathy or any other disorder according to which endoscopy and/or biopsies are contraindicated, if they have participated in a clinical trial with another not approved drug within 30 d before entering the study and in case of pregnancy

Sample size calculations

A total of 114 patients were screened in 3 active study centres. Analyses of the sensitivity and specificity of the modified UBT for *H. pylori* infection with new test meal were performed for exploratory purposes. Sample size calculations were based on previous experience with the modified UBT for *H. pylori* infection^[14]. This experience showed a sensitivity of at least 90% after 29 d of PPI medication. Although rare cases of false positive breath tests may occur in *H. pylori* negative patients, if other urea active bacteria than *H. pylori* such as *Proteus mirabilis* or *Staphylococcus aureus* colonize gastric lumen in patients with extensive atrophy or intestinal metaplasia^[15]. However, specificity of 90% was still assumed. With a sample size of 43, a two-sided 95%CI for a single proportion using the large sample normal approximation would extend 9 percentage-points from the observed proportion for an expected proportion of 90% (width of the 95%CI of 18%). With a sample size of $n = 43$ *H. pylori* positive patients and $n = 43$ actual *H. pylori* negative patients, sufficient precision for assessing sensitivity and specificity was expected. The actual sample sizes chosen to be used in the study were slightly larger [*H. pylori* positive: 63 in intention-to-treat (ITT) population, 53 in the PP population; *H. pylori* negative: 51 in ITT population, 49 in PP population]. Concerning

Table 1 Demographic data *n* (%)

Demographic variable	<i>n</i> = 114
Age (yr), mean ± SD	51.07 ± 14.4
Height (cm), mean ± SD	168.90 ± 9.3
Weight (kg), mean ± SD	73.43 ± 14.9
BMI (kg/m ²), mean ± SD	25.66 ± 4.3
Ethnic group	
Caucasian	114 (100.0)
Other	0 (0.0)
Gender	
Female	76 (66.7)
Male	38 (33.3)

Table 2 Diagnostic investigation for *Helicobacter pylori* (*n* = 114)

Hp diagnostic test	<i>n</i> (%)
UBT with standard test meal	
Positive	63 (55.3)
Negative	51 (44.7)
Upper endoscopy	
Yes	110 (96.5)
No	4 (3.5)
Culture	
Positive	60 (54.5)
Negative	36 (32.7)
Not evaluated	14 (12.7)
Histology	
Positive	53 (48.2)
Negative	47 (42.7)
Not evaluated	10 (9.1)
Rapid urease test	
Positive	63 (57.3)
Negative	47 (42.7)

Hp: *Helicobacter pylori*.

the primary variable of sensitivity, the asymptotic confidence intervals were quite close to the exact Clopper-Pearson intervals (ITT: asymptotic 95%CI: 64.39%-85.61%, exact 95%CI: 62.60%-84.98%; PP: asymptotic 95%CI: 85.38%-99.56%, exact 95%CI: 81.79%-97.91%). Concerning the secondary variable of specificity, the asymptotic confidence intervals deviate a bit more from the exact Clopper-Pearson intervals (ITT: asymptotic 95%CI: 94.12%-100%, exact 95%CI: 89.35%-99.95%; PP: asymptotic 95%CI: 94.00%-100%, exact 95%CI: 89.15%-99.95%) due to the proximity to 100% of the estimated specificity.

Ethics

The study was carried out in accordance with national laws and regulations, the ICH Guideline E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95), and with the Declaration of Helsinki, revised version, 48th WMA General Assembly, Somerset West, October 1996. Permission of the national regulatory authority was a prerequisite for initiation of the study.

Each patient was supplied with full and adequate verbal and written information on the objectives and procedures of the study as well as potential benefits,

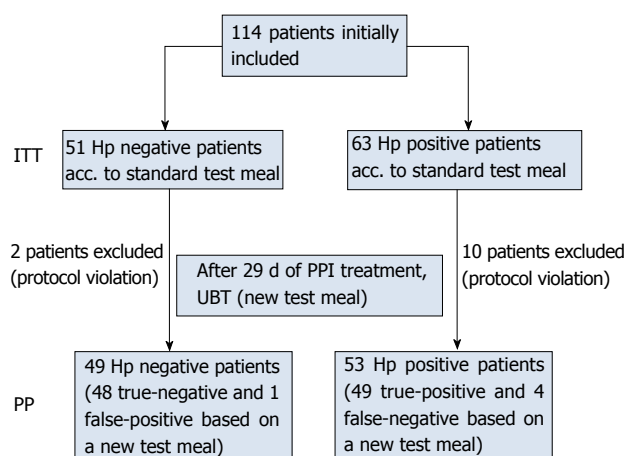


Figure 1 Flow diagram. The patient population and results of *Helicobacter pylori* diagnosis (based on study criteria and UBT with standard test meal and new test meal after 29 d of PPI treatment). UBT: Urea breath test; ITT: Intention-to-treat; PPI: Proton pump inhibitors; Hp: *Helicobacter pylori*.

discomforts and risks involved prior to inclusion in the study.

Statistical analysis

The sensitivity and specificity of the UBT with the new test meal on day 30 was assessed using relative frequencies and 95%CI (two-sided). Descriptive statistical methods were applied.

RESULTS

One hundred and fourteen patients were initially included in three centres. Twelve patients were excluded for not fulfilling the inclusion criteria (7 took Nexium 40 mg on day 30, three patients took antibiotics during the study period and two patients did not return on day 30). Altogether, 102 patients were eligible for PP analysis (Figure 1). Demographic data are presented in Table 1. The results of the diagnostic tests are presented in Table 2.

The primary variable in this study was the sensitivity of the ¹³C-UBT test using the new test meal for *H. pylori* in patients with dyspepsia taking PPI with a one day break in medication. The sensitivity of the ¹³C-UBT test was assessed using relative frequency and 95%CI (two-sided).

In our study, the cut-offs were set at 3.0‰, 2.5‰ and 2.0‰. The best sensitivity and specificity were achieved by using cut-off points of 2.5‰ and 2.0‰ combined with a break in PPI intake of one day before performing the UBT (Table 3).

The sensitivity of the ¹³C-UBT test was assessed using relative frequency and 95%CI (two-sided). The sensitivity of the ¹³C-UBT was found to be 92.45% (95%CI: 81.79%-97.91%) for the PP population (Table 4).

In the PP population, in patients with a positive *H. pylori* infection, 92.5% also had positive ¹³C-UBT results, and 7.5% showed (false) negative results.

Table 3 Thirty days of proton pump inhibitors medication, different cut-off points 2‰, 2.5‰, 3‰, sampling time 30 min

Cut-off	Sensitivity	Specificity	PPV	NPV	Accuracy
2‰	92.45%	97.96%	98.00%	92.31%	95.10%
2.5‰	92.45%	97.96%	98.00%	92.31%	95.10%
3‰	86.79%	97.96%	97.87%	87.27%	92.16%

In patients negative for *H. pylori* infection, 98.0% of the patients had negative ^{13}C -UBT results, and 2.0% showed (false) positive results.

As a secondary variable, the specificity was analysed for the ^{13}C -UBT test using the new test meal for *H. pylori* in patients with dyspepsia and taking PPI with a one day break in medication. The specificity was found to be 97.96% for the PP population (95%CI: 89.15%-99.95%)(Table 5).

The analysis of ROC curves considers in principle all cut-offs in order to identify a value with high efficiency. The chosen cut point of 2.5‰ based on the data leads to an excellent sensitivity and specificity. A well the excellent overall performance of the new UBT is substantiated by the ROC curve with a maximum Youden value of 0.90412, corresponding to a measured $\Delta\delta$ -value of 2.588‰ (Figure 2).

However, a value that is based on only one study with a limited possible number of cases may lead to slightly higher sensitivity and specificity. Our simulation study for different sample sizes and up to 1000 repeats of the diagnostic test provided an expected average sensitivity of 90%, which is only 2.5% lower than the observed sensitivity of 92.5% in our study. The expected average specificity is 99%, and therefore, higher than the observed value (97.96%). In all simulations, the study shows an expected maximum bias potential of 2.5%.

The accuracy of the method can be derived from 95% confidence limits based on the simulation study. These limits are defined by the 2.5% and 97.5% percentiles of the calculated distributions of the sensitivity and specificity. When reviewing 1000 repeats and a simulated sample size of 400, the limits were 86.8% to 92.5% (sensitivity) and 96.8% to 100% (specificity).

Following adverse events (AEs) are observed: Overall 8 patients (7.0%) experienced AEs, and all AEs were assessed and considered to be related to PPI medication. No serious adverse events, AEs leading to permanent discontinuation of the study medication, or fatal AEs were documented during this study. Mild AEs were reported for 3 patients (2.6%) and moderate AEs for 5 patients (4.4%) related to PPI medication.

DISCUSSION

^{13}C -UBT is the non-invasive method of choice for the detection of *H. pylori* infection in a test and treat strategy as well as for the assessment of the success

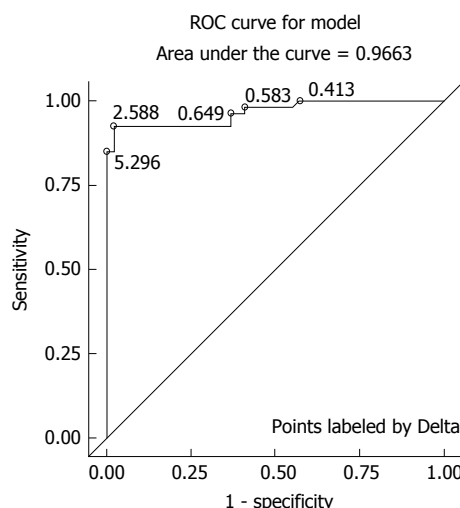


Figure 2 Empirical receiver operating characteristic curve by using cut-off values of 2.0‰, 2.5‰ and 3.0‰ (Per-protocol population, $n = 102$).

of *H. pylori* eradication^[1].

However, the sensitivity of the test decreases to unacceptable levels if patients are on PPI treatment^[1,2]. Currently available breath and stool tests are recommended to be performed 14 d after discontinuation of the PPI^[16]. This delay results in additional costs and the inconvenience of an additional visit. Dyspeptic symptoms may occur in patients due to acid rebound after the withdrawal of PPI therapy. In this study, we report on the diagnostic performance of a novel ^{13}C -UBT based on a special mixture of acid components in a test meal.

PPIs have a direct antibacterial effect on *H. pylori* and have been reported to inhibit *H. pylori* urease activity^[17-19]. False negative results, therefore, are a limitation in the use of the standard UBT in patients on PPIs. Up to 40% of individuals taking PPIs had a false negative test result^[5,6,19-22]. While 5 d on a PPI had no significant effect on *H. pylori* and the UBT, one-third of volunteers had a negative UBT after being on PPI therapy for 7 d (omeprazole 20 mg)^[12]. The UBT was positive again in all but one patient 4 d after stopping the PPI and in all patients 14 d after stopping the PPI. In all these studies, 2 g of citric acid was used as the test meal for UBT. Patients with a negative ^{13}C -UBT were also negative for *H. pylori* in antrum biopsies and had reduced *H. pylori* scores in corpus biopsies^[23].

The role of citric acid in the UBT test meal is to acidify the gastric contents and retard gastric emptying^[11,12,24-27]. The effect of citric acid on enhancing intragastric urease activity is dose dependent for doses between 1 and 4 g in 200 mL of water^[28]. Gastric pH, therefore, plays a major role in *H. pylori* urease activity.

In a study measuring gastric juice pH in 109 patients on chronic PPI therapy, 74% of the patients presented with gastric hypochlorhydria ($\text{pH} > 4$), and 26% of patients with presented with a $\text{pH} \leq 4$ ^[28]. False-negative RUT results were prevalent in patients with a $\text{pH} > 4$, whereas with a gastric pH of 2-4 (due

Table 4 Sensitivity of the ¹³C-urea breath test using the new test meal for *Helicobacter pylori* n (%)

Population	Result of UBT with new test meal	Diagnosis of Hp infection		Sensitivity	95%CI
		Positive	Negative		
ITT (n = 114)	Positive	50 (78.1)	1 (2.0)	78.13%	66.03%-87.49%
	Negative	13 (20.3)	48 (96.0)		
	Not performed	1 (1.6)	1 (2.0)		
PP (n = 102)	Positive	49 (92.5)	1 (2.0)	92.45%	81.79%-97.91%
	Negative	4 (7.5)	48 (98.0)		

UBT: Urea breath test; ITT: Intention-to-treat; PP: Per-protocol; Hp: *Helicobacter pylori*.

Table 5 Specificity of the ¹³C-urea breath test using the new test meal for *Helicobacter pylori* n (%)

Population	Result of UBT with new test meal	Diagnosis of Hp infection		Specificity	95%CI
		Positive	Negative		
ITT (n = 114)	Positive	50 (78.1)	1 (2.0)	96.00%	86.29%-99.51%
	Negative	13 (20.3)	48 (96.0)		
	Not performed	1 (1.6)	1 (2.0)		
PP (n = 102)	Positive	49 (92.5)	1 (2.0)	97.96%	89.15%-99.95%
	Negative	4 (7.5)	48 (98.0)		

UBT: Urea breath test; ITT: Intention-to-treat; PP: Per-protocol; Hp: *Helicobacter pylori*.

to inadequate PPI effect), RUT results were positive^[29].

The proton-gated inner membrane urea channel, *H. pylori* Urel, is essential for the survival of the *H. pylori* bacteria in the acidic environment of the stomach (pH < 2)^[30]. This channel is closed at neutral pH and opens at low pH, allowing urea access to urease. *H. pylori* urease forms NH₃ and CO₂, which neutralize incoming protons and thus buffer the periplasmic space to pH approximately 6, even in gastric juice at a pH < 2.0^[7,31-35].

To compensate for the unfavourable gastric pH due to PPI therapy, the concentration of citric acid for UBT has been raised maximally to 4.2 g in 200 mL water^[28]. This concentration of citric acid is poorly tolerated and induces symptoms. To avoid inconvenience for patients and to overcome the negative impact of PPIs on *H. pylori* urease, we used a highly concentrated mixture of organic acids (5.5 g in 200 mL water: tartaric acid, malic acid and citric acid) to reduce patient complaints^[14]. Agha *et al.*^[13] showed that an enhancement in urease activity can similarly be obtained for citric and malic acid.

H. pylori urease is a nickel-containing enzyme^[36], and preliminary data have suggested that changes in intracellular *H. pylori* nickel levels may influence urease activity^[37]. *H. pylori* urease and the membrane-bound hydrogenase enzyme are both *H. pylori* metalloenzymes, which are nickel-dependent. Moreover, the nickel transporter NixA and accessory proteins such as HypA and HypB serve to increase intracellular *H. pylori* nickel levels and enhance urease activity^[37].

Citric acid, tartaric acid and malic acid are organic acids that bind many trace metals, including nickel, and they can increase *H. pylori* urease activity both by lowering pH as well as by providing nickel to *H. pylori*.

The new test meal, Refex, has a unique 5.5 g powder mixture of three organic acids: citric, malic and tartaric acid dissolved in 200 mL water (pH 1.8). This highly concentrated organic acid mixture increases the acidity of the stomach for a short period of time and permits an increase in the bacterial urease activity to the point that urease activity becomes detectable in patients on PPIs.

We could not compare the new test meal Refex with the classic meal with 2.0 g of citric acid, because according to the UBT protocol the second test should be performed earliest one day later in order to avoid a false positive result. This means second UBT meal can be performed earliest after two days break of PPI treatment instead one day what will implement great bias in the study.

For optimizing the sensitivity of the UBT on Refex, we adjusted the cut-off point. We analysed the cut-off point of 4‰ for the standard test meal and for the new test meal (Refex), but we also investigated cut-off points of 3.0‰, 2.5‰ and 2.0‰. The best sensitivity and specificity were achieved with cut-off points at 2.5‰ and 2.0‰. With these modifications, we were able to reach a sensitivity of 92.5% (95%CI: 81.79%-97.91%) and specificity of 97.96% (95%CI: 89.15%-99.95%) for the PP population.

The UBT test using the new test meal Refex was well tolerated, with 7.2% of patients reporting dyspeptic effects during test meal intake. No severe side effects were noted.

With good patient compliance (PP population), we were able to demonstrate that the new UBT Refex can be reliable enough to be used in everyday clinical practice in patients who cannot stop their PPI therapy for more than one day.

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COMMENTS

Background

The urea breath test (UBT) is recommended as the test of choice for determining the success of eradication treatment. The UBT is highly sensitive and specific, except in patients taking proton pump inhibitors (PPIs) where the UBT can be false negatives in 10%-40% of patients. Current guidelines recommend stopping these medications for 14 d before the UBT or stool test.

Research frontiers

PPIs have a direct antibacterial effect on *Helicobacter pylori* (*H. pylori*) and can inhibit *H. pylori* urease activity. The *H. pylori* colonisation of the stomach can also be reduced, especially in antrum. Citric acid test meal is used to acidify the gastric contents and retard gastric emptying. The effect of citric acid on enhancing intragastric urease activity is dose dependent for doses between 1 and 4 g in 200 mL of water. High concentration of citric acid is poorly tolerated and induces symptoms.

Innovations and breakthroughs

A highly concentrated mixture of organic acids (5.5 g in 200 mL water: tartaric acid, malic acid and citric acid; pH 1.8) was used to reduce patient complaints and increase the accuracy of the UBT. This mixture can increase the *H. pylori* urease activity by the influence of low pH on the Urel channel, or by providing additional nickel to *H. pylori*. With the adjustment of the cut-off point to 2.5‰ sensitivity of UBT with new test meal can be improved.

Applications

The new UBT test meal can be used as the *H. pylori* diagnostic test in patients on PPI who can not stop their PPI therapy for two weeks or more. Recommendations for the use of UBT after antimicrobial therapy should not be changed.

Peer-review

This work is good and it will help us in further clinical work in the detection of *H. pylori* positive and negative dyspepsia patients on proton pump inhibitors.

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Prospective Study

Real time endoscopic ultrasound elastography and strain ratio in the diagnosis of solid pancreatic lesions

Hussein Okasha, Shaimaa Elkholy, Ramy El-Sayed, Mohamed-Naguib Wifi, Mohamed El-Nady, Walid El-Nabawi, Waleed A El-Dayem, Mohamed I Radwan, Ali Farag, Yahya El-sherif, Emad Al-Gemeie, Ahmed Salman, Mohamed El-Sherbiny, Ahmed El-Mazny, Reem E Mahdy

Hussein Okasha, Shaimaa Elkholy, Mohamed Naguib Wifi, Mohamed El-Nady, Ali Farag, Ahmed Salman, Mohamed El-Sherbiny, Ahmed El-Mazny, Internal Medicine Department, Cairo University, Cairo 11311, Egypt

Ramy El-Sayed, Waleed A El-Dayem, Mohamed I Radwan, Department of Tropical Medicine, Zagazig University, Elsharkiah 44519, Egypt

Walid El-Nabawi, Internal Medicine Department, Beni Suf University, Beni Suf 71515, Egypt

Yahya El-sherif, Tropical Medicine Department, El Manial Specialized Hospital, Cairo University, Cairo 11311, Egypt

Emad Al-Gemeie, Pathology Department, National Cancer Institute, Cairo University, Cairo 11311, Egypt

Reem E Mahdy, Internal Medicine Department, Assiut University, Assiut 71515, Egypt

Author contributions: Okasha H is the main endosonographer who performed all the cases and participated in the study design; Elkholy S, Mahdy RE and El-Sayed R participated in Manuscript writing and data analysis; Wifi MN, El-Nady M, El-Dayem WA and Radwan MI participated in design and oversight of the study; El-Nabawi W, Farag A and El-sherif Y participated in design of the study and data collection; Salman A, El-Sherbiny M and El-Mazny A were involved with data collection, and follow up of the patients; Al-Gemeie E is the main histopathologist who performed histopathological analysis for the specimens; all the above mentioned authors have read and approved the final manuscript.

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Correspondence to: Shaimaa Elkholy, MD, Lecturer, Department of Internal Medicine, Faculty of Medicine, Cairo University, Kasralainy street Cairo, Cairo 11311, Egypt. shaimaa.elkholy@cu.edu.eg
Telephone: +2-10-60407761
Fax: +2-37-493563

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Abstract

AIM

To evaluate the accuracy of the elastography score combined to the strain ratio in the diagnosis of solid pancreatic lesions (SPL).

METHODS

A total of 172 patients with SPL identified by endoscopic

ultrasound were enrolled in the study to evaluate the efficacy of elastography and strain ratio in differentiating malignant from benign lesions. The semi quantitative score of elastography was represented by the strain ratio method. Two areas were selected, area (A) representing the region of interest and area (B) representing the normal area. Area (B) was then divided by area (A). Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were calculated by comparing diagnoses made by elastography, strain ratio and final diagnoses.

RESULTS

SPL were shown to be benign in 49 patients and malignant in 123 patients. Elastography alone had a sensitivity of 99%, a specificity of 63%, and an accuracy of 88%, a PPV of 87% and an NPV of 96%. The best cut-off level of strain ratio to obtain the maximal area under the curve was 7.8 with a sensitivity of 92%, specificity of 77%, PPV of 91%, NPV of 80% and an accuracy of 88%. Another estimated cut off strain ratio level of 3.8 had a higher sensitivity of 99% and NPV of 96%, but with less specificity, PPV and accuracy 53%, 84% and 86%, respectively. Adding both elastography to strain ratio resulted in a sensitivity of 98%, specificity of 77%, PPV of 91%, NPV of 95% and accuracy of 92% for the diagnosis of SPL.

CONCLUSION

Combining elastography to strain ratio increases the accuracy of the differentiation of benign from malignant SPL.

Key words: Endoscopic Ultrasound; Elastography; Strain Ratio; Real Time; Pancreatic lesions

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Core tip: This prospective study included 172 patients with solid pancreatic lesions (SPL) to evaluate the value of combining the elastography score to strain ratio for differentiating benign from malignant lesions. Adding both elastography to strain ratio resulted in a sensitivity of 98%, specificity of 77%, positive predictive value (PPV) of 91%, negative predictive value (NPV) of 95% and accuracy of 92% for the diagnosis of SPL. The best cut-off level of strain ratio was 7.8 with a sensitivity of 92%, specificity of 77%, PPV of 91%, NPV of 80% and an accuracy of 88%. So, adding both diagnostic tools increases the yielding of diagnosis.

Okasha H, Elkholy S, El-Sayed R, Wifi MN, El-Nady M, El-Nabawi W, El-Dayem WA, Radwan MI, Farag A, El-sherif Y, Al-Gemeie E, Salman A, El-Sherbiny M, El-Mazny A, Mahdy RE. Real time endoscopic ultrasound elastography and strain ratio in the diagnosis of solid pancreatic lesions. *World J Gastroenterol* 2017; 23(32): 5962-5968 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i32/5962.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i32.5962>

INTRODUCTION

Solid pancreatic lesions (SPL) are mostly malignant with 5-year survival rates of less than 5%^[1]. Endoscopic ultrasound guided fine needle aspiration (EUS-FNA) is a very good tool for the diagnosis of malignant SPL, with sensitivity and specificity rates of 91% and 94%, respectively^[2,3], but it shows false negative results in approximately 15% of cases^[4]. Strict follow up for negative FNA lesions is mandatory and may necessitate the use of invasive techniques to reach a full diagnosis, such as diagnostic laparoscopy^[5].

The elastic properties of the tissues were used to assist in diagnosis by comparing color images in the B mode before and after compression^[6,7]. This was used in endosonography to calculate the elastography of the lesion without using other invasive techniques^[8,9]. A 5-scored system was developed by Giovannini *et al*^[10] and colleagues to distinguish between benign and malignant lesions, yet it was very subjective. Then, the strain ratio was developed as a semi quantitative method by dividing the area of interest by the normal tissue to improve objectivity and reach a better diagnosis^[11].

In this prospective study, we investigate the efficacy of endosonographic elastography and strain ratio for the differentiation of benign from malignant lesions.

MATERIALS AND METHODS

Patients

Patients with SPL identified by EUS were enrolled in this prospective study. It included patients that were referred to the endoscopy units of both Cairo and Zagazig University Hospitals for endosonographic evaluation. The inclusion criteria were as follows: patients with identified SPL from prior radiological imaging; patients with extrahepatic biliary obstruction showing negative imaging results and referred for EUS; and patients above 18 years old. The exclusion criteria included: patients who declined to participate in the study, patients with a contraindication to the procedure, such as patients unfit for propofol sedation or coagulopathy, and patients lost to follow up or in whom the final diagnosis could not be reached. The ethical committee approved the study protocol and informed consents were obtained from all patients prior to the procedure.

Methods

The study was designed as a prospective study to evaluate the efficacy of elastography and strain ratio in diagnosing SPL. Eligible patients who agreed to participate in the study were appointed to the endoscopy room on the day of the procedure for EUS examination under conscious sedation with IV propofol administration. An EUS examination was performed on all patients with a linear Echoendoscope

Table 1 Location of the Solid pancreatic Lesions

Location of pancreatic lesions	Number of cases = 172
Head of the pancreas	118
Uncinate process	7
Body of the pancreas	22
Tail of the pancreas	4
Diffuse involvement (pan-pancreatic lesion)	21

Table 2 Final diagnosis of solid pancreatic lesions

Nature of the lesion	Final diagnosis	Number of cases = 172
Benign lesions (49 cases)	Pancreatitis	49
	-Chronic pancreatitis	40
	-Autoimmune pancreatitis	9
Malignant lesions (123 cases)	Ductal adenocarcinoma	97
	Mucinous neoplasm	22
	Neuroendocrine tumors	2
	Lymphoma	1
	Metastasis	1

Pentax EG3830UT (HOYA Corporation, PENTAX Lifecare Division, Showanomori Technology Center, Tokyo, Japan) connected to a Hitachi EUB-7000 HV ultrasound unit (Hitachi Medical Systems, Tokyo, Japan). All examinations were performed by one endosonographer. For EUS-FNA biopsies, we used the Cook needle 22G (Echotip®; Wilson-Cook, Winston Salem, NC, United States). Elastography was applied to evaluate the SPL. Elastography is the sound wave technique to measure tissue deformation in response to compression. Theoretically, malignant lesions are harder than inflammatory ones. The hardness of the lesion is reflected by the degree of deformation represented by a color map (red-green-blue colors represent soft to hard tissue, respectively). Quantitative scores and strain ratios were determined during the procedure. EUS-FNA was performed after the elastography.

Qualitative score

"Elastic score" reported by Giovannini *et al.*^[10] was used. A score of 1 was defined as homogeneous soft tissue (green) and interpreted as normal tissue. A score of 2 was given to heterogeneous soft tissue (green, yellow, and red), and interpreted as fibrosis or inflammation as shown in Figure 1. A score of 3 represented mixed hard and soft tissues (mixed colors) or a honeycombed elastography pattern, interpreted as indeterminate for malignancy as shown in Figures 2 and 3. A score of 4 was given for hard (blue) lesions with a soft (green) central area, interpreted as malignant, hypervascularized lesions. Finally, a score of 5 represents predominantly hard (blue) lesions with dispersed heterogenic soft (green, red) areas, interpreted as advanced malignant lesions with necrotic areas as shown in Figure 4.

The semi quantitative score of elastography was

represented by the strain ratio method. Two areas were selected, area (A) representing the region of interest and area (B) representing the normal area. Area (B) was then divided by area (A). For pancreatic lesions with a homogeneous pattern of elasticity, area A was chosen from any region, but in heterogeneous regions, area A was chosen to cover as much heterogeneous area as possible. Both areas were manually selected by these criteria. The means of strain ratios were calculated and used as final results for each patient as shown in Figures 5 and 6. Subsequently, the best cut-off value was selected from the receiver operating characteristic (ROC) curve and was used for the calculation of diagnostic value. The best cut-off value of strain ratio was also combined with the results of elastography for the calculation of diagnostic value.

Statistical analysis

Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were calculated by comparing diagnoses made by elastography, strain ratio and final diagnoses.

The final diagnosis of the SPL was obtained from the positive cytopathological examination of aspirate taken by EUS-FNA, the excisional biopsy of surgically removed tumors, and the presence of metastases or the follow up of benign lesions for at least one year.

RESULTS

From January 2013 to April 2016, 172 patients with pancreatic lesions were enrolled in this study. There were 120 males and 52 females with mean age of 55.7 years. The site, final diagnosis of pancreatic lesions, and elastography score are presented in Tables 1-3.

Scores 1 and 2 were considered benign while scores 3 to 5 were considered malignant. Elastography alone had a sensitivity of 99%, specificity of 63%, PPV of 87%, NPV of 96%, and accuracy of 88% (Table 4).

The mean value of the strain ratio for benign lesions is 5.58 while the mean value for malignancy is 31.25; this difference was statistically significant at a p value of 0.01.

Based on the results of the ROC curve that was used for analysis, the best cut-off level of strain ratio to obtain the maximal area under the curve was 7.8 with a sensitivity of 92%, specificity of 77%, PPV of 91%, NPV of 80% and accuracy of 88%. Another cut off level of strain ratio was calculated at a level of 3.8 and demonstrated very high sensitivity (99%) and NPV (96%), but less specificity (53%), PPV (84%), and accuracy (86%). Adding elastography to strain ratio resulted in a sensitivity of 98%, specificity of 77%, PPV of 91%, NPV of 95% and accuracy of 92% for the diagnosis of SPL (Table 4).

DISCUSSION

The percentage of benign SPL in our study is 28%, which is similar to a study carried out by Pradernchai

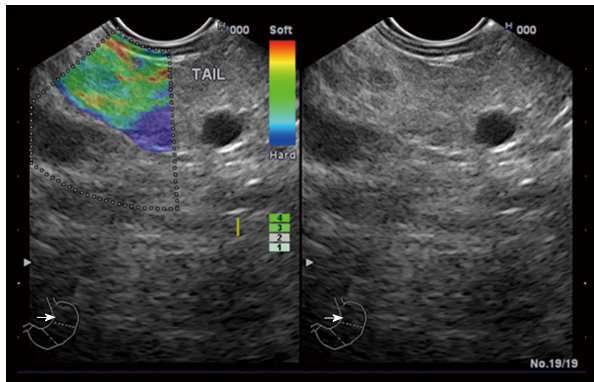


Figure 1 A patient with chronic pancreatitis showing heterogeneous soft tissue (green, yellow, and red), and interpreted as fibrosis or inflammation.

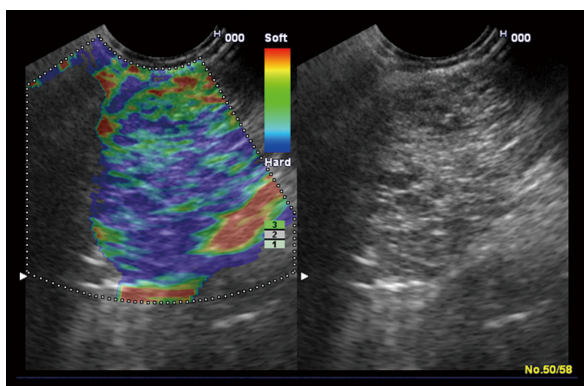


Figure 2 A patient with elasticity score 3 showing mixed hard and soft tissues (mixed colors) or a honeycombed elastography pattern, interpreted as indeterminate for malignancy.

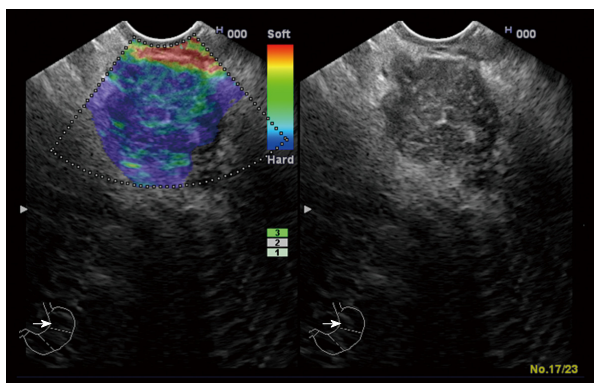


Figure 3 A patient with autoimmune pancreatitis showing elasticity score 3.

Kongkam and colleagues^[12] that reported a percentage of 23 and is similar to a meta-analysis that presented a close figure of 26.5%^[13].

The diagnostic value of EUS-FNA has always been questioned due to the high false negative rates encountered; these rates can reach up to 15%-17%^[2,5]. These false negative findings are manifested mostly in focal lesions in patients with chronic pancreatitis due to a similar hypoechoic pattern when compared to the

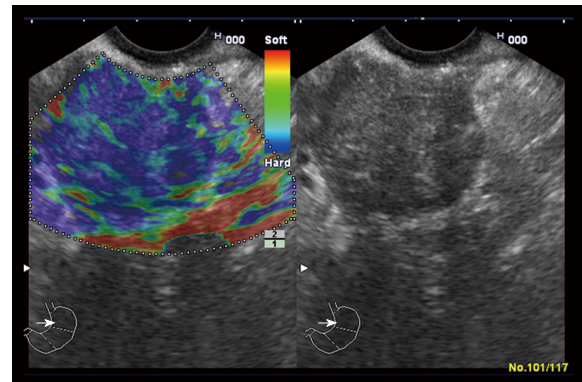


Figure 4 A patient with advanced malignant lesions with necrotic areas (elasticity score 5) showing predominantly hard (blue) lesion with dispersed heterogenic soft (green) areas.

Table 3 Qualitative analysis by elastography distribution

Diagnosis (n = 172)	Score 1	Score 2	Score 3	Score 4	Score 5
Pancreatitis	6	25	12	-	6
Chronic pancreatitis	6	21	8	-	5
Autoimmune pancreatitis	-	4	4	-	1
Ductal adenocarcinoma	-	-	28	-	69
Mucinous neoplasm	-	-	3	-	19
Neuroendocrine tumors	-	-	1	-	1
Lymphoma	-	-	1	-	-
Metastasis	-	-	-	-	1

Table 4 Diagnostic values of elastography and strain ratio

	Elasticity score	SR 7.8	SR 3.8	Elasticity score and SR 7.8
Sensitivity	99%	92%	99%	98%
Specificity	63%	77%	53%	77%
PPV	87%	91%	84%	91%
NPV	96%	80%	96%	95%
Accuracy	88%	88%	86%	92%

PPV: Positive predictive value; NPV: Negative predictive value.

surrounding area^[14].

EUS-FNA also has many drawbacks, including the need for multiple needle passes to obtain an adequate sample, iatrogenic complications^[15], a learning curve and the need to evaluate many cases to obtain better efficacy.

These drawbacks raised the need to develop other techniques for the diagnosis of SPL with fewer complications and better efficacy. Dawwas and colleagues reported a sensitivity of 100% for EUS elastography but with a very low specificity of 16.7%^[16]. This was in contrast to previous published studies^[17,18] and was not in concordance with our study that showed a specificity of 63%. Still, a problem appeared when using the elastic score due to its subjectivity. In our study, 36.7% (18/49) of patients with chronic pancreatitis had scores of 3 and 5 which is supposed to indicate malignancy. This may be

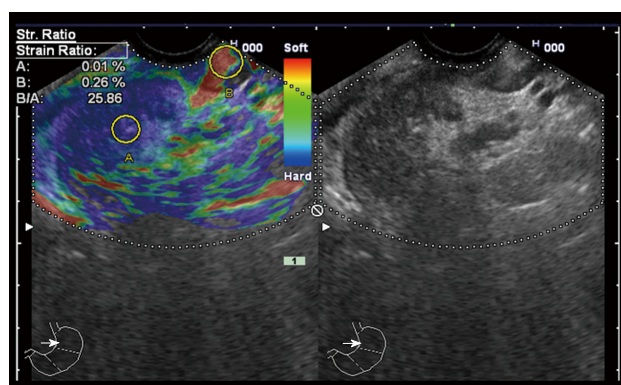


Figure 5 A patient with pancreatic head malignancy showing high strain ratio (25.86).

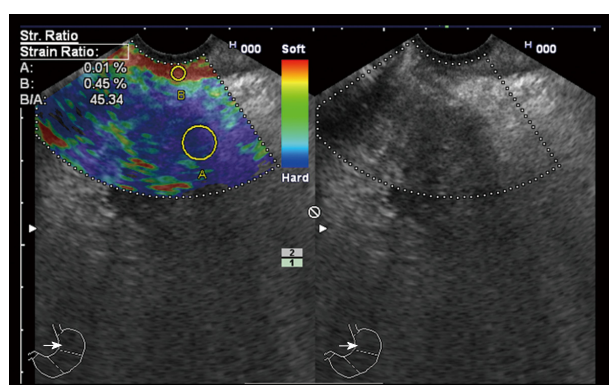


Figure 6 A patient with pancreatic head malignancy showing very high strain ratio (45.34).

attributed to the presence of calcifications and fibrous strands, which increases the score. Additionally, 6 patients out of 40 with chronic pancreatitis scored 1 although this score is supposed to reflect normal pancreatic tissue. Considering that chronic pancreatitis is a well-known and established risk factor for the development of pancreatic cancer^[19], SPL in patients with chronic pancreatitis is a worrisome feature that may indicate the development of malignancy on top of a chronic inflammatory condition. In a study of 373 patients with chronic pancreatitis, 4 of them developed pancreatic malignancy after a follow up period of 2 years^[20]. Fifty percent of neuroendocrine tumors scored 2 instead of 4 and 71% of ductal adenocarcinomas had scores of 4 instead of 3 according to the scale. This was similar to a study published by Itokawa and colleagues in which only 33% of neuroendocrine tumors were scored 4 and 22% had a score of 1^[9]. In our study, the 2 cases with neuroendocrine tumors were scored as 3 and 5 and not 4, which may explain why none of our cases had an elasticity score of 4. In a study done by Giovannini *et al*^[21]. Sixteen point one of the lesions that had scores of 1 or 2 were adenocarcinoma. This renders elastography less specific although it has high sensitivity in our study sensitivity was 99% despite low specificity (63%).

As an elastography score is a very subjective tool and depends on the operator in most of the cases, another tool was added to increase its specificity to reach a better diagnosis^[22-24]. The strain ratio with different cut off levels was mentioned in many studies^[16,17,21]. We had a cut off level of 3.8 that had a sensitivity, specificity, PPV, NPV and accuracy of 99%, 53%, 84%, 86% and 96%, respectively. This was similar to the study done by Pradermchai Kongkam and colleagues^[12] that identified a cut off value of 3.17 that gave a better specificity of 66.7%, but lower values in sensitivity, PPV, NPV, and accuracy 86.2%, 89.3%, 60%, and 81.6%, respectively. In our study, the best cut off value to differentiate benign from malignant SPL was 7.8, it has a sensitivity of 92%, specificity of 77%, PPV of 91%, NPV of 80% and accuracy of 88%.

Other studies have analyzed the usefulness of quantitative EUS-elastography. Iglesias-Garcia *et al*^[25] published the strain ratio results of 86 consecutive patients with pancreatic solid lesions (49 adenocarcinomas, 27 inflammatory masses, 6 malignant neuroendocrine tumors, 2 metastatic oat cell lung cancers, 1 pancreatic lymphoma, and 1 pancreatic solid pseudopapillary tumor) and 20 controls. The strain ratio was significantly higher among patients with malignant pancreatic tumors than those with inflammatory masses. Normal pancreatic tissue showed a mean strain ratio of 1.68 (95%CI: 1.59-1.78). Inflammatory masses exhibited a strain ratio (mean 3.28; 95%CI: 2.61-3.96) that was significantly higher than that of the normal pancreas ($P < 0.001$), but lower than that of pancreatic adenocarcinoma (mean 18.12; 95%CI: 16.03-20.21) ($P < 0.001$). The highest strain ratio was found among endocrine tumors (mean 52.34; 95%CI: 33.96-70.71). The sensitivity and specificity of the strain ratio for the detection of pancreatic malignancies with a cut-off value of 6.04 were 100% and 92.9%, respectively, exceeding the accuracy obtained with qualitative elastography. Another publication retrospectively evaluated 109 patients with solid pancreatic masses using the same methodology. A total of 20 patients were diagnosed with chronic pancreatitis (6 without and 7 with focal inflammatory masses, and 7 with autoimmune pancreatitis), 72 were diagnosis with pancreatic cancer, 9 with pancreatic neuroendocrine tumors, and 8 with a normal pancreas. In the qualitative evaluation, all pancreatic cancers showed an intense blue coloration, whereas the inflammatory masses presented mixed colorations (green, yellow, and low-intensity blue). The mean strain ratio was 23.66 ± 12.65 for the inflammatory masses and 39.08 ± 20.54 for pancreatic cancer ($P < 0.05$)^[9].

To increase the efficacy of the diagnosis of SPL, we combined elastography with the strain ratio level of 7.8 to have a sensitivity of 98%, a specificity of 77%, an accuracy of 92%, a PPV of 91% and an NPV of 95%

and increased the accuracy compared to the use of each tool alone.

SPL should be investigated thoroughly to identify their type. The use of elastography combined with strain ratio increases the accuracy of differentiation between malignant and benign SPL.

COMMENTS

Background

Different real time elasticity scores were developed to distinguish between benign and malignant lesions, yet they are very subjective, which is an important drawback. Strain ratio is a semi-quantitative method developed by dividing the area of interest by the normal tissue to improve objectivity and reach a better diagnosis.

Research frontiers

Accurate diagnosis of the nature of pancreatic masses aids a lot in the proper management. In this study, there is a suggestion that adding strain ratio to elastography increase the accuracy of diagnosis.

Innovations and breakthroughs

The literature suggests that adding strain ratio to elastography score would add to proper diagnosis and differentiation of pancreatic masses. This study suggests a new cut off value for strain ratio to differentiate between benign and malignant pancreatic lesions being 7.8.

Applications

The study adds additional evidence of using two non-invasive techniques being elastography score and strain ratio for diagnosis solid pancreatic masses.

Terminology

Strain ratio: a quantitative method for proper diagnosing of the nature of lesions, calculated by dividing the area of interest by the normal tissue.

Peer-review

The authors have performed a good study, the manuscript is interesting.

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Prospective Study

Efficacy and safety of sofosbuvir and daclatasvir in treatment of kidney transplantation recipients with hepatitis C virus infection

Yan Xue, Li-Xin Zhang, Lei Wang, Tao Li, Yun-Dong Qu, Feng Liu

Yan Xue, Li-Xin Zhang, Lei Wang, Tao Li, Yun-Dong Qu, Feng Liu, Department of Infectious Diseases and Hepatology, the Second Hospital of Shandong University, Jinan 250033, Shandong Province, China

ORCID number: Yan Xue (0000-0001-6185-2424); Li-Xin Zhang (0000-0001-9795-6487); Lei Wang (0000-0002-8240-9795); Tao Li (0000-0002-2548-5476); Yun-Dong Qu (0000-0003-4119-4422); Feng Liu (0000-0001-7060-3710).

Author contributions: Xue Y and Zhang LX contributed equally to this work; Wang L, Li T, Qu YD and Liu F conceived the study; Wang L, Xue Y and Zhang LX designed the research; Wang L, Xue Y and Zhang LX collected the data; Xue Y, Zhang LX and Qu YD analyzed the data; Xue Y wrote the article; Xue Y, Zhang LX and Li T revised the manuscript for final submission; Wang L participated in the study supervision.

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Institutional review board statement: The study protocol was approved by the Ethical Committee of the Second Hospital of Shandong University.

Informed consent statement: Written informed consent was obtained from all patients.

Conflict-of-interest statement: All authors have no conflicts of interest.

Data sharing statement: There are no additional data available in relation to this manuscript.

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Correspondence to: Lei Wang, PhD, MD, Professor, Department of Infectious Diseases and Hepatology, the Second Hospital of Shandong University, 247 Beiyuan Road, Jinan 250033, Shandong Province, China. wlcbr@sdu.edu.cn
Telephone: +86-5318-5875122

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Abstract

AIM

To assess the efficacy and safety of sofosbuvir and daclatasvir regimens for kidney transplantation (KT) patients with hepatitis C virus (HCV) infection.

METHODS

This study enrolled a prospective cohort of consecutive Chinese KT patients with HCV infection. They were given sofosbuvir combined with daclatasvir, with or without ribavirin. They were monitored regularly during and after the treatment.

RESULTS

Six patients were recruited in our prospective study cohort. All patients were male and naive to direct-acting antiviral treatment. The treatment duration was

12 wk. Most patients (4/6) were infected with HCV genotype 1b. HCV RNA was undetectable at week 4 after treatment and at the end of treatment in all patients. Sustained virological response rate at 12 wk was 100% (6/6). Two patients had to accept a half dose of sofosbuvir due to serum creatinine elevation during treatment. Kidney function in the remaining patients was stable. No serious adverse events (AEs) were observed. No patient discontinued antiviral therapy due to side effects.

CONCLUSION

Sofosbuvir and daclatasvir for treatment of KT recipients with HCV infection are highly efficient and safe. Patients tolerated the medications well, and no serious AEs were observed. Larger prospective cohort studies are needed to validate these results.

Key words: Hepatitis C virus; Sofosbuvir; Daclatasvir; Kidney transplantation; Direct-acting antivirals

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Core tip: This is a prospective study to assess the efficacy and safety of sofosbuvir and daclatasvir regimens for kidney transplantation (KT) patients with hepatitis C virus (HCV) infection. This study enrolled a prospective cohort of consecutive Chinese KT patients with HCV infection. The recipients were given sofosbuvir combined with daclatasvir with or without ribavirin. Sofosbuvir and daclatasvir treatments are highly efficient and safe. Patients tolerated the regimens and no serious adverse events were observed. Larger prospective cohort studies are needed to validate these results.

Xue Y, Zhang LX, Wang L, Li T, Qu YD, Liu F. Efficacy and safety of sofosbuvir and daclatasvir in treatment of kidney transplantation recipients with hepatitis C virus infection. *World J Gastroenterol* 2017; 23(32): 5969-5976 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i32/5969.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i32.5969>

INTRODUCTION

Hepatitis C virus (HCV) infection is common in kidney transplantation (KT) recipients. The worldwide prevalence of HCV infection in patients on hemodialysis is 13.5%, compared with 3% in the general population^[1]. The prevalence is significantly higher in patients with KT than in the general population^[2]. HCV infection in KT recipients increases the risk of graft loss, liver fibrosis, hepatocellular carcinoma, and death^[3,4]. To date, data on the efficacy and safety of direct-acting antivirals (DAAs) in the treatment of KT patients with HCV infection have been limited. New-generation

DAAs [e.g., NS5B inhibitor sofosbuvir (SOF) combined with NS5A inhibitor daclatasvir (DCV), with or without ribavirin (RBV)] have been shown to be highly efficient in treating HCV infection in cirrhotic and non-cirrhotic immunocompetent patients^[5]. SOF revolutionized the treatment of HCV infection, leading to high rates of sustained virological response (SVR) with few side effects^[6]. However, the use of SOF is restricted to patients with an estimated glomerular filtration rate (eGFR) ≥ 30 mL/min per 1.73 m², as it has not been studied in patients with an eGFR < 30 mL/min per 1.73 m². In other words, these limitations are not based on current clinical data. GS331007, the active metabolite of SOF, is eliminated by the kidney. Levels of SOF and GS331007 are substantially higher in patients with severe renal impairment (eGFR < 30 mL/min per 1.73 m²)^[7]. Premarket animal testing has raised concerns for cardiovascular and hepatobiliary toxicity at higher levels of SOF dosing, but toxicity of the drug and metabolite levels in humans remains unknown^[7]. DCV has been recommended for treatment of patients with severe renal disease, as its components are metabolized mainly by the liver. Currently, few data on the treatment of patients post KT are available so far. The aim of this pilot study was to assess the efficacy and safety of SOF combined with DCV for HCV RNA-positive KT patients.

MATERIALS AND METHODS

Patients and study design

This study enrolled a prospective cohort of consecutive Chinese KT patients with HCV infection from March to September 2016. They were given SOF combined with DCV, with or without RBV therapy at the Department of Infectious Diseases and Hepatology, the Second Hospital of Shandong University, Jinan, China. Written informed consent was obtained from all patients, and the study protocol was approved by the Ethical Committee of the Second Hospital of Shandong University. All patients were non-cirrhotic [diagnosed by either ultrasonography, CT or determination of liver stiffness (FibroScan; cut-off for cirrhosis: 12.5 kPa)]. They were all naive to treatment, and their baseline eGFR was above 30 mL/min per 1.73 m². All patients received therapy for 12 wk. Patients with coexisting hepatitis B virus infection, human immunodeficiency virus infection, alcoholism, autoimmune hepatitis, or malignancy were excluded. Clinical assessment, conventional liver and kidney biochemistry parameters, serum HCV RNA, as well as the types of immunosuppressive drugs and their doses were assessed routinely as follows: at the beginning of treatment; 2, 4 and 12 wk post treatment; at the end of treatment (EOT); and at 12 wk after therapy was completed. Prothrombin time, alpha-fetoprotein and abdominal ultrasonography were tested when necessary.

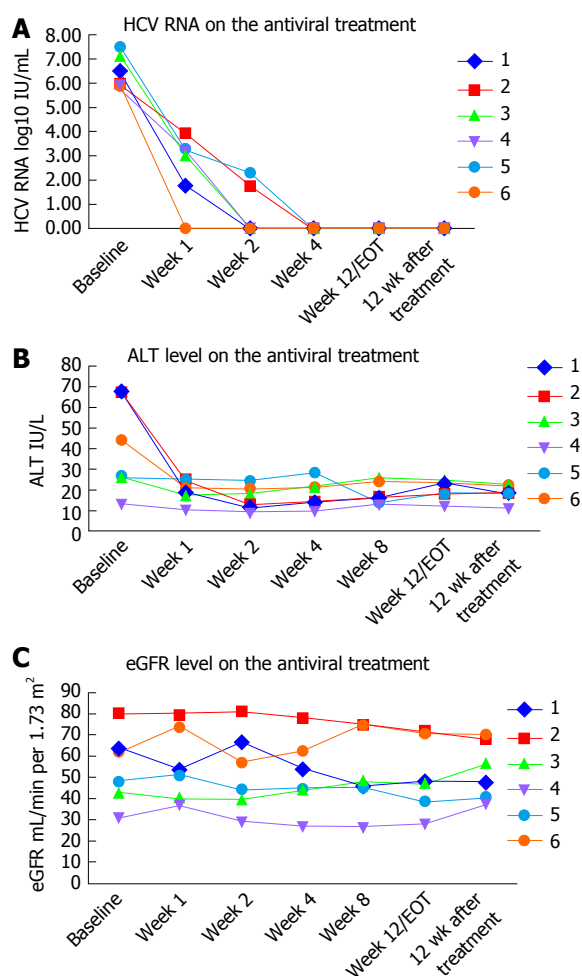


Figure 1 Outcomes of hepatitis C virus RNA (A), alanine amino transferase levels (B) and estimated glomerular filtration rates (C) at different treatment time points in the six patients. EOT: End of therapy; ALT: Alanine aminotransferase; eGFR: Estimated glomerular filtration rate; HCV: Hepatitis C virus.

Biochemical response was identified as normalization of transaminases. Virological response was identified as rapid virological response (RVR, negative HCV RNA at 4 wk on treatment) and SVR (SVR12, negative HCV RNA at 12 wk after EOT).

Adverse events (AEs) were surveilled during the treatment period.

Biochemistry and laboratory methods

Liver and kidney biochemical parameters were tested with a Beckman UniCel DXC 800 Chemistry Analyzer (Beckman Coulter, Fullerton, CA, United States). HCV RNA was detected by quantitative real-time PCR assay using the Cobas Taqman HCV test v 2-(LLOQ 15 IU/mL). Serum anti-HCV antibodies were measured by enzyme-linked immunosorbent assay with a diagnostic kit for HCV (Zhuhai Livzon Diagnostics Inc, China). HCV genotypes were determined by direct sequencing of amplicons of the HCV gene using PCR. The eGFR was calculated based on the serum creatinine measurement prior to the initiation of treatment using the Chronic Kidney Disease Epidemiology Collaboration

formula^[8].

Statistical analysis

Statistical analyses were performed using SPSS 17.0 (SPSS, Chicago, IL, United States). HCV RNA levels were logarithmically transformed for further analysis. Continuous variables are expressed as either mean \pm SD or as median and range. Frequencies were used for categorical variables. Quantitative variables were compared using the *t* test or the Mann-Whitney *U* test for variables according to different characteristics of distribution when necessary. Categorical data were compared using the Pearson χ^2 test or Fisher's exact test when necessary. *P* < 0.05 (two-tailed) was considered statistically significant.

RESULTS

Baseline patient characteristics

Our study cohort included a total of six Chinese KT recipients with HCV infection. One of them (1/6) had received two kidney transplants. All six patients were male and their mean age was 45.3 (40-49) years. None had cirrhosis. They were infected with HCV genotype 1 (4/6 GT1b), genotype 3 (1/6 GT3a) and genotype 6 (1/6 GT6a). Viral load was measured with a range between 0.514 and 29.5 million IU/mL. eGFR was > 30 mL/min per 1.73 m² at the beginning of treatment. They were all naive to treatment, and all received 12 wk of therapy. Treatment started at 400 mg of SOF and 60 mg of daclatasvir daily in all patients. Dosage of SOF was adjusted to 200 mg daily in two patients due to elevated serum creatinine levels, one on day 2 of treatment, and the other on day 15. One of the patients received RBV (weight-based) in addition to 400 mg of SOF and 60 mg of daclatasvir. The others were treated without RBV. All patients suffered from hypertension. Their antihypertensive medications were switched from calcium channel blockers to angiotensin receptor antagonists or angiotensin converting enzyme inhibitors. The baseline clinical characteristics of the six patients are summarized in Table 1.

Virological response

All six patients completed antiviral treatment, and were followed for at least 12 wk post treatment. There were no discontinuations of therapy and none were lost to follow-up. All six patients achieved RVR, of whom four had undetectable viral load by week 2 of treatment. All patients had undetectable HCV viral load at the end of treatment. SVR12 rate was achieved in 100% (6/6) of the recipients. Timelines of virological responses are depicted in Figure 1A.

Liver biochemistry parameters and other laboratory values

Serum alanine aminotransferase (ALT), aspartate aminotransferase and γ -glutamyl transferase levels

Table 1 Baseline clinical characteristics of patients after kidney transplantation treated with sofosbuvir and daclatasvir

Patient number	1	2	3	4	5	6
Age/gender	47/M	40/M	40/M	48/M	48/M	49/M
Pre-treatment serum HCV viral load (IU/mL)	3.2E+6	8.6E+5	1.4E+7	5.14E+5	2.95E+7	8.08E+5
HCV genotype	1b	1b	3a	1b	6a	1b
Anti-HCV	Negative	Positive	Positive	Positive	Negative	Positive
Cirrhosis	No	No	No	No	No	No
Number of kidney transplantations	Two	One	One	One	One	One
Prior antiviral therapy	No	No	No	No	No	No
Baseline serum Cr ($\mu\text{mol/L}$) ($n = 53\text{--}115$)	75.5	84.2	146.6	175.6	128	89.3
Baseline eGFR (mL/min)	63.63	80.04	42.86	30.94	48.29	61.85
Baseline Hgb (g/dL)	113	136	121	111	167	137
Baseline ALT (IU/L)	68	67	26	13	26	44
Baseline AST (IU/L)	161	143	42	27	42	54
Baseline $\gamma\text{-GT}$ (IU/L)	162	621	55	25	60	54
Baseline TB ($\mu\text{mol/L}$)	30	29.2	9.6	8	18.7	12.5
Baseline (Hb g/L)	113	136	121	94	167	137
Complication	Hypertension	Hypertension	Hypertension	Hypertension	Hypertension/ Diabetes	Hypertension
Antiviral regimen	Sofosbuvir 400 mg daily + daclatasvir 60 mg daily	Sofosbuvir 400 mg daily + daclatasvir 60 mg daily	Sofosbuvir 400 mg daily + daclatasvir 60 mg daily + ribavirin 0.6 g daily	Sofosbuvir 400 mg daily + daclatasvir 60 mg daily	Sofosbuvir 400 mg daily + daclatasvir 60 mg daily	Sofosbuvir 400 mg daily + daclatasvir 60 mg daily
Treatment duration (wk)	12	12	12	12	12	12
Baseline immunosuppressive regimen	Mycophenolatemofetil 500 mg bid	Mycophenolate mofetil 540 mg bid	Mycophenolate mofetil 540 mg bid	Mycophenolate mofetil 750 mg bid	Cyclosporin A 75 mg bid	Mycophenolate mofetil 720 mg bid
	Tacrolimus (FK506) 0.5 mg bid	Tacrolimus (FK506) 1.5 mg bid	Tacrolimus (FK506) 2 mg bid	Tacrolimus 2 mg bid	Mycophenolate mofetil 540 mg bid	Tacrolimus (FK506) 0.5 mg bid
	Methylprednisolone 4 mg qd	Prednisone 5 mg qd	Prednisone 5 mg qd	Prednisone 5 mg qd	Prednisone 5 mg qd	Methylprednisolone 4 mg qd
Baseline anti-hypertension regimen	Metoprolol 12.5 mg qd	Benazepril 10 mg qd	Benazepril 10 mg bid	Valsartan 80 mg bid	Irbesartan 150 mg bid	None
		Valsartan 80 mg qd				
Other regimens	Benzbromarone tablets 12.5 mg bid			Recombinant human erythropoietin injection 10000 U, IH, biw		

Cr: Creatinine; eGFR: Estimated glomerular filtration rate; HCV: Hepatitis C virus; HD: Hemodialysis; Hb: Hemoglobin; IU: International units; MMF: Mycophenolate mofetil; MPGN: Membranoproliferative glomerulonephritis; N/A: Not applicable; IH: Hypodermic injection; qd: Once daily; bid: Twice daily; biw: Twice weekly.

significantly improved with the antiviral treatment, especially during the initial two weeks. Hemoglobin levels were stable during treatment. Timelines of the ALT values are shown in Figure 1B.

AEs

No serious AEs were reported during the treatment process. Common AEs included fatigue (1/6), diarrhea (1/6), tinnitus (1/6), abdominal discomfort (1/6), discomfort of transplanted kidney region (1/6), refractory hypertension (1/6), elevation of serum creatinine (2/6), and unstable blood pressure (3/6). Patient 6 suffered from fatigue and diarrhea one week after therapy, but he continued DAA treatment, and his diarrhea gradually ameliorated. Patient 4

suffered from fatigue 40 d after therapy began, along with tinnitus, and discomfort of the abdomen and transplanted kidney regions. However, symptoms spontaneously disappeared during subsequent treatment. Antihypertensive medication was modified in patients 2, 3 and 5 to avoid drug interactions with DCV, causing unstable blood pressure during the first two weeks of treatment. No patient had renal transplant complications related to antiviral treatment, and there were no kidney rejection episodes. Antiviral therapy was not discontinued due to side effects in any patient. Patient 4 had to accept a half-dose SOF on the second day of treatment because his serum creatinine level increased from 175.6 $\mu\text{mol/L}$ to 209 $\mu\text{mol/L}$, and his eGFR fell to less than 30 mL/min per 1.73 m^2 .

Table 2 Adverse events reported

	Event	Patients
Any adverse event leading to discontinuation	-	0
Serious adverse events	Gastrointestinal bleeding/Portal vein thrombosis and Streptococcus bacteremia/Sinus bradycardia and first degree A-V block with syncope	0
Common adverse events	Fatigue	2
	Diarrhea	1
	Tinnitus	1
	Elevation in serum creatinine	2
	Discomfort of abdomen	1
	Discomfort of transplanted kidney region	1
	Unstable blood pressure	3
	Rash	0
	Insomnia	0
	Headache	0

His serum creatinine levels remained stable for the rest of the course. Patient 5 was hospitalized twice for elevated serum creatinine levels in the process of treatment, and the dose of SOF was reduced to 200 mg daily starting in the third week. Kidney function remained stable in the remaining patients, including patient 3, whose baseline serum creatinine level was 146.6 $\mu\text{mol/L}$. Kidney function remained stable in the remaining patients. Other AEs, such as nausea, headache and myalgia/arthritis, were not reported. Table 2 displays all AEs reported. eGFR values are shown in Figure 1C.

Immunosuppression

All six patients were on immunosuppressive agents. Five patients received tacrolimus and mycophenolate mofetil. The remaining patient received cyclosporine and mycophenolate mofetil (Table 3). Other agents included prednisone or methylprednisolone, and azathioprine. During antiviral treatment, blood concentrations of tacrolimus, mycophenolate mofetil, and cyclosporine varied within goal trough levels according to the risk of immune rejection. Immunosuppressive agents were adjusted in three patients. The other patients' blood concentrations of immunosuppressive agents remained stable throughout the course of antiviral therapy. All dose adjustments of immunosuppressive agents during treatment are displayed in Table 3.

DISCUSSION

With a global prevalence rate of approximately 3%, affecting over 170 million individuals, chronic HCV infection is a leading cause of chronic liver disease and hepatocellular carcinoma worldwide^[9]. Importantly, for patients with KT, HCV infection is associated with an increased rate of liver fibrosis, graft loss, hepato-

cellular carcinoma and death^[10-12]. To date, treatment recommendations for patients with HCV infection of American Association for the Study of Liver Diseases and European Association for Study of Liver have been modified four times. With these recommendations, new IFN-free DAA therapy may prove to be the treatment of choice.

SOF is an inhibitor of the NS5B polymerase. As a nucleotide analogue, it causes chain termination during the replication of viral genomic RNA. SOF has a pan-genotypic activity and a high resistance barrier. It may only be given to patients with a glomerular filtration rate above 30 mL/min per 1.73 m² due to its renal elimination.

Daclatasvir is an NS5A inhibitor that has high antiviral activity against genotypes 1 to 4 both *in vivo* and *in vitro*, and is also active against genotypes 5 and 6. DCV does not require renal dose adjustment and thus provides a promising option for the "difficult-to-treat" cohort.

The combination of SOF + DCV \pm RBV has been investigated in treatment-naïve patients with genotype 1, 2 and 3 without cirrhosis in several clinical studies. The results showed high SVR rates between 93%-100% regardless of treatment duration and addition of RBV. The ALLY-1 study investigated SOF + DCV + RBV for 12 wk in patients with cirrhosis ($n = 60$). For genotype 1, patients with cirrhosis achieved an SVR rate of 82%^[13]. Initially, only a 24-wk treatment was evaluated in genotypes 2 and 3 patients. The SVR rates were 92% in genotype 2 and 89% in genotype 3^[14]. ALLY-3 study genotype 3 patients were treated with SOF + DCV for 12 wk without RBV. Naïve patients without cirrhosis achieved a high SVR rate of 97%, while in case of cirrhosis, the SVR rate was lower (only 58%)^[15]. However, the efficacy and safety of DAAs, especially the combination of SOF + DCV \pm RBV, in patients post KT are rarely reported^[16].

In our study, all KT patients with HCV infection who received SOF and DCV regimens achieved RVR, EOT virological response (HCV RNA were undetectable at the EOT) and SVR12. Liver biochemistry parameters ameliorated significantly during antiviral treatment. The RVR rate and SVR12 rate of this group differed from those in several similar studies. For KT patients with HCV infection, Lin *et al*^[17] reported an overall SVR12 rate of 91% (21/23). Patients in their study were given SOF plus simeprevir, with or without RBV, SOF plus ledipasvir, with or without RBV, or SOF plus RBV. Only two patients who relapsed post treatment had traditionally unfavorable treatment profiles. The two patients were African-American, had genotype 1a infection, high pre-treatment HCV viral load, and underlying advanced liver disease/cirrhosis, were previously treatment-experienced with interferon and RBV, and did not achieve RVR^[17]. Kamar *et al*^[18] reported an RVR rate of 88% (22/25) in their study, and their SVR12 rate was 100% (25/25). The three

Table 3 Adjustment of immunosuppression regimen in patients after renal transplantation treated with sofosbuvir and daclatasvir

	KT patient 1	KT patient 2	KT patient 3	KT patient 4	KT patient 5	KT patient 6
Baseline immunosuppressive regimen	Prednisone 5 mg qd Mycophenolate mofetil 1.5 g bid	Prednisone 5 mg qd Mycophenolate mofetil 540 mg bid	Prednisone 5 mg qd Mycophenolate mofetil 540 mg bid	Methylprednisolone 4 mg qd Mycophenolate mofetil 750 mg bid	Prednisone 5 mg qd Mycophenolate mofetil 540 mg bid	Methylprednisolone 4 mg qd Mycophenolate mofetil 720 mg bid
1 st adjustment	Tacrolimus (FK506) 0.5 mg bid FK506 0.5 mg qd + 1 mg qn (3 wk after the treatment for his blood drug concentration of FK506 was 3.7 ng/mL).	Tacrolimus (FK506) 1.5 mg bid No	Tacrolimus (FK506) 2 mg bid No	Tacrolimus (FK506) 2 mg bid Mycophenolate mofetil 1000 mg bid FK506 3 mg bid (11 wk after the treatment for his blood drug concentration of FK506 was 5.4 ng/mL).	Cyclosporine 75 mg bid Cyclosporine 75 mg qd + 50 mg qn (5 d after the treatment for his blood drug concentration of cyclosporine rose to 277.9 ng/mL).	Tacrolimus (FK506) 0.5 mg bid No
2 nd adjustment	FK506 4 mg bid (8 wk after the treatment for his blood drug concentration of FK506 was 6.4 ng/mL).	No	No	No	Mycophenolate mofetil 720 mg bid (45 d after the treatment was begun).	No
3 rd adjustment	Mycophenolate mofetil 1000 mg bid (one month after the treatment)	No	No	No	No	No

patients who did not achieve RVA had an METAVIR fibrosis score of F2. Two of the three patients were infected with HCV genotype 1b and were given SOF + simeprevir + RBV or SOF + ledipasvir without RBV. The third patient was infected with genotype 4 and treated with SOF + ledipasvir. The combination of SOF + DCV ± RBV in our study showed higher RVR and SVR12 rates than other studies. Possible reasons for this include: (1) our patients are all Asian; (2) they were all given SOF combined with DCV with or without RBV; (3) all patients were non-cirrhotic; (4) they were all naive to treatment; (5) they were younger than those in other studies; and (6) they were all compliant.

To date, data regarding efficacy and safety of DAAs in the treatment of KT patients with HCV infection have been limited. Nazario *et al.*^[19] reported few AEs with full dose SOF and simeprevir in patients with end-stage renal diseases, although only 11/17 patients completed the 12-wk post-treatment follow-up. Beinhardt *et al.*^[20] reported some serious AEs, such as photosensitivity/sunburn, spontaneous bacterial peritonitis, hemolytic anemia, re-listing for transplantation due to graft failure, as well as common AEs, such as headache and myalgia/arthritis. We did not observe these in our study.

The majority of patients in our study tolerated SOF and DCV regimens well. Some of the more serious AEs may be related to the disease itself. Hemoglobin levels were stable during treatment, consistent with the results of other reports^[18]. Notably, 67% (4/6) of our patients tolerated full-dose SOF well. Only two patients, whose baseline serum creatinine levels were higher than normal, received half-dose SOF. Three patients suffered from unstable blood pressure during

the first two weeks, as reported in another study^[20]. Common AEs including fatigue, diarrhea, tinnitus, discomfort of abdomen and transplanted kidney region, refractory hypertension, and elevation in serum creatinine levels were reported during treatment, just as reported in another study^[20].

Dabbous *et al.*^[21] reported one recipient death one week following treatment inception, due to unresolved hepatic encephalopathy. Some AEs, such as gastrointestinal bleeding, portal vein thrombosis and *Streptococcus* bacteremia, sinus bradycardia and first degree A-V block with syncope, shortness of breath, gout flare, headache, dizziness, pain in the lower extremity, photosensitivity, rash, and insomnia, were reported by Lin *et al.*^[17].

Anti-HCV assay by enzyme immunoassay (EIA) technique is the most common screening tool for HCV infection due to its simplicity, availability and low cost. The second generation EIA (EIA-2) assay was frequently associated with false negative results in patients with end stage renal diseases on dialysis, with a reported rate of 2.6%-7%^[15,20]. In our cohort, two patients were false negative for anti-HCV. Therefore, we recommend that HCV RNA should be determined in patients following KD.

The main limitation of our study is its small sample size. However, in the current clinical environment of increased need for treatment in the "difficult-to-treat" group, we believe that our study results offer proof of concept and feasibility data for future larger studies.

In conclusion, even though HCV patients with KT are considered "difficult-to-treat", SOF plus daclatasvir is an attractive therapeutic option. The regimens appear to be safe, well-tolerated and efficacious,

resulting in high rates of SVR for up to 12 wk following completion of treatment. The optimal dose of SOF should be adjusted according to the creatinine clearance rate and eGFR. With these adjustments, even patients with elevated baseline serum creatinine levels can achieve satisfactory results.

COMMENTS

Background

Hepatitis C virus (HCV) infection is common in kidney transplantation (KT) recipients. HCV infection in KT recipients increases the risk of graft loss, liver fibrosis, hepatocellular carcinoma and death. To date, there are limited data regarding the efficacy and safety of direct-acting antiviral regimens (DAAs) in the treatment of KT patients with HCV infection. New-generation DAAs [*i.e.*, sofosbuvir (SOF) combined with daclatasvir (DCV), with or without ribavirin (RBV)] have been shown to be highly efficient in treating HCV infection in cirrhotic and non-cirrhotic immunocompetent patients. The study was designed to assess the efficacy and safety of SOF combined with DCV for HCV RNA-positive KT patients.

Research frontiers

HCV patients post KT are considered "difficult-to-treat". Outcomes of our study show that SOF plus DCV regimens appear to be safe, well-tolerated and efficacious, resulting in high rates of sustained virological response at 12 wk after treatment completion for these "difficult-to-treat" patients.

Innovations and breakthroughs

Sofosbuvir plus daclatasvir regimens are free of interferon. This study showed that the majority of patients after KT tolerated SOF and DCV regimens well. Thus, it is an attractive option to treat HCV patients after KT.

Applications

This study demonstrates a safe, well-tolerated, efficacious and attractive option to treat HCV patients after KT.

Peer-review

The authors have performed a good study, the study is well designed, and the results are interesting.

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Randomized Controlled Trial

New botanical drug, HL tablet, reduces hepatic fat as measured by magnetic resonance spectroscopy in patients with nonalcoholic fatty liver disease: A placebo-controlled, randomized, phase II trial

Jae Yoon Jeong, Joo Hyun Sohn, Yang Hyun Baek, Yong Kyun Cho, Yongsoo Kim, Hyeonjin Kim

Jae Yoon Jeong, Joo Hyun Sohn, Department of Internal Medicine, Hanyang University Guri Hospital, Hanyang University College of Medicine, Guri 11923, South Korea

Yang Hyun Baek, Department of Internal Medicine, Dong-A University Hospital, Dong-A University College of Medicine, Busan 49201, South Korea

Yong Kyun Cho, Department of Internal Medicine, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Seoul 03181, South Korea

Yongsoo Kim, Department of Radiology, Hanyang University Guri Hospital, Hanyang University College of Medicine, Guri 11923, South Korea

Hyeonjin Kim, Department of Radiology, Seoul National University Hospital, Seoul 03080, South Korea

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Correspondence to: Joo Hyun Sohn, MD, PhD, Professor of Medicine, Department of Internal Medicine, Hanyang University Guri Hospital, Hanyang University College of Medicine, 153 Gyeongchun-ro, Guri 11923, South Korea. sonjh@hanyang.ac.kr
Telephone: +82-31-5602225
Fax: +82-31-5552998

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Abstract

AIM

To evaluate the efficacy and safety of HL tablet extracted from *magnolia officinalis* for treating patients

with nonalcoholic fatty liver disease (NAFLD).

METHODS

Seventy-four patients with NAFLD diagnosed by ultrasonography were randomly assigned to 3 groups given high dose (400 mg) HL tablet, low dose (133.4 mg) HL tablet and placebo, respectively, daily for 12 wk. The primary endpoint was post-treatment change of hepatic fat content (HFC) measured by magnetic resonance spectroscopy. Secondary endpoints included changes of serum aspartate aminotransferase, alanine aminotransferase (ALT), cholesterol, triglyceride, free fatty acid, homeostasis model assessment-estimated insulin resistance, and body mass index (BMI).

RESULTS

The mean HFC of the high dose HL group, but not of the low dose group, declined significantly after 12 wk of treatment (high dose *vs* placebo, $P = 0.033$; low dose *vs* placebo, $P = 0.386$). The mean changes of HFC from baseline at week 12 were $-1.7\% \pm 3.1\%$ in the high dose group ($P = 0.018$), $-1.21\% \pm 4.97\%$ in the low dose group ($P = 0.254$) and $0.61\% \pm 3.87\%$ in the placebo group (relative changes compared to baseline, high dose were: $-12.1\% \pm 23.5\%$, low dose: $-3.2\% \pm 32.0\%$, and placebo: $7.6\% \pm 44.0\%$). Serum ALT levels also tended to decrease in the groups receiving HL tablet while other factors were unaffected. There were no moderate or severe treatment-related safety issues during the study.

CONCLUSION

HL tablet is effective in reducing HFC without any negative lipid profiles, BMI changes and adverse effects.

Key words: Botanical drug; Nonalcoholic fatty liver disease; Magnetic resonance spectroscopy; Randomized controlled trial; *Magnolia officinalis*

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Core tip: The prevalence of nonalcoholic fatty liver disease (NAFLD) is growing gradually with the increase in population age and obesity, while effective and safe drugs for NAFLD are not yet available. HL tablet, a new botanic drug extracted from *Magnolia officinalis*, is effective in reducing hepatic fat content without any negative lipid profiles, body mass index changes and adverse effects.

Jeong JY, Sohn JH, Baek YH, Cho YK, Kim Y, Kim H. New botanical drug, HL tablet, reduces hepatic fat as measured by magnetic resonance spectroscopy in patients with nonalcoholic fatty liver disease: A placebo-controlled, randomized, phase II trial. *World J Gastroenterol* 2017; 23(32): 5977-5985 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i32/5977.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i32.5977>

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease worldwide, and includes a spectrum of diseases from simple steatosis to nonalcoholic steatohepatitis (NASH), and liver cirrhosis^[1]. Some patients with NAFLD have NASH and some of these may progress to liver cirrhosis and develop hepatocellular carcinoma^[2]. Also, the prevalence of NAFLD is growing gradually with the increase in population age and obesity^[3,4]. Life-style change has been the most effective treatment for NAFLD^[5-7], while effective and safe drugs for NAFLD are not yet available^[1]. Therefore the demand for new drugs for NAFLD is increasing worldwide.

In NAFLD, visceral adipose tissue emits multiple signals that alter lipid and glucose metabolism, causing fat accumulation and a proinflammatory environment in the liver^[1]. These effects lead to injury to the liver and other tissues. Oxidative stress, lipotoxicity, and endoplasmic reticulum and mitochondrial apoptotic pathways contribute to liver damage and promote liver fibrosis^[1].

Magnolia officinalis (MO) is a traditional medicinal plant that has been used to treat liver diseases and other diseases^[8]. Some constituents extracted from MO have been reported to have anti-inflammatory and antioxidant effects^[9,10], and MO can be effective against NAFLD. *In vitro* honokiol, extracted from MO, induced apoptosis of the activated hepatic stellate cells responsible for hepatic fibrosis^[11]. Honokiol and magnolol extracted from MO significantly inhibited hepatic toxicity induced by galactosamine, generation of intracellular reactive oxygen species and glutathione depletion^[9]. Also, MO inhibited lipid accumulation in free fatty acid (FFA)-exposed hepatocytes^[12]. In another study, MO ameliorated body fat accumulation, insulin resistance, and adipose inflammation in high fat-fed mice^[13].

We hypothesized that MO would improve steatosis and inflammation in patients with NAFLD. HL tablet is a new botanical drug extracted from MO. The aim of this study was thus to evaluate the efficacy and safety of HL tablet in the treatment of patients with NAFLD.

MATERIALS AND METHODS

Study design and population

Our study was a multi-center, randomized allocation, double-blind, placebo controlled, 3 group parallel, phase II trial between November 2013 and May 2015 in 3 hospitals in the Republic of Korea. It was conducted in patients with NAFLD diagnosed by ultrasonographic examination. The inclusion criteria were (1) age 19-75 years; (2) ultrasonographic features of nonalcoholic fatty liver; and (3) serum alanine aminotransferase (ALT) or aspartate aminotransferase

(AST) above the upper normal limits. Exclusion criteria were (1) serum AST/ALT > 2; (2) type 1 diabetes; (3) other liver diseases (viral hepatitis, autoimmune hepatitis or biliary obstruction, *etc.*); (4) excessive alcohol consumption ≥ 30 g/d for men and ≥ 20 g/d for women; (5) use of steatogenic medications (amiodarone, methotrexate, systemic glucocorticoids, tetracycline or tamoxifen, *etc.*) within the past 3 mo; (6) serious underlying diseases (liver cirrhosis, malignancy, severe metabolic disease, serious kidney disease, serious cardiovascular disease or serious lung disease, *etc.*); (7) history of bariatric surgery within the past 6 mo; (8) contraindication for magnetic resonance spectroscopy (MRS), such as history of pacemaker implantation or shunt operation; and (9) pregnancy, breastfeeding or hypersensitivity to MO. This study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committee of each hospital, and was registered at ClinicalTrials.gov (NCT02491905).

After all the included patients had provided written informed consent, they were randomly assigned (1:1:1) to groups given high dose (400 mg) HL tablet, low dose (133.4 mg) HL tablet or placebo daily for 12 wk. Patients visited the clinic at baseline and after 4, 8, and 12 wk of treatment. At each visit, a physical examination was conducted, adverse drug events were recorded, and compliance with medication was assessed by pill counts. MRS was performed at baseline and after 12 wk of treatment. Body mass index (BMI) and blood tests such as serum AST, ALT, lipid profiles [total cholesterol, triglycerides, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, very low density lipoprotein (VLDL) cholesterol and FFA], and homeostasis model assessment-estimated insulin resistance (HOMA-IR) were performed at baseline and after 8 and 12 wk of treatment. A follow-up phone call related to safety was performed at 16 wk.

Assessment of hepatic fat content by MRS

MRS of the liver was performed to assess HFC at baseline and after 12 wk of treatment. MRS data were collected on 3.0T human scanners (Philips (Philips Medical Systems, Best, Netherlands) at Hanyang University Guri Hospital (site I) and Kangbuk Samsung Hospital (site II), and on a General Electric scanner (GE Healthcare, Waukesha, United States) at Dong-A University Hospital (site III). Patients were placed inside the magnet in a supine position. Prior to data collection, scout images were acquired along the three orthogonal axes in the end-exhalation breath-hold condition. To account for potential liver heterogeneity, two MRS voxels were defined in the liver (one from S5, 6 and the other from S7, 8 for each patient) avoiding major blood vessels. The edges of the voxels were at least 1 cm from the borders of the liver. A point-resolved spectroscopy (PRESS) sequence was used^[14].

The sequence parameters for all three sites were: repetition time (TR) = 2000 ms, spectral bandwidth = 2500 Hz, 2048 data points, and voxel size = 2.5 cm × 2.5 cm × 2.5 cm. At sites I and II, data were collected under the end-exhalation breath-hold condition using echo times (TEs) = 36 and 100 ms, number of receiver channels (NRC) = 32, and number of signal averages (NSA) = 4. At site III, data were collected under the shallow breathing condition using TE = 30 and 100 ms, NRC = 20, and NSA = 8. The data acquired at the two different TEs were used for T2 collection. Immediately after each scan, the quality of the spectrum was examined based on the signal intensities and line shapes (including line widths) of the water (about 4.7 ppm) and fat (about 1.3 ppm) signals. In cases where a spectrum was considered contaminated (*e.g.*, due to patient movement), the scan was repeated with the consent of the patient. After the MRS scan, the raw data were stored. The voxels in the post-treatment MRS scan were carefully co-registered with those in the pre-treatment, baseline scan by referring to the previous data (voxel locations superimposed on the scout images in the three orthogonal directions).

The MRS data were processed with a jMRUI (v5.0)^[15]. First, they were Fourier-transformed, line-broadened and phase-corrected, and the peak areas of the water (about 4.7 ppm) and lipid (methylene at about 1.3 ppm) resonances were obtained using AMARES for the individual spectra acquired at two different TEs^[16]. Then, the T2's of water and lipid were corrected by assuming single exponential decay. From the T2-corrected water and lipid signal intensities, a hepatic fat fraction (HFF) was calculated as lipid/(water + lipid) × 100 for each voxel. Finally, the mean HFF value over the two voxels was used as the HFC value for each patient.

Endpoints and safety evaluation

The primary endpoint was the post-treatment change in HFC measured by MRS. Secondary endpoints included post-treatment changes of serum AST, ALT, cholesterol, triglycerides, FFA, HOMA-IR, and BMI. Safety assessments included adverse events, laboratory findings, vital signs and electrocardiograms. Adverse events were classified as mild, moderate or severe, and as certainly, probably/likely, possibly, not likely, and not due treatment, or not known.

Statistical analysis

The 18 subjects in each group provided a power of 90% for identifying a difference of 4.6% in liver fat decrease between placebo and the low dose HL group. In previous studies, pioglitazone was shown to produce a 10% reduction in liver fat compared to baseline and 0% in the placebo^[17]. Also, the 8% weight loss group in a weight loss program was shown to have a 4.6% reduction in liver fat compared to baseline^[18]. Therefore, we considered that the high dose HL group

Table 1 Baseline characteristics of the study population

Characteristic	High dose (<i>n</i> = 22)	Low dose (<i>n</i> = 23)	Placebo (<i>n</i> = 23)	<i>P</i> value	
				High dose vs placebo	Low dose vs placebo
Age, yr	39.1 ± 9.5	45.5 ± 11.5	42.7 ± 11.2	0.474	0.355
Males, <i>n</i> (%)	20 (90.9)	14 (60.9)	20 (87.0)	1.000 ¹	0.044 ¹
Height, cm	170.1 ± 7.1	166.7 ± 10.3	170.4 ± 8.4	0.916	0.184
Weight, kg	82.4 ± 12.1	78.4 ± 16.0	82.9 ± 13.1	0.891	0.301
BMI, kg/m ²	28.2 ± 3.4	28.2 ± 4.0	28.4 ± 3.7	0.805	0.855
Above moderate steatosis in US, <i>n</i> (%)	18 (81.8)	16 (69.6)	16 (69.6)	0.491 ¹	1.000
MRS liver fat, %	16.1 ± 7.1	13.3 ± 7.1	12.0 ± 7.5	0.065	0.526
AST, IU/L	52.0 ± 19.1	58.4 ± 24.9	46.3 ± 20.5	0.461	0.077
ALT, IU/L	89.8 ± 34.8	90.3 ± 50.9	67.4 ± 32.7	0.109	0.078
Total cholesterol, mg/dL	205.0 ± 40.3	208.1 ± 31.2	194.5 ± 36.2	0.155	0.179
Triglyceride, mg/dL	190.3 ± 80.6	210.8 ± 137.9	286.7 ± 216.1	0.308	0.162
HDL cholesterol, mg/dL	46.3 ± 7.1	49.3 ± 10.9	43.6 ± 9.9	0.337	0.071
LDL cholesterol, mg/dL	136.0 ± 36.3	133.6 ± 29.7	118.6 ± 30.7	0.053	0.099
VLDL cholesterol, mg/dL	22.7 ± 11.2	25.3 ± 16.3	32.4 ± 21.5	0.263	0.214
Free fatty acid, μEq/L	526.2 ± 209.5	523.7 ± 341.6	581.9 ± 634.7	0.135	0.700
HOMA-IR	2.6 ± 1.6	2.5 ± 1.0	3.5 ± 2.4	0.167	0.326 ²

¹Fisher's exact test; ²Wilcoxon's rank sum test. Continuous data are expressed as mean ± SD. Categorical data are expressed as *n* (%). BMI: Body mass index; US: Ultrasonography; MRS: Magnetic resonance spectroscopy; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HDL: High density lipoprotein cholesterol; LDL: Low density lipoprotein cholesterol; VLDL: Very low density lipoprotein cholesterol; HOMA-IR: Homeostasis model assessment-estimated insulin resistance.

would have a 10% reduction in liver fat compared to baseline and the low dose HL group a 4.6% reduction. To detect these levels of change, a sample size of 23 subjects in each group was needed, assuming a 20% drop-out rate.

Analyses were conducted principally by full analysis set and were supplemented by per protocol set. Continuous variables are given as mean values with SD or median values with inter-quartile ranges. Categorical variables are given as frequencies or percentages. Baseline characteristics of the placebo group and each HL group (high dose vs placebo, low dose vs placebo) were evaluated using a two sample *t*-test or Wilcoxon's rank sum test for continuous variables and Pearson's χ^2 test or Fisher's exact test for categorical variables. Differences between variables were evaluated using two-sample *t*-tests or Wilcoxon's rank sum tests. Changes of HFC from baseline after 12 wk of treatment within each group were evaluated using paired *t* tests or Wilcoxon signed rank tests. *P* values < 0.05 in 2-sided tests were considered statistically significant. SPSS 19.0 for Windows (SPSS Inc, Chicago, IL, United States) was used for all statistical analyses.

RESULTS

Population

A total of 104 patients were screened in this study. Of these, 74 patients with NAFLD diagnosed by ultrasonographic examination were randomly assigned to groups receiving high dose HL (*n* = 24), low dose HL (*n* = 26), and placebo (*n* = 24), respectively. Finally, a safety analysis set, full analysis set and per protocol set were analyzed in 73, 68, and 60 patients, respectively (Figure 1). Most of the enrolled patients

completed the study (60/74; 81.1%).

Baseline characteristics were mostly similar in the 3 groups, as shown in Table 1. Patients in this study had a mean age of 39.1 ± 9.5 years in the high dose HL group, 45.5 ± 11.5 years in the low dose HL group and 42.7 ± 11.2 years in the placebo group. The low dose HL group was significantly low in males compared to the placebo group (*P* = 0.044). Hepatic fat by MRS showed a tendency to be higher in the high dose HL group than the placebo group (high dose vs placebo: 16.1% ± 7.1% vs 12.0% ± 7.5%, *P* = 0.065).

Primary end point

The mean HFC of the high dose HL group, but not of the low dose group, declined significantly after 12 wk of treatment (high dose vs placebo, *P* = 0.033; low dose vs placebo, *P* = 0.386) (Table 2). The mean changes of HFC from baseline at week 12 were -1.7% ± 3.1% in the high dose group (*P* = 0.018), -1.21% ± 4.97% in the low dose group (*P* = 0.254) and 0.61% ± 3.87% in the placebo group (relative changes compared to baseline, high dose were: -12.1% ± 23.5%, low dose: -3.2% ± 32.0%, and placebo: 7.6% ± 44.0%) (Table 2 and Figure 2). The per protocol analysis yielded similar results for the primary end points in the HL groups.

Secondary end points

Secondary end points are shown in Table 3. There was a modest but non-significant decrease in serum ALT level in both HL groups (vs high dose: -12.73 ± 29.30 IU/L, *P* = 0.097; vs low dose: -13.65 ± 39.56 IU/L, *P* = 0.153). Serum AST levels were similar in all three groups.

Levels of triglycerides decreased significantly in the placebo group (-86.91 ± 157.20 mg/dL) compared

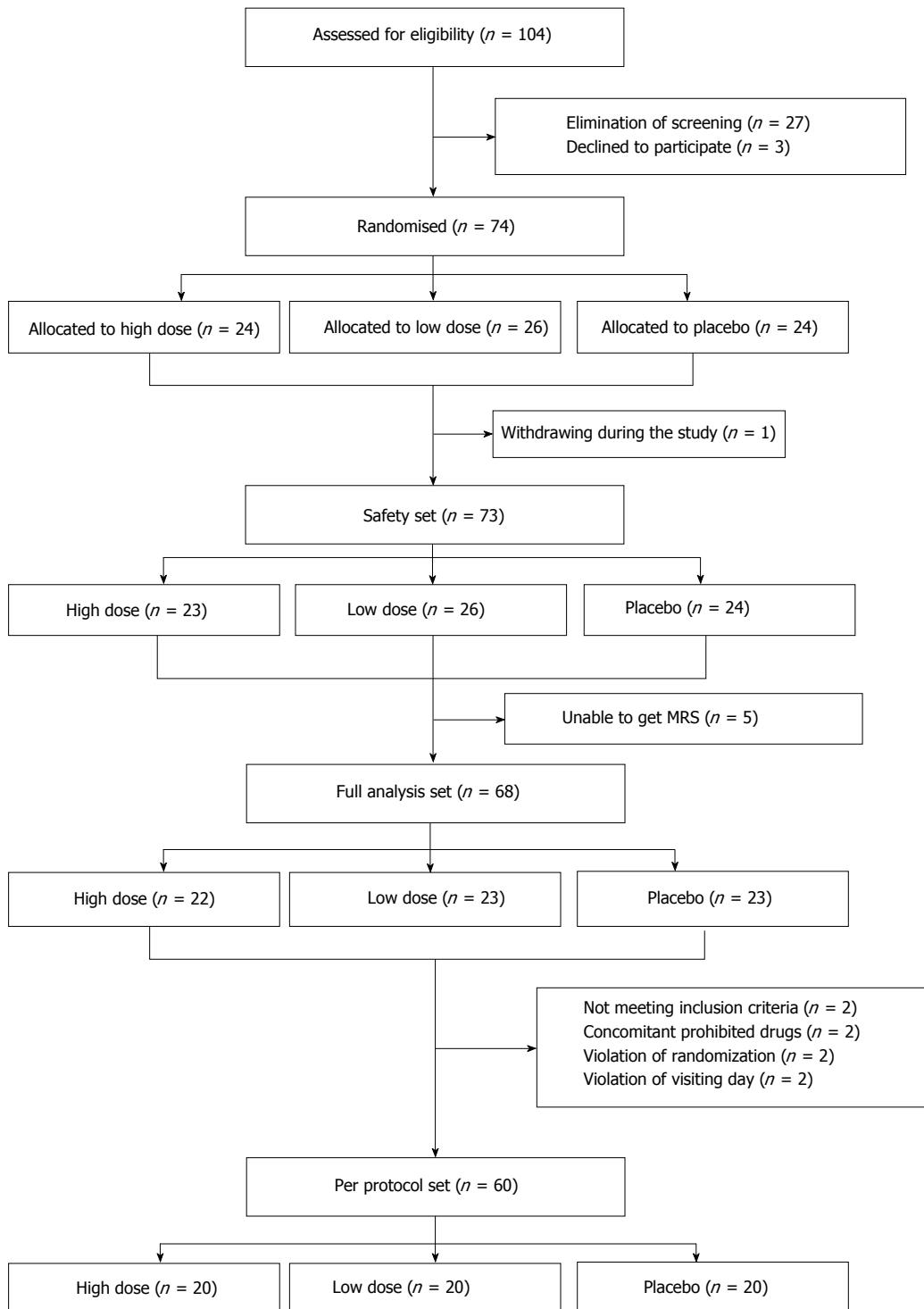


Figure 1 Flow chart of the study participants.

with each HL group (vs high dose: -16.50 ± 52.47 mg/dL, $P = 0.041$; vs low dose: 29.57 ± 186.19 mg/dL, $P = 0.031$). And levels of VLDL cholesterol also decreased significantly in the placebo group (-9.77 ± 17.47 mg/dL) compared with the HL groups (vs high dose: 0.43 ± 12.05 mg/dL, $P = 0.047$; vs low dose: 9.52 ± 27.85 mg/dL, $P = 0.007$). Other lipid profiles (total cholesterol, HDL cholesterol, LDL cholesterol, and FFA) were similar in the three groups, as was

BMI. A small decrease in HOMA-IR, was observed in the placebo group whereas no change was seen in the low HL group ($P = 0.019$). However, there was no difference in HOMA-IR between placebo and the high HL group. Per protocol analysis yielded a similar pattern for secondary end points (Supplement Table 1).

Safety

Four patients (17.4%) treated with high dose HL,

Table 2 Change of hepatic fat content from baseline after 12 wk of treatment

Treatment group	Full analysis set				Per protocol set			
	<i>n</i>	Baseline	12 wk	<i>P</i> value	<i>n</i>	Baseline	12 wk	<i>P</i> value
Placebo, %	23	11.96 ± 7.46	12.57 ± 8.34		20	12.59 ± 7.70	13.59 ± 8.47	
Low dose, %	23	13.33 ± 7.11	12.12 ± 6.33	0.386 ¹	20	12.99 ± 6.86	11.37 ± 6.02	0.164 ¹
High dose, %	22	16.05 ± 7.05	14.34 ± 7.19	0.033 ²	20	16.12 ± 7.19	14.77 ± 7.15	0.039 ²

¹Two sample *t*-test; ²Wilcoxon's rank sum test. Continuous data are expressed as mean ± SD.

Table 3 Secondary endpoint changes from baseline after 12 wk of treatment (full analysis set)

Variable	High dose (<i>n</i> = 22)	Low dose (<i>n</i> = 23)	Placebo (<i>n</i> = 23)	<i>P</i> value	
				High dose vs placebo	Low dose vs placebo
AST, IU/L	-7.82 ± 20.59	-2.74 ± 33.79	-2.96 ± 21.19	0.865	0.397
ALT, IU/L	-12.73 ± 29.30	-13.65 ± 39.56	-0.17 ± 19.58	0.097	0.153
Total cholesterol, mg/dL	-7.86 ± 27.92	2.22 ± 32.89	-0.61 ± 22.47	0.532	0.613
Triglyceride, mg/dL	-16.50 ± 52.47	29.57 ± 186.19	-86.91 ± 157.20	0.041	0.031
HDL cholesterol, mg/dL	1.32 ± 14.98	-1.78 ± 8.71	1.83 ± 8.62	0.289	0.567
LDL cholesterol, mg/dL	9.05 ± 27.78	-5.35 ± 23.63	5.74 ± 19.98	0.088	0.253
VLDL cholesterol, mg/dL	0.43 ± 12.05	9.52 ± 27.85	-9.77 ± 17.47	0.047	0.007
Free fatty acid, μEq/L	-73.23 ± 283.52	15.70 ± 304.68	-73.87 ± 666.93	0.146	0.921
HOMA IR	-0.01 ± 1.41	0.18 ± 0.77	-1.32 ± 2.44	0.174	0.019
BMI, kg/m ²	0.01 ± 0.81	-0.06 ± 0.97	-0.20 ± 0.80	0.608	0.904

Continuous data are expressed as mean ± SD. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HDL: High density lipoprotein cholesterol; LDL: Low density lipoprotein cholesterol; VLDL: Very low density lipoprotein cholesterol; HOMA-IR: Homeostasis model assessment-estimated insulin resistance.

Table 4 Treatment-related adverse events during the study (*n* (%))

System organ class preferred term	High dose (<i>n</i> = 23)	Low dose (<i>n</i> = 26)	Placebo (<i>n</i> = 24)
Gastrointestinal disorders			
Abdominal pain upper	0	1 (3.85)	0 (0)
Nausea	0	0 (0)	1 (4.17)
Nervous system disorders			
Dizziness	0	1 (3.85)	0 (0)

10 patients (38.5%) treated with low dose HL and 9 placebo patients (37.5%) experienced at least one adverse event (Supplementary Table 2). Treatment-related adverse events are shown in Table 4. All the treatment-related adverse events were mild, and no patient was withdrawn because of a treatment-related events.

DISCUSSION

In this randomized, double-blind and placebo-controlled study, we evaluated the efficacy and safety of HL tablet extracted from MO for hepatic fat reduction in patients with NAFLD. Treatment with HL tablet for 12 wk lowered hepatic fat measured by MRS and did not result in any negative lipid profiles or changes of BMI. The HL tablet was well-tolerated.

At the present time, no effective and safe pharmacologic therapy for patients of NAFLD is available^[1]. Weight loss due to life style modifications such as diet

and excise has been the most effective therapy for NAFLD, leading to reduction of intrahepatic fat content; however, maintaining such benefits is difficult^[1,19]. Pioglitazone, a PPAR-γ agonist, significantly decreased HFC, FFA levels and insulin resistance in patients with NASH, and improved histologic findings such as hepatic steatosis and inflammation^[17,20,21]. However long-term administration of pioglitazone carries risks of weight gain, postmenopausal bone loss or malignancies such as bladder cancer^[20-24]. Vitamin E is an antioxidant, and high dose RRR-α-tocopherol (800 IU/d) improves serum AST levels, serum ALT levels and histologic findings^[20], but safety concerns have also been raised about long-term administration of vitamin E^[1]. Obeticholic acid (OCA, 6α-ethyl-cheno-deoxycholic acid), a farnesoid X receptor agonist, is receiving attention for patients with NAFLD^[25]. In phase II b randomized trial (the FLINT study), OCA led to histological improvement, including reduced hepatic fibrosis in non-cirrhotic patients with NASH^[26]. However, histological improvement was seen in only 45% of those treated with OCA, and pruritus, the main side effect, occurred in 23% of the patients^[26].

The reason for progression from simple steatosis to NASH is not clear. However, the most likely idea is that triglycerides accumulate in hepatocytes due to increased peripheral insulin resistance ("first hit" theory), and then oxidative stress causes fat peroxidation, liver cell damage and inflammatory cytokine activation ("second hit" theory)^[27]. This implies that fat accumulation in hepatocytes is the initial cause of NASH. Given that

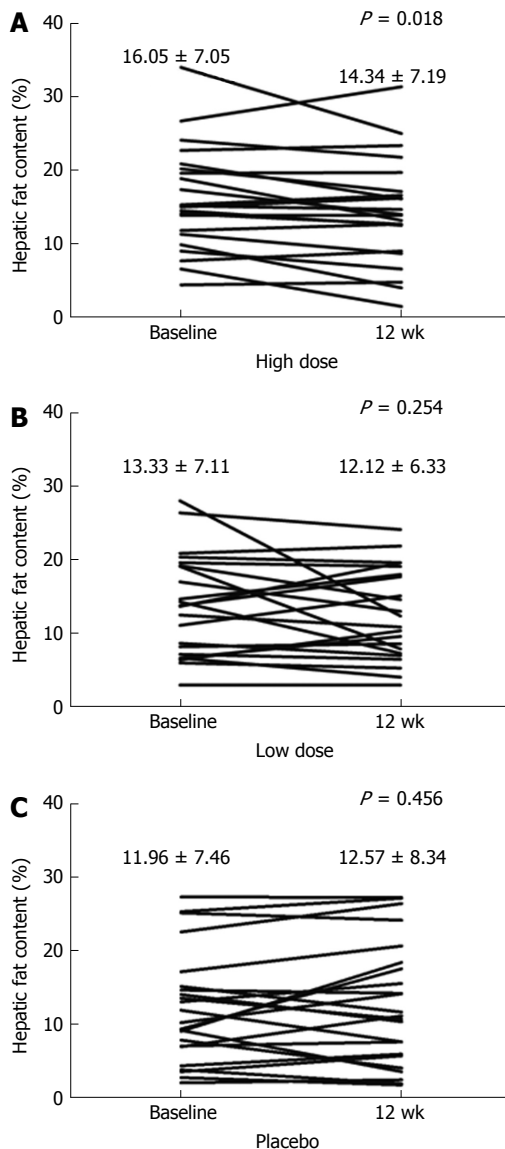


Figure 2 Changes of hepatic fat content from baseline after 12 wk of treatment.

fat accumulation in hepatocytes can lead to NASH, reducing intrahepatic fat by treatment with HL tablet would be a key to preventing the "first hit" leading to NAFLD and this would prevent the transition to NASH. Furthermore, despite treatment for only 12 wk, there was a significant reduction of mean HFC in the high HL group, and we anticipate that treatment for 24 wk would have a greater effect.

In patients with NAFLD, elevation in serum ALT may correlate with HFC and insulin resistance^[28]. HOMA-IR has been extensively used as a marker of insulin resistance in large population epidemiology studies. In the present study there was no improvement of HOMA-IR in the high HL group but serum ALT levels showed a modest though non-significant decrease in the HL groups. Though we did not carry out any liver biopsies in this study and HOMA-IR is not the gold standard for measuring insulin resistance^[29], our

results suggest that HL tablet may improve not only insulin resistance but also NASH. Longer-term studies of insulin resistance with use of higher doses of HL tablet are needed.

We observed no significant difference in most lipid profiles between the placebo group and the HL groups. However, surprisingly, levels of triglyceride and of VLDL cholesterol were significantly lower in the placebo group than either HL group. Because this study was a randomized, double-blind and placebo-controlled study and routine treatment was simultaneously performed, we recommended life style modifications to the patients as NAFLD treatment. Also, the enrolled patients knew that they suffered from NAFLD, and had the good sense to undertake life style modifications. Although this is not certain, we presume that these unexpected results occurred because the placebo group complied better with the suggested life style modifications than either HL tablet group.

HL tablet was well-tolerated by the patients. All treatment-related adverse events were mild. Because HL tablet does not cause weight gain, unlike pioglitazone, this will lead to few harmful effects in patients with metabolic syndrome such as diabetes and dyslipidemia^[30].

This study had several limitations. First, it involved a relatively small number of patients and a short duration of treatment, because it was a phase II clinical trial. Second, since liver biopsies were not performed, we do not know how many patients with NASH were included in the study and cannot discuss any histologic findings. However, since patients with NAFLD in this study were excluded those with serum AST/ALT ratio > 2 and had higher baseline serum ALT level than serum AST level (AST/ALT ratio < 1), we think that more patients with simple steatosis than with advanced liver fibrosis were included^[31].

In conclusion, HL tablet is effective in reducing HFC in short-term treatment (12 wk) without causing any negative lipid profiles, BMI changes and serious adverse effects. It may be a new and effective drug for treating NAFLD. Larger trials are warranted to assess the long-term efficacy of HL tablet in patients with NAFLD.

COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease worldwide and the prevalence of NAFLD is growing gradually with the increase in population age and obesity. But, effective and safe drugs for NAFLD are not yet available.

Research frontiers

The demand for new drugs for NAFLD is increasing worldwide. *Magnolia officinalis* (MO) is a traditional medicinal plant that has been used to treat liver diseases and other diseases. Some constituents extracted from MO are reported to have anti-inflammatory and antioxidant effects and MO can be effective against NAFLD. So, the authors hypothesized that MO would improve steatosis and inflammation in patients with NAFLD.

Innovations and breakthroughs

The authors evaluated the efficacy and safety of HL tablet, a new botanical drug extracted from MO, in the treatment of patients with NAFLD. The high dose (daily 400 mg) HL tablet is effective in reducing hepatic fat content (HFC) in short-term treatment (12 wk) without causing any negative lipid profiles, body mass index (BMI) changes and adverse effects.

Applications

HL tablet is effective in reducing HFC without any negative lipid profiles, BMI changes and adverse effects. Larger trials are warranted to assess the long-term efficacy of HL tablet in patients with NAFLD.

Terminology

HL tablet is a new botanical drug extracted from MO. Magnetic resonance spectroscopy data were collected on 3.0T human scanners.

Peer-review

This is a study of phase II trial of HL tablet as a therapy for NAFLD. HL tablet is effective in reducing HFC without any negative lipid profiles, BMI changes and adverse effects. The results of this study are particularly of interest for the reader of the journal.

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Randomized Clinical Trial

Randomized clinical trial comparing fixed-time split dosing and split dosing of oral Picosulfate regimen for bowel preparation

Jae Hyuck Jun, Koon Hee Han, Jong Kyu Park, Hyun Il Seo, Young Don Kim, Sang Jin Lee, Baek Gyu Jun, Min Sik Hwang, Yoon Kyoo Park, Myeong Jong Kim, Gab Jin Cheon

Jae Hyuck Jun, Koon Hee Han, Jong Kyu Park, Hyun Il Seo, Young Don Kim, Sang Jin Lee, Baek Gyu Jun, Min Sik Hwang, Yoon Kyoo Park, Gab Jin Cheon, Department of Internal Medicine, Gangneung Asan Hospital, University of Ulsan College of Medicine, Gangneung 25440, South Korea

Myeong Jong Kim, Catholic Kwandong University, Gangneung 25440, South Korea

ORCID number: Jae Hyuck Jun (0000-0002-8005-8215); Koon Hee Han (0000-0003-1844-1712); Jong Kyu Park (0000-0002-5474-6794); Hyun Il Seo (0000-0003-1844-1712); Young Don Kim (0000-0001-9003-9862); Sang Jin Lee (0000-0001-6297-7966); Baek Gyu Jun (0000-0003-4693-9542); Min Sik Hwang (0000-0003-2661-7087); Yoon Kyoo Park (0000-0002-9371-7064); Myeong Jong Kim (0000-0001-5204-1312); Gab Jin Cheon (0000-0001-5937-5999).

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Correspondence to: Koon Hee Han, Associate professor, Department of Internal Medicine, Gangneung Asan Hospital, University of Ulsan College of Medicine, 38 Bangdong-gil, Sacheon-myeon, Gangneungsi 25440, South Korea. 9292@gnah.co.kr
Telephone: +82-33-6103139
Fax: +82-33-6108130

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Abstract

AIM

To compare the efficacy of fixed-time split dose and split dose of an oral sodium picosulfate for bowel preparation.

METHODS

This study was prospective, randomized controlled study performed at a single Institution (2013-058). A total of 204 subjects were assigned to receive one of two sodium picosulfate regimens (*i.e.*, fixed-time split or split) prior to colonoscopy. Main outcome measurements were bowel preparation quality and subject tolerability.

RESULTS

There was no statistical difference between the fixed-time split dose regimen group and the split dose regimen group (Ottawa score mean 2.57 ± 1.91 vs 2.80 ± 2.51 , $P = 0.457$). Cecal intubation time and physician's satisfaction of inspection were not significantly different between the two groups ($P = 0.428$, $P = 0.489$). On subgroup analysis, for afternoon procedures, the fixed-time split dose regimen was equally effective as compared with the split dose regimen (Ottawa score mean 2.56 ± 1.78 vs 2.59 ± 2.27 , $P = 0.932$). There was no difference in tolerability or compliance between the two groups. Nausea was 21.2% in the fixed-time split dose group and 14.3% in the split dose group ($P = 0.136$). Vomiting was 7.1% and 2.9% ($P = 0.164$), abdominal discomfort 7.1% and 4.8% ($P = 0.484$), dizziness 1% and 4.8% ($P = 0.113$), cold sweating 1% and 0% ($P = 0.302$) and palpitation 0% and 1% ($P = 0.330$), respectively. Sleep disturbance was two (2%) patients in the fixed-time split dose group and zero (0%) patient in the split dose preparation ($P = 0.143$) group.

CONCLUSION

A fixed-time split dose regimen with sodium picosulfate is not inferior to a split dose regimen for bowel preparation and equally effective for afternoon colonoscopy.

Key words: Colonoscopy; Bowel preparation; Split dose preparation; Sodium picosulfate; Ottawa Bowel Preparation Scale

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Core tip: Fixed-time split dose bowel preparation was not inferior to a split dose regimen in bowel cleansing using the Ottawa Bowel Preparation Scale. The average score using the Ottawa Scale was 2.57 ± 1.91 in the fixed-time split dose group and 2.80 ± 2.51 in the split dose group ($P = 0.457$). There was no statistical difference in mean Ottawa score between the two groups when the procedure was performed in the morning or afternoon (2.56 ± 1.78 vs 2.59 ± 2.27 , $P = 0.932$). Therefore fixed-time split dosing with sodium picosulfate is as effective as split dosing for subjects scheduled for colonoscopy in the afternoon.

Jun JH, Han KH, Park JK, Seo HL, Kim YD, Lee SJ, Jun BK, Hwang MS, Park YK, Kim MJ, Cheon GJ. Randomized clinical trial comparing fixed-time split dosing and split dosing of oral

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INTRODUCTION

Currently, colorectal cancer is the third most common cancer in the world and its incidence is rapidly increasing in Asian countries^[1]. Colonoscopy is an indispensable procedure for the diagnosis and treatment of various colon diseases^[2]. Colonoscopy also prevents colorectal cancer by detecting and eliminating precancerous lesions^[3-5]. For an accurate colonoscopy, however, it is necessary to perform an appropriate colon preparation^[6-8]. Unfortunately, about 20% to 25% of colonoscopies have been reported to occur following inadequate bowel preparation^[5-7]. In 27% of patients who had poor bowel preparation, more than 10 mm of polyps were not observed on the first colonoscopy. Therefore the importance of bowel preparation was further emphasized^[3].

A variety of bowel preparation agents have been developed to reduce the large amount of fluid consumption and bad taste that can occur during bowel preparation for colonoscopy^[9,10]. One mixture of sodium picosulfate and magnesium citrate, available in Korea (Picolight® powder, Pharmbio Korea Co., Ltd, Seoul, Korea), is low-volume bowel cleansing agent with low toxicity in children and adults^[11]. This formulation is widely used because of its good compliance with dosage, and excellent bowel cleansing effect^[12]. According to the latest guidelines, sodium picosulfate can be taken with only 2 liters of water, which can increase patient compliance^[13,14]. The original cleansing protocol entailed two sachets of sodium picosulfate, one at 5 pm and one at 10 pm on the day before colonoscopy. However, a two-day split dose of one sachet at 7 pm on the day prior to colonoscopy and a second sachet four hours before colonoscopy, is also reported as a good regimen for bowel preparation and is nearly equivalent to the use of a polyethylene glycol (PEG) solution^[15,16]. In addition, according to a recent report, it is most effective to finish the bowel cleansing three-to-four hours before the scheduled time of the procedure^[12].

However as this regimen is based on western diet and lifestyle, it is not necessarily the right method for Koreans, who typically eat more high fiber vegetables than westerners. In the case of Gangneung Asan Hospital located in Gangwon Province, the accessibility of patients is deteriorated by the surrounding mountainous environment. Therefore, it is difficult for some patients to adjust their preparation to colonoscopy time. For the medical staff (doctor, nurse, pharmacist), it is also inconvenient to explain the proper regimen for effective bowel preparation. Additionally, in patients

who have to undergo abdominal ultrasound and esophagogastroduodenoscopy at the same time, the results may not be accurate.

We hypothesized that sufficient water intake and effective low residue diet, combined with a fixed-time split dose intake of two, or plus one sachets of sodium picosulfate would result in non-inferior bowel preparation and patient compliance to the split dose regimen. We compared the efficacy of bowel cleansing between the split dose group (last split dose of sodium picosulfate was assigned four hours prior to the colonoscopy) and the fixed-time split dose group (last split dose of picosulfate was assigned at 5:00 am in cases in which the colonoscopy was performed in the morning (09:30 to 11:30 h) and at 6:00 am in cases in which the colonoscopy was performed in the afternoon (13:00 to 15:00 h) using the Ottawa Bowel Preparation Scale^[8]. Colonoscopies are often scheduled in the afternoon, and the split dose may not leave a clean colon by then. Therefore, we further compared the bowel cleansing effect between the morning and afternoon examination groups. We also assessed patient compliance and tolerability to the two bowel preparation regimens.

MATERIALS AND METHODS

Patients

This study was designed as a prospective single center, single blind, randomized control study of ambulatory outpatients at the Gangneung Asan Hospital, Republic of Korea.

From August 2014 to November 2015, a total of 240 consecutive patients, between the ages of 18 and 76 years who were scheduled to undergo colonoscopy were included. The indications for colonoscopy included colon cancer screening; a family history of colorectal cancer or lower GI symptoms such as constipation or change of bowel habits.

Exclusion criteria included: (1) pregnant women; (2) acute abdomen; (3) history of dehydration and/or drug allergy; (4) congestive heart failure; (5) chronic liver disease or renal insufficiency; (6) history of colon resection or abdominal surgery within six months; and (7) participation in other clinical studies within four weeks prior to randomization. Patients who refused to provide informed consent were also excluded. Following agreement to participate, patients were randomized by computer-generated random numbers to assign them to one of the two preparation regimens. All patients were provided written instructions by the clinical staff. A total of two expert endoscopists who reviewed the Ottawa Bowel preparation Scale and who were blinded to the method of bowel preparation participated in the study. This study was approved by Institutional Review Board of the Gangneung Asan Hospital (IRB: 2013-058), and all participating patients, or their legal guardian provided informed

written consents prior to study enrollment.

Preparation instructions

Patients were randomized to the fixed-time split dose and split dose of the sodium picosulfate group. Each group received three sachets of sodium picosulfate (Picolight[®] powder, Pharmbio Korea Co., Ltd, Seoul, Korea), each containing sodium picosulfate hydrate 10 mg, magnesium oxide 3.5 g, and citric acid 12 g. On the third day prior to the colonoscopy, the patients were advised to ingest a low residue diet by doctor and nurse. In the fixed-time split dose group, patients who scheduled to undergo colonoscopy in the morning (9:30-11:30 am) were instructed to dissolve one sachet of sodium picosulfate in 250 mL of water and drink it at 7:00 pm on the day before colonoscopy and at 5:00 am on the day of colonoscopy. Each time, they were to drink 1.25 L of water successively. The patients who scheduled in the afternoon (13:00-15:00), were instructed to take their first dose at 8:00 pm on the day before colonoscopy and their second dose at 6:00 am on the day of colonoscopy in the same method. In the split dose group, the patients were instructed to take the first sachet of sodium picosulfate at 7 pm on the day before colonoscopy and then four hours before the colonoscopy on the day of procedure, in the same method. In both groups, in case of any signs of incomplete bowel preparation (*i.e.*, stool residue or unclear fluid was noted after defecation) observed one hour after the second dose of sodium picosulfate, they also instructed to take an additional one sachet of sodium picosulfate dissolved in 250 mL of water and successive 0.75 L of water.

Colonoscopy

Colonoscopies were performed with the patients under conscious sedation by a consultant gastroenterologist. All colonoscopies were performed between 9:30 am and 3:00 pm (morning session between 9:30 and 11:30 am, and afternoon session between 1:00 pm and 3:00 pm). Intravenous midazolam 2 mg was used for sedation in patients in whom there was no contraindication; half of that dose was used in patients over the age of 60 years.

Additional sedation was used if required and permissible. Pulse, blood pressure, and oxygen saturation were measured in all patients before, during, and after the procedure.

Data collection

Before the colonoscopy, a questionnaire survey was conducted on the compliance of the subjects and side effects of the sodium picosulfate. We evaluated the compliance with dosing time and regimen, and the degree of discomfort felt by the patients was recorded separately as sleep disorder, nausea, vomiting, abdominal pain, abdominal discomfort, dizziness, cold sweating, and/or palpitation. We also evaluated

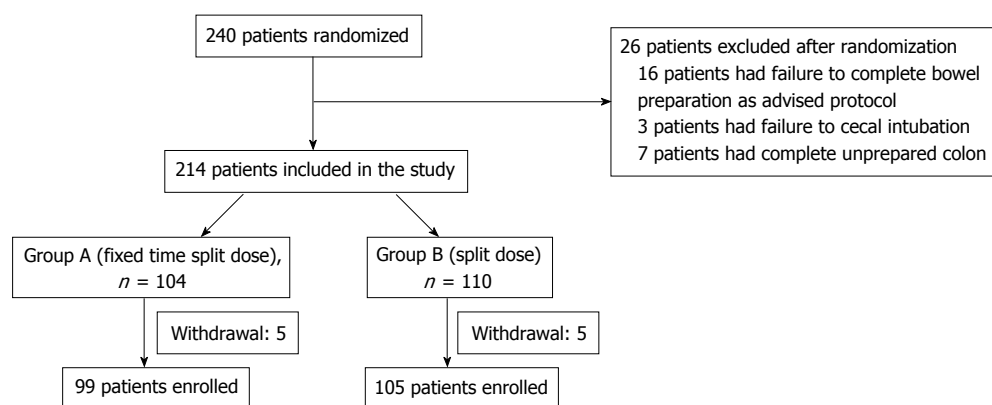


Figure 1 Study design: Group randomization.

the amount of consumed water. Colonoscopy was performed by two endoscopists who did not know the used regimen. The Ottawa Bowel Preparation Scale was used to evaluate the degree of bowel preparation^[8]. This scale assesses cleanliness and volume state, separately. Cleanliness was assessed further separately for the right colon (cecum - ascending colon), mid-colon (transverse - descending colon), and rectosigmoid colon on a five-point scale (no liquid = 0, minimal liquid, no suctioning required = 1, suction required to see mucosa = 2, wash and suction = 3, solid stool, not washable = 4). The degree of residual fluid in the entire colon was classified from 0 to 2. A score of 0 indicates excellent bowel preparation, and a score of 14 indicates that colonoscopy is impossible^[8,17]. The cecal intubation time was also measured and compared. Starting and cecal intubation time was recorded. After the colonoscopy, the bowel preparation quality and satisfaction of the procedure (via a 10-point physician's satisfaction of inspection) was rated by two investigators who were blinded to the type of preparation. The results were recorded on a standardized form.

Statistical analysis

In this study, the level of significance was assumed to be 5% with regards to the number of effective subjects. The second type error was 0.2, the power was maintained at 80%, and the effect size was 10%. The total number of subjects necessitated at least 91 subjects in each group. A total of 240 subjects (120 subjects per group) were required for a follow-up loss of about 30%.

This study was designed as a one-sided χ^2 test for non-inferiority. Statistical analysis was performed using IBM SPSS ver. 23.0 for Windows (IBM, Armonk, NY, United States). The variables expressed as percentages (characteristics of the study subject, adverse effects) were used in χ^2 test (Fisher's exact test). The means of the two groups (Ottawa Bowel Preparation Scale value, satisfaction, insertion time, and degree of disgust) was used in Student's *t*-test.

RESULTS

In this prospective, randomized, investigator-blinded study, we enrolled 240 patients between August 2014 and November 2015. Of the 240 patients randomized, 36 were excluded due to the following: failure to complete bowel preparation as advised ($n = 16$); failure to cecal intubation due to abdominal pain or vomiting, colon cancer ($n = 3$); withdrawal of consent ($n = 10$, fixed-time split dose group; 5, split dose group; 5); and a completely unprepared colon ($n = 7$, fixed-time split dose group; 4, split dose group; 3). Ultimately, 99 patients in the fixed-time split dose group and 105 in the split dose group completed the study and were analyzed (Figure 1). The characteristics of the patients in the two groups are shown in Table 1. There were no statistical difference between the two groups with respect to gender, height, weight, mean age, BMI, and/or history of abdominal surgery and DM (Table 1).

Quality of bowel preparation

Using the Ottawa Bowel Preparation Scale, the mean Ottawa score was 2.57 ± 1.91 in the fixed-time split dose preparation and 2.80 ± 2.51 in the split dose preparation ($P = 0.457$)(Figure 2).

Cecal intubation time and physician's satisfaction of the inspection were not significantly different between the two groups ($P = 0.428$, $P = 0.489$)(Table 2).

On subgroup analysis of fixed-time split dose preparation, there was no difference of mean Ottawa score between the morning colonoscopy (9:30 to 11:30 am) and the afternoon (1:00-3:00 pm) colonoscopy groups (2.56 ± 1.78 vs 2.59 ± 2.27 , $P = 0.932$)(Figure 3).

Tolerability of the preparation and sleep disturbance

Nausea was complained of in 21.2% of the patients with the fixed-time split dose preparation and in 14.3% with split dose preparation ($P = 0.136$). Vomiting was reported by 7.1% and 2.9% ($P = 0.164$), abdominal discomfort by 7.1% and 4.8% ($P = 0.484$), dizziness

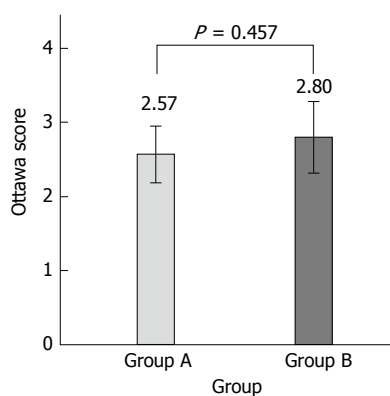


Figure 2 Comparison of Ottawa Score between the fixed-time split dose (group A) and split dose (group B) preparation.

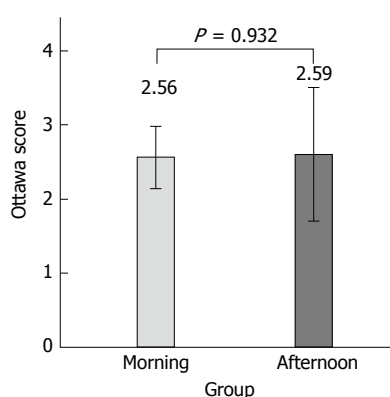


Figure 3 Comparison of Ottawa Score between the morning and afternoon preparation of fixed-time split dose regimen.

by 1% and 4.8% ($P = 0.113$), cold sweating by 1% and 0% ($P = 0.302$) and palpitation by 0% and 1% ($P = 0.330$), respectively. Sleep was disturbed in two (2%) patients in the fixed-time split dose preparation and zero (0%) patients in the split dose preparation ($P = 0.143$) groups (Table 2).

DISCUSSION

Colonoscopy is the most effective tool for diagnosing colon diseases, including inflammation bowel disease and colorectal cancers. Good quality of bowel preparation can lead to good colonoscopy results, while incomplete bowel preparations decreases cecal intubation and adenoma detection rates^[18]. It also increases patient's discomfort and procedure costs^[5]. Sodium picosulfate is safe and effective for bowel preparation with good tolerability and fewer side effects than the standard 4L PEG solution^[15,19-21]. It is known from a meta-analysis of 29 studies that the use of a split dose on the day before and on the day of the procedure can induce more effective bowel preparations than a dosing regimen on the day before the procedure^[7]. Recently, the use of a split dose regimen of sodium picosulfate was reported more superior than a previous-day regimen and the Ottawa

Table 1 Patients characteristics

	Group A (fixed time split dose preparation) n = 99	Group B (split dose preparation) n = 105	P value
Male: Female	61:38	57:48	NS
Mean age, r	54.6 ± 11.5	54.3 ± 9.4	NS
Age ≥ 65	20 (20.2)	16 (15.2)	NS
Weight (kg)	64.7 ± 11.7	65.4 ± 11.3	NS
BMI (kg/m ²)	23.8 ± 3.05	24.2 ± 2.88	NS
BMI ≥ 30	4 (4)	4 (3.8)	NS
History of abdominal surgery	7 (7.1)	7 (6.7)	NS
History of Diabetes mellitus	8 (8.1)	12 (11.4)	NS

NS: Nonspecific.

Bowel Preparation Scale score was the lowest at three-to-four hours after the last cleansing^[12].

Although sodium picosulfate is a low-volume agent for bowel preparation that has been available in Korea since December 2011, we often experienced indications that this agent showed limited bowel preparation capacity. It is often inconvenient for medical staff members to explain the effective bowel preparation time to the patients because each patient has a different examination time. In addition, in patients with poor compliance, such as elderly patients or those who live far away from the hospital due to their local characteristics, it is often difficult to maintain the effective bowel cleansing time and thus the bowel cleansing often becomes inadequate. Therefore, we hypothesized that if the patient has adequate dietary control and water intake before colonoscopy, it is possible to obtain effective bowel preparations by instructing them regarding a fixed dosing time varied according to a morning or afternoon test time.

First, we compared the Ottawa Bowel Preparation Scale score in the fixed-time split dose group and the split dose group to confirm the appropriate bowel preparations. There was no significant difference between the two groups (Table 2 and Figure 2). Additionally Ottawa scores according to each segment were no significantly different between the experimental group and the control group (Table 2).

Sixty-seven patients (68% of fixed-time split dose group) and 70 patients (67% of split dose group) completed bowel preparation with two sachets of sodium picosulfate (Table 3). We think these results are due to the Korean life style and high fiber diet, which include foods such as "Kimchi" and seaweed, and two sachets of sodium picosulfate are insufficient for bowel preparation. Therefore we recommend three sachets of sodium picosulfate and adequate modification of water intake.

The prospecting randomized clinical trials to compare the preference and efficacy of sodium picosulfate in Korea proved that three sachets of sodium picosulfate regimen were as effective as conventional high volume

Table 2 Results of bowel cleansing and adverse effects

	Group A (fixed time split dose preparation) <i>n</i> = 99	Group B (split dose preparation) <i>n</i> = 105	<i>P</i> value
Mean total Ottawa preparation score ¹	2.57 ± 1.91	2.80 ± 2.51	0.457
Age ≥ 65	3.40 ± 2.03	3.38 ± 2.98	0.976
Right colon, mean (0-4)	0.92 ± 0.71	0.96 ± 0.82	0.692
Mid colon, mean (0-4)	0.76 ± 0.74	0.76 ± 0.83	0.969
Rectosigmoid colon, mean (0-4)	0.73 ± 0.68	0.74 ± 0.85	0.886
Residual fluid, mean (0-2)	0.17 ± 0.43	0.33 ± 0.56	0.023
Adverse GI symptoms, <i>n</i> (%)			
Nausea	21 (21.2)	14 (13.3)	0.136
Vomiting	7 (7.1)	3 (2.9)	0.164
Sleep disturbance	2 (2)	0 (0)	0.143
Abdominal discomfort	7 (7.1)	5 (4.8)	0.484
Dizziness	1 (1)	5 (4.8)	0.113
Cold sweating	1 (1)	0 (0)	0.302
Palpitation	0 (0)	1 (1)	0.330
Mean cecal intubation time, min ± SD	5.02 ± 3.42	4.70 ± 2.27	0.428
Physician's satisfaction of inspection	8.17 ± 1.21	8.04 ± 1.51	0.489

¹With the Ottawa bowel preparation scale, lower scores indicate better preparation.

Table 3 Comparison of Ottawa Score between the fixed time split dose (group A) and split dose (group B) preparation

	Group A (fixed time split dose preparation) <i>n</i> = 99	Group B (split dose preparation) <i>n</i> = 105	<i>P</i> value
Sodium picosulfate			
2 sachets	2.43 ± 1.90 (<i>n</i> = 67)	3.00 ± 2.68 (<i>n</i> = 70)	0.158
3 sachets	2.84 ± 1.95 (<i>n</i> = 32)	2.40 ± 2.10 (<i>n</i> = 35)	0.375
2 or 3 sachets	2.57 ± 1.91 (<i>n</i> = 99)	2.80 ± 2.51 (<i>n</i> = 105)	0.457

PEG solution. And sodium picosulfate groups reported superior palatability and tolerability^[22-24].

But patients with renal insufficiency, uncontrolled cardiovascular problems, liver disease, metabolic disease and admitted patients were excluded from this study. Therefore, these results are inapplicable to high-risk or admitted patients and additional studies are warranted.

Second, we compared the bowel cleansing effects of the sodium picosulfate between the morning colonoscopy and afternoon colonoscopy groups. There was no significant difference between the two groups (Figure 3). According to this results, fixed-time dosing regimen may be convenient for patients for bowel preparation who are undergoing other procedures, such as abdominal ultrasound and esophagogastroduodenoscopy on the same day. However, this result is in conflict with a previous study that shows the lowest Ottawa Bowel Preparation Scale score when endoscopy was performed three-to-four hours after the completion of bowel preparation^[12,17,25-27]. The European Society of Gastrointestinal Endoscopy also recommends that the last preparation to colonoscopy interval should be minimized and should be no longer than four hours. In cases of the afternoon colonoscopy group, the gap between last dosing and colonoscopy time was six to nine hours.

We believe that there are several reasons for these result. In this study, we suggests that if the patients has an accurate understanding of bowel cleansing time and method, that this may be more important than other additional factors. Considering the Korean life style and diet pattern, we emphasized that patients should take relatively more water (3 L to 4 L) than typical, and should consume low residue diet starting three days before colonoscopy. The second consideration is that the previous study was mainly focused PEG solution rather than sodium picosulfate, so the bowel cleansing result of sodium picosulfate observed in this study may be different from that in the previous study^[17]. In addition, previous studies conducted with sodium picosulfate were mainly performed involving westerners, and these individuals may consume diets different from that of an Asian's diet, therefore further studies on Asians are needed^[12,28].

Our study has several limitations. First, because all enrolled patients were Koreans who eat a more high-fiber diet than most westerners do, our protocol consisted of recommending a relatively large amount of fluid (3 L to 4 L) intake and suggesting low residue diet three days before colonoscopy. Therefore this study's results may differ from western data due to the inclusion of different dietary patterns and other ethnic groups.

Second, although there was no difference in bowel preparation between the morning colonoscopy and the afternoon colonoscopy groups (Figure 3), the number of patients enrolled in the afternoon group was smaller (74 vs 25). To obtain more accurate results, analysis of a larger number of patients is needed.

Lastly, the lower success rate for bowel preparation with two sachets of sodium picosulfate and 3L of water intake (68% of the fixed-time split dose group), led us to recommend three sachets of sodium picosulfate

and adequate water intake (Table 3). Because dietary factors and amount of water intake can greatly affect the success of bowel preparation, to find the optimal conditions for effective bowel preparation, further studies on these factors are needed.

In conclusion, the fixed-time split dose bowel preparation is equally effective as compared with the conventional split dose bowel preparation. This method is also convenient for medical staffs (*i.e.*, doctors, nurses and pharmacists) as a means to simplify explaining the bowel cleansing protocol to patients, and may possibly increase the compliance of patients in bowel preparation.

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COMMENTS

Background

Sodium picosulfate is a widely used bowel cleansing agent, but there is no standard recommendation for the adequate bowel cleansing time. Studies have shown that the split preparation is better than the conventional previous evening preparation for bowel preparation quality and patient's compliance. The split dose option is also endorsed by the American College of Gastroenterology and is considered an optimal choice for colonoscopy. Previous studies have proven that the bowel cleansing effect is the best when taking the sodium picosulfate three to four hours before colonoscopy. However, this regimen is based on data from western countries, which have different dietary patterns from that of many Asians including Koreans. Additionally, this protocol is not easy to ensure in patients who have low accessibility to hospitals. Patients who are scheduled to undergoing ultrasound and esophagogastroduodenoscopy on the morning of the same day morning also may have inaccurate results. Considering the Korean high fiber dietary pattern, the authors compared the quality of bowel preparation between a fixed -time split dose group and split group after training them to intake a sufficient amount of water and consume a low residue diet beginning three days before colonoscopy. The primary endpoint was the quality of bowel preparation.

Research frontiers

The quality of bowel preparation and optimal time of colonoscopy is important for successful colonoscopy. Compliance of patients is also considered modifiable factor for good results. Various studies are being conducted to determine the optimal time of colonoscopy after last dose of bowel preparation.

Innovations and breakthroughs

This is the first randomized controlled study to evaluate the efficacy of fixed-time split dosing of oral picosulfate for bowel preparation. In this study there was no statistical difference in the quality of bowel preparation between the fixed-time split dose bowel preparation and the split dose bowel preparation. Therefore, fixed-time split dose bowel preparation can be an alternative regimen for successful colonoscopy.

Applications

This study would be used to improve compliance for colonoscopy and convenience for the medical staff in instructing patients on the regimen of bowel cleansing agents.

Terminology

Fixed-time bowel preparation: the patients take the last split dose of laxative at a set time according to the morning and afternoon colonoscopy.

Peer-review

This article presents an important issue. This is the first study to compare the bowel cleansing effect when the last split dosing time was fixed. Methods and study population are adequate, and conclusions are reasonable and of possible practical use. Overall this study is timely and interesting to the readership.

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Systematic review and meta-analysis of colon cleansing preparations in patients with inflammatory bowel disease

Sophie Restellini, Omar Kherad, Talat Bessissow, Charles Ménard, Myriam Martel, Maryam Taheri Tanjani, Peter L Lakatos, Alan N Barkun

Sophie Restellini, Talat Bessissow, Peter L Lakatos, Alan N Barkun, Division of Gastroenterology, Department of Medicine, McGill University Health Center, Montreal, QC H3G1A4, Canada

Omar Kherad, Division of Internal Medicine, Department of Medicine, La Tour Hospital and University of Geneva, 1217 Geneva, Switzerland

Charles Ménard, Division of Gastroenterology, Department of Medicine, University of Sherbrooke, Sherbrooke, QC J1K2R1, Canada

Myriam Martel, Alan N Barkun, Department of Clinical Epidemiology, McGill University and the McGill University Health Center, Montreal, QC H3G1A4, Canada

Maryam Taheri Tanjani, Department of Family Medicine, Queens University, Kingston, ON K7L 3G2, Canada

ORCID number: Sophie Restellini (0000-0003-0646-3439); Omar Kherad (0000-0002-6003-0776); Talat Bessissow (0000-0003-2610-1910); Charles Ménard (0000-0001-9487-2499); Myriam Martel (0000-0001-8317-613X); Maryam Taheri Tanjani (0000-0001-5297-6097); Peter L Lakatos (0000-0002-3948-6488); Alan N Barkun (0000-0002-1798-5526).

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Correspondence to: Alan N Barkun, MD, Full Professor, Division of Gastroenterology, McGill University Health Center, Montreal General Hospital site, 1650 Cedar Avenue, room D7-346, Montréal, QC H3G 1A4, Canada. alan.barkun@muhc.mcgill.ca
Telephone: +1-514-9348309
Fax: +1-514-8348531

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Abstract

AIM

To performed a systematic review and meta-analysis to determine any possible differences in terms of effectiveness, safety and tolerability between existing colon-cleansing products in patients with inflammatory

bowel disease.

METHODS

Systematic searches were performed (January 1980-September 2016) using MEDLINE, EMBASE, Scopus, CENTRAL and ISI Web of knowledge for randomized trials assessing preparations with or without adjuvants, given in split and non-split dosing, and in high (> 3 L) or low-volume (2 L or less) regimens. Bowel cleansing quality was the primary outcome. Secondary outcomes included patient willingness-to-repeat the procedure and side effects/complications.

RESULTS

Out of 439 citations, 4 trials fulfilled our inclusion criteria ($n = 449$ patients). One trial assessed the impact of adding simethicone to polyethylene glycol (PEG) 4 L with no effect on bowel cleansing quality, but a better tolerance. Another trial compared senna to castor oil, again without any differences in term of bowel cleansing. Two trials compared the efficacy of PEG high-volume *vs* PEG low-volume associated to an adjuvant in split-dose regimens: PEG low-dose efficacy was not different to PEG high-dose; OR = 0.84 (0.37-1.92). A higher proportion of patients were willing to repeat low-volume preparations *vs* high-volume; OR = 5.11 (1.31-20.0).

CONCLUSION

In inflammatory bowel disease population, PEG low-volume regimen seems not inferior to PEG high-volume to clean the colon, and yields improved willingness-to-repeat. Further additional research is urgently required to compare contemporary products in this population.

Key words: Inflammatory bowel disease; Polyethylene glycol; Colonoscopy; Meta-analysis

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Core tip: This is the first meta-analysis addressing the issue of bowel preparations in Inflammatory bowel disease (IBD) patients aiming to determine any differences in terms of effectiveness, safety and tolerability between existing colon-cleansing products. This work is especially timely considering that colonoscopy is used frequently in IBD patients for both diagnosis and surveillance, and that recommendation on how to prepare these patients prior to colonoscopy are based mostly on expert opinion. The results suggest that low-volume polyethylene glycol (PEG) preparation with adjuvants in split-dosing may represent a valid alternative to standard high-volume PEG with at least a similar efficacy and better acceptability.

Restellini S, Kherad O, Bessissow T, Ménard C, Martel M, Taheri Tanjani M, Lakatos PL, Barkun AN. Systematic review and meta-analysis of colon cleansing preparations in patients with inflammatory bowel disease. *World J Gastroenterol* 2017;

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INTRODUCTION

Adequacy of the bowel preparation is one of the most important predictors of colonoscopic quality^[1]. Recent guidelines in bowel preparations strongly endorse the use of split-dose regimens, irrespective of the type of product, as this regimen results in greater proportions of patients with adequate preparation^[2]. However, many specific clinical situations need to be explored as some patients requiring specific considerations are often excluded in clinical trials because of safety concerns.

Inflammatory bowel disease (IBD), consisting of ulcerative colitis (UC) and Crohn's disease (CD), is an increasingly prevalent intestinal disorder with significant co-morbidities^[3]. Colonoscopy is used frequently in this patient population for both diagnosis and surveillance as patients with UC and extensive colonic CD, are at a higher risk of developing colonic malignancy after 8-10 years of disease^[4].

Safety is also a major concern in this population as bowel-preparation-induced mucosal inflammation has been reported^[5]. This risk seems higher with sodium phosphate (Nap) and picosulfate (PICO) than with polyethylene glycol (PEG)^[6]. Erosions, aphthoid lesions (ulcer < 5 mm), and ulcers (> 5 mm) as result of the preparation are often multiple^[7]. Some UC patients have also reported flare symptoms after colonoscopy^[8]. Furthermore, IBD patients with inadequate levels of bowel cleansing may need examinations rescheduled which may increase anxiety and interfere with follow-up that is critical in their clinical management^[9]. Some publications have found that IBD patients reported low satisfaction from the bowel preparation compared to other patients, while personal and anecdotal experiences suggests increased difficulty with bowel preparation in some patients with IBD^[10].

Unfortunately, only few data exist on colonoscopy preparations in IBD patients^[11-13]; the recommendations of how to prepare these patients prior to colonoscopy are thus based mostly on expert opinions^[4,14-17]. The aim of this systematic review was to summarize available evidence in order to determine any existing differences in effectiveness, willingness-to-repeat, and safety between contemporary colon-cleansing products in the IBD population.

MATERIALS AND METHODS

Search strategy

Systematic searches were performed (January 1980-September 2016) using MEDLINE, EMBASE, Scopus, CENTRAL and ISI Web of knowledge. Citation selection utilized a highly sensitive search strategy

identifying randomized trials^[18] with MeSH headings relating to (1) colonoscopy; (2) gastrointestinal agents; (3) bowel preparation; (4) generic and brand names; and (5) IBD (Annex 1). Recursive searches, cross-referencing and subsequent hand-searches were completed.

Trial selection and patient population

All fully published randomized trials in French or English with at least one arm administering a product as defined as PEG, NAP, PICO, or OSS (oral sulfate sodium) in a study population made up exclusively of IBD patients were included. Trials comprising pediatric and in-patients were also included. Authors of unpublished abstracts were contacted in order to access the full paper.

Choice of outcomes

The primary outcome measure was bowel cleanliness defined as the proportion of patients with an adequate preparation. Secondary outcomes were patient willingness-to-repeat the preparation, as a proxy for patient tolerance and satisfaction, as well as polyp or adenoma detection rates and side effects or complications (flare of disease, increase in SCAL score, ulceration).

Validity assessment

Two investigators assessed citation eligibility with discrepancies resolved by an independent reviewer. Quality of trials were graded using the Cochrane risk bias tool and Jadad score^[19] (with, one extra point for reported *a priori* sample size calculations).

Sources of possible heterogeneity - both clinical and statistical

Possible sources of clinical heterogeneity were noted across trials in keeping with pre-planned sensitivity or subgroup analyses. Identification and handling of statistical heterogeneity is described below.

Statistical methods and sensitivity analyses

For each outcome and in every comparison, effect size was calculated as odds ratios for categorical variables and weighted mean differences (WMDs) for continuous variables. The Mantel-Haenszel method for fixed effect models determined corresponding overall effect sizes with confidence intervals, except when statistical heterogeneity was noted in which case a random-effects model was used according to the DerSimonian and Laird method^[20]. WMDs were manipulated using the inverse variance approach. Statistical heterogeneity across studies was defined using a χ^2 test of homogeneity with 0.10 significance level. The Higgins I^2 statistic was calculated to quantify the proportion of variation in treatment effects attributable to between-study heterogeneity^[21].

Values for intention-to-treat (ITT) were preferred to

per protocol when both were presented. We included non-compliant patients or withdrawals in the ITT analysis to minimize bias^[22]. Publication bias was evaluated by assessing the funnel plots, if more than 3 studies were to be included in the meta-analysis.

All percentages of outcomes reported in the trials were converted to absolute numbers and no attempt at determining extractable values from graphics or figures was undertaken to avoid possible subjectivity.

All statistical analyses were completed using Meta package in R version 2.13.0, (R Foundation for Statistical Computing, Vienna, Austria, 2008) and Review Manager Version 5.3. Copenhagen: The Nordic Cochrane Centre, the Cochrane Collaboration, 2014.

We adopted the terminology of no difference purposefully rather than using possibly misleading or statistically incorrect terminologies such as non-inferiority or equivalence.

RESULTS

Included studies

Out of 43 citations, 4 trials fulfilled our inclusion criteria ($n = 449$ patients)^[12,13,23,24] (Figure 1 and Table 1). Twenty-five citations were excluded as they were not randomized controlled trials; 352 did not assess colonoscopy or bowel preparations, 24 had incorrect comparators, 21 an incorrect patient population, 2 were published in another language than French or English and 11 for other reasons such as one was not fully published and the abstract's authors did not respond to our query^[25].

Inter-rater, heterogeneity, publication bias and study quality

Moderate to strong heterogeneity was noted for the main outcome analyses. Publication bias was observed across the main outcome analyses.

The Jadad modified quality scores ranged from 0 to 6 points (mean of 4.3 ± 1.0). The Cochrane risk bias tool revealed a low potential for selection bias across studies for detection, attrition, reporting and other bias. Selection bias was unclear for several trials as the random sequence generation and allocation concealment was not described. Performance bias was high as the majority of the trials were single blinded (the endoscopist) (Figure 2).

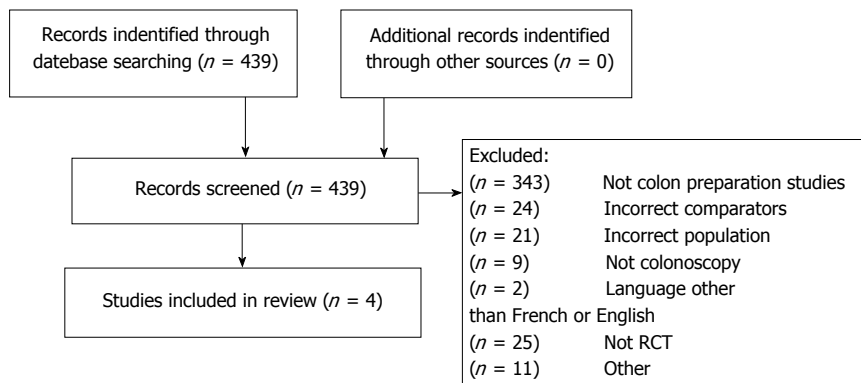
Primary outcome: bowel cleanliness

All four trials ($n = 449$) assessed the bowel cleanliness specifically in an IBD population (Table 2). One trial^[12] assessed the impact of the addition of Simethicone to PEG 4 L with no effect on bowel cleansing quality. However, the addition of simethicone showed a significant reduction in the formation of bubbles. Gould *et al.*^[23] 1982 compared the efficacy of the equivalent of 75 mg of senna to castor oil with no difference demonstrated between the two preparations.

Table 1 Recapitulative table with all included studies

Ref.	Arm 1 Time to FD and LD to endoscopy	Arm 2 Time to FD and LD to endoscopy	Overall IBD type	Time of endoscopy
Gould <i>et al</i> ^[23] 1982 (United Kingdom) outpatient elective Score: 3	Castor oil 30 min (<i>n</i> = 23) LD: 24 h before	Senna 75 mg (<i>n</i> = 23) LD: 24 h before	RCH = 46 CD = 0 Active disease = 7	-
Lazzaroni <i>et al</i> ^[12] 1993 (Italy) outpatient elective Score: 4	PEG 4 L + placebo (<i>n</i> = 56) FD: 2 L afternoon before LD: 2 L 6 am day of	PEG 4 L + simethicone 120 mg (<i>n</i> = 59) FD: 2 L afternoon before LD: 2 L 6 am day of	RCH = 61 CD = 44 Active disease = 0	-
Manes <i>et al</i> ^[13] 2015 (Italy) outpatient elective Score: 5	PEG 4 L (<i>n</i> = 108) <i>Whole dose</i> (<i>n</i> = 48) FD: 4 pm day before <i>Split dose</i> (<i>n</i> = 60) FD: 2 L day before	PEG 2 L + bisacodyl 10 mg (<i>n</i> = 108) <i>Whole dose</i> (<i>n</i> = 35) FD: 4 pm day before <i>Split dose</i> (<i>n</i> = 73) FD: 2 L day before	RCH = 216 CD = 0 Active disease = 116	Between 8 am and 2 pm
Kim <i>et al</i> ^[24] 2017 (Korea) outpatient elective Score: 5	LD: 2 L between 5 am and 7 am day of PEG 4 L (<i>n</i> = 55) FD: 2 L 8 pm day before; LD: 2 L morning day of If colonoscopy scheduled in the afternoon, 4 L between 6 am and 8 am	LD: 2 L between 5 am and 7 am day of PEG 2 L + ascorbate (<i>n</i> = 57) FD: 2 L 8 pm day before; LD: 2 L morning day of If colonoscopy scheduled in the afternoon, 4 L between 6 am and 8 am	RCH = 112 CD = 0 Active disease = 0	Between 9 am and 5 pm

FD: First dose; LD: Last dose; IBD: Inflammatory bowel disease; PEG: Polyethylene glycol; CD: Crohn's disease.

**Figure 1** STROBE diagram.

Two studies compared the efficacy of PEG high-volume (4 L) vs PEG low-volume (2 L) with an adjuvant, both mainly in split regimens. Manes *et al*^[13] 2015 assessed the efficacy of a low-dose iso-osmotic preparation based on low-dose PEG (2 L) associated to bisacodyl vs a PEG high-volume (4 L) preparation alone. The quality of colon cleansing was similar in the two groups (83% vs 75%, $P = 0.37$). Of note, simethicone was added to PEG low-volume with bisacodyl. Kim *et al*^[24] 2017 compared 4 L PEG with 2 L PEG plus ascorbic acid in terms of efficacy in patients with inactive UC. Successful cleansing was equally achieved in most patients from both groups with no significant differences noted (96.2% vs 92.9%, $P = 0.67$)^[24]. In an analysis restricted to a comparison between PEG high-volume to PEG low-volume with adjuvant in split dose regimens, there was no clinically relevant difference in bowel preparation irrespective of the type of adjuvant used [OR = 0.84 (0.37-1.92)] (Figure 3).

Secondary outcomes: willingness-to-repeat

Three studies reported the willingness-to-repeat the same preparation as a proxy of acceptance with only two studies with analyzable data ($n = 320$)^[13,24]. The drug combination of PEG with simethicone induced a significantly better acceptance among patients when compared with the use of PEG plus placebo^[12]. Manes *et al*^[13] 2015 reported that willingness to repeat the same preparation in case of a new endoscopy was higher in PEG low-volume plus bisacodyl vs PEG high-volume alone (94.3% vs 61.9%, $P < 0.001$). In the study of Kim *et al*^[24] 2017, participants in the PEG low-volume with ascorbate group reported that they were more willing to repeat bowel preparation with the same agent for the future colonoscopy than those in the PEG high-volume (4 L) group (82.1% vs 64.2%, respectively, $P = 0.034$)^[24].

In a pooled analysis restricted to comparison between PEG high-volume vs PEG low-volume, a significant higher proportion of patients were willing to

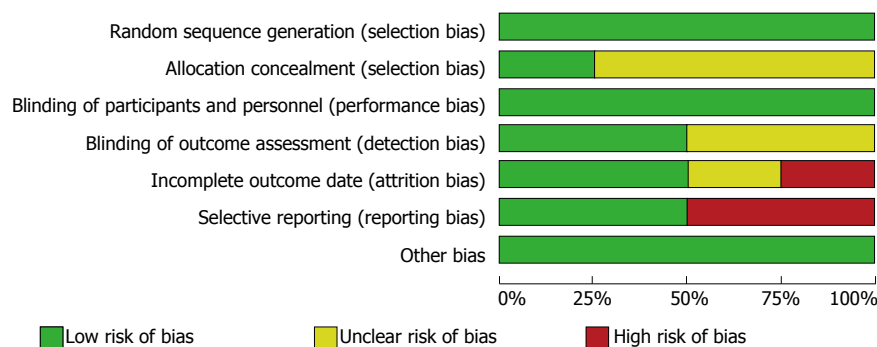


Figure 2 Cochrane risk bias tool.

Table 2 Primary outcome: bowel cleanliness

	Numbers of studies	ITT patients	OR (95%CI)	Heterogeneity <i>P</i> value	<i>I</i> ²
Primary outcome					
Bowel cleanliness	2	325	1.19 (0.52-2.71)	0.27	19%
Secondary outcome					
Willingness to repeat	2	320	5.11 (1.31-20.00)	0.03	78%
Procedure related outcome					
Rates of cecal intubation	2	320	0.91 (0.32-2.53)	0.54	0%
Mayo endoscopic score = 0	2	320	1.09 (0.69-1.70)	0.38	0%
Mayo endoscopic score = 1	2	320	0.84 (0.54-1.33)	0.61	0%
Mayo endoscopic score = 2	1	109	1.17 (0.46-3.00)	-	-
Mayo endoscopic score = 3	2	320	2.89 (0.30-28.24)	1.00	0%
Side effects or complications					
Flare of disease	1	109	0.62 (0.10-3.85)	-	-
Increase SCA II score	1	109	1.14 (0.47-2.75)	-	-
Dizziness	1	109	1.44 (0.23-9.00)	-	-
Abdominal pain/cramps	1	109	0.30 (0.03-3.01)	-	-
Bloating	1	109	0.72 (0.26-1.98)	-	-
Nausea	1	109	0.39 (0.16-0.94)	-	-
Vomiting	1	109	0.55 (0.17-1.81)	-	-
Insomnia	1	109	1.72 (0.70-4.23)	-	-

ITT: Intention-to-treat.

repeat low-volume preparations with adjuvants vs high-volume; OR 5.11 (1.31-20.0).

Secondary outcomes: side effects and polyp and adenoma detection rates

Side effects were reported in all studies but with different patterns and classifications, precluding meta-analysis. Globally, severe side effects such as flare of disease in IBD patients undergoing colonoscopy were very uncommon without significant differences between different preparations as reported in the 4 studies. Gould *et al*^[23] 1982 failed to incriminate castor oil as a more likely cause of exacerbation of colitis than an equally effective dose of senna. On the contrary, bowel disturbances were more common following senna than castor oil (48% vs 26%). Of note, authors used high doses of senna (equivalent of 75 mg). Lazzaroni *et al*^[12] 1993 reported significantly better results for patients treated with the drug combination of PEG plus simethicone regarding reduction of general malaise (19% vs 44%, *P* = 0.01) and sleep disturbances (19% vs 44%, *P* = 0.01). Manes *et al*^[13]

2015 noted that PEG low-volume (2 L) with bisacodyl was better tolerated than PEG high-volume (4 L) as evidenced by a significantly higher number of patients who described no or mild discomfort (83% vs 44.8%, *P* < 0.001). No severe adverse events were reported in the 2 groups. In the study by Kim *et al*^[24] 2017, overall adverse events during preparation were observed more frequently in the PEG high-volume group than in the PEG low-dose plus ascorbate, although the difference was not statistically significant (50.9% vs 36.4%, respectively, *P* = 0.127). However, patients in the PEG high-volume group reported significantly more nausea than those in the PEG low-volume plus ascorbate (35.8% vs 17.9%, respectively, *P* = 0.034). Finally, no study reported the polyp or adenoma detection rates or dysplasia findings.

DISCUSSION

The aim of this systematic review was to summarize existing evidence on bowel cleansing, specifically in IBD patients. Surprisingly, only four randomized controlled

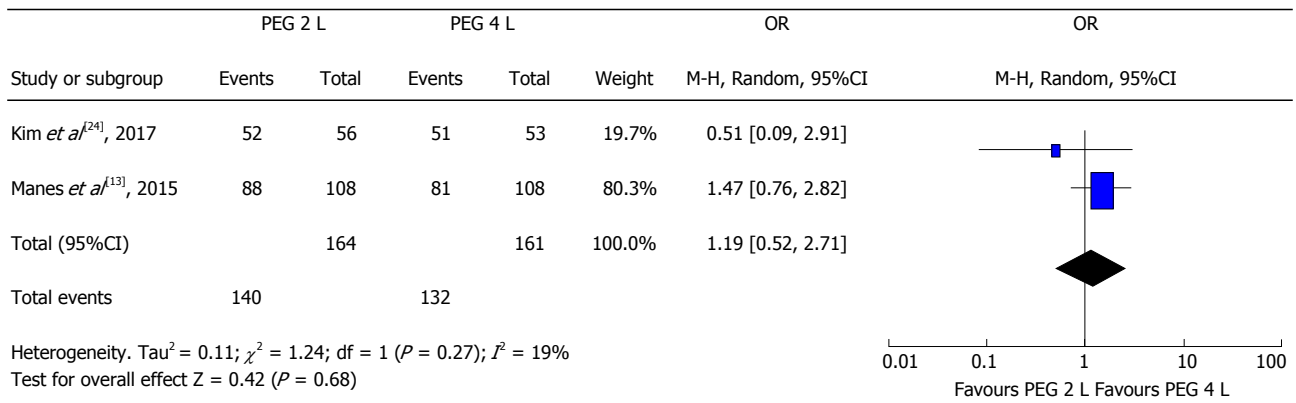


Figure 3 Forest plot quality of preparation comparing polyethylene glycol high-dose vs polyethylene glycol low-dose with adjuvant.

studies specifically addressed bowel cleansing in this important patient population, precluding any firm conclusions. The main results suggest that PEG-based preparations appear safe in IBD patients, with equivalent efficacy when comparing PEG low-volume vs PEG high-volume in split and non-split regimens, yielding improved tolerance for the former. Adjuvant therapies (osmotic and stimulant laxatives) were systematically associated to low-volume preparation without safety concerns. Severe side effects such as flare of the disease or preparation-induced ulcerations occurred in less than < 6%^[23,24].

The majority of included studies utilized PEG-based preparations, reflecting current practice guidelines that support the use of this safe solution for bowel cleansing in patients with IBD^[4,14,16,17]. Indeed, PEG preparations that are non-absorbable iso-osmotic solutions, minimizing the water and electrolytes imbalance, have long been proven to be well tolerated even in frail populations such as the elderly or patients with chronic kidney disease^[26,27]. Interestingly, none of the existing society recommendations provide any guidance about the use of low-volume preparations in an IBD population^[14,16,17]. In a general population undergoing colonoscopy, low-volume PEG preparations have been introduced to improve tolerability and acceptability before colonoscopy^[2]. The pooled analysis in this current study meta-analysis in IBD patients that includes the two most recently published studies on the topic suggests that PEG low-volume preparations are equivalent to PEG high-volume when associated to an adjuvant [OR = 0.84 (0.37-1.92)], while yielding improved tolerance as patient were more willing to repeat the low-volume preparation OR 5.11 (1.31-20.0). Of note, the majority of patients in these two studies took the preparations in a split-dose regimen with a better efficacy on bowel cleansing. Professional guidelines addressing the IBD population recommend the use of split-dose preparations based on the overwhelming evidence in non IBD populations^[2,17]. However, caution is advised in patients with partial bowel obstruction, gastroparesis, or known delayed intestinal motility, because of an increased risk for

gastric retention and aspiration^[17].

Furthermore, PEG low-volume preparations were systematically associated with an adjuvant. They were based on the combination of 2-L PEG with a stimulant laxative (bisacodyl), or an osmotically active agent (ascorbic acid). The impact of adjuvants on bowel preparations remains debated, with differing US and European society recommendations, but a recent meta-analysis suggests that adjuvants may improve tolerance when used with low-volume PEG preparations^[28]. PEG low-dose associated to either bisacodyl or ascorbate may thus represent a potentially ideal low-volume cleansing product for patients with IBD^[16]. Furthermore, simethicone remains an interesting adjuvant, decreasing luminal bubbles as reported by Lazzaroni *et al*^[12] 1993 in IBD patients mirroring same results in overall population^[12,29]. Society guidelines are disparate on this topic^[14,16] but a recent meta-analysis suggest the addition of simethicone may further improve bowel preparation quality^[28].

There is always a trade-off between cleansing efficacy and tolerance, especially when comparing high- to low-dose preparations^[30]. In general population, PEG high-volume leads to cleaner preparations compared to PEG low-volume with an adjuvant when restricting the analysis to split-dosing, but with a lower tolerance^[28]. Compliance may be limited by the inability of patients to ingest such a large volume of solution. Tolerability and compliance with bowel preparations are however both crucial particularly in patients with IBD. Indeed, IBD patients require repeated colonoscopies throughout their lifetime, so that a bad experience with the preparation may affect the acceptability and uptake of future colonoscopy, particularly in IBD surveillance protocols^[17]. Conversely, mucosal findings in IBD such as dysplastic and non-polypoid flat lesions may be very subtle and can be detected only in a perfectly prepared colon^[4], with the assistance of chromoendoscopy^[31]. Further studies comparing high-volume vs low-volume preparations in split-dosing, including more contemporary one such as PICO are urgently needed. Meanwhile, clinicians should probably engage the

discussion with the patient in a shared decision process in order to choose between an effective high-volume preparation or a more acceptable low-volume on a case-by-case basis.

Safety is also an important issue to be considered when prescribing a preparation for colonoscopy in IBD. Indeed, some procedures are performed in patients suffering from severe symptoms such as diarrhea and abdominal pain with possible preexisting electrolytic imbalances. Patients with IBD may develop complications either from the colonoscopy or from the cleansing procedure^[13]. These patients experience significantly more embarrassment and burden during the preparation when compared with patients undergoing colonoscopy for other indications^[9]. Furthermore, clinicians periodically express concern that bowel preparation may precipitate a relapse of colitis, based on personal experience^[17]. Additionally the preparation may cause ulcerations that can mimic IBD-related mucosal lesions, interfering with correct disease diagnosis and staging^[17]. Bowel preparations may indeed induce colonic mucosal damage through crypt cell apoptosis and increased oxidative stress^[32,33]. Reassuringly, side effects reported in this systematic review were not more frequent than in the general population, and were severe in < 6%^[23,24]. Relapse of colitis or flare of the disease requiring corticosteroid therapy occurred after colonoscopy in 2% to 4% and is often self-limited^[24]. This inconvenient side effect was mostly reported with Nap that has been removed from market anyway due to rare but significant renal toxicity, particularly in patients with kidney failure^[34]. Nap was indeed significantly associated with colonic mucosal inflammation suggesting it may have a transient role in provoking relapse in patients with ulcerative colitis^[32,33] and causing IBD-like mucosal damage^[17].

Limitations

The limited number of published randomized controlled studies represents a critical issue precluding firm conclusions from this meta-analysis. The lack of research in bowel preparation in inflammatory colonic conditions is surprising, as colonoscopy plays a key role in the management of IBD patients. Furthermore, it should be noted that the majority patients included in these studies had quiescent or relatively inactive colitis and that colonoscopies were usually undertaken electively in the context of cancer screening. Furthermore, patients with CD were almost absent in these studies, including fistulizing or stenotic diseases, limiting the generalizability of results to this subgroup population. It remains unclear whether PEG-based preparations can be administered to patients with more active disease in whom tap water enemas may represent an alternative for what is often a partially empty colon^[35].

Notwithstanding the limited published data, our results confirm that PEG preparations can be used in

a safe manner in patients with UC. Side effects were not more frequent than in the general population. In patients without contraindications, low-volume PEG preparation with adjuvants in split-dosing may represent a valid alternative to standard high-volume PEG with at least a similar efficacy and a better acceptability. Further research is required comparing low-dose contemporary products in this specific patient population, particularly patients with CD, so that the most adequate preparation can be chosen and authoritative recommendations can be confidently issued, based on best evidence.

COMMENTS

Background

Adequacy of the bowel preparation is one of the most important predictors of colonoscopic quality. Colonoscopy is performed frequently in an inflammatory bowel disease (IBD) patient population for both diagnosis and surveillance as these patients are at higher risk of developing a colonic malignancy. Recommendations on how to prepare these patients prior to colonoscopy are based mostly on expert opinion.

Research frontiers

Recent guidelines endorse the use of split-dose regimens preparations before colonoscopy, but only few data are available in IBD patients who are often excluded from trials for safety concerns.

Innovations and breakthroughs

This is the first meta-analysis addressing the issue of bowel preparations in IBD patients aiming to determine any differences in terms of effectiveness, safety and tolerability between existing colon-cleansing products.

Applications

The results of this meta-analysis suggest that low-volume polyethylene glycol (PEG) preparation with adjuvants in split-dosing may represent a valid alternative to standard high-volume PEG with at least similar efficacy and better acceptability. However, the limited number of published randomized controlled studies precludes firm conclusions and further research is required comparing low-dose contemporary products in an IBD population, so that the most adequate preparation can be chosen and authoritative recommendations can be confidently issued, based on best evidence.

Terminology

Split dose refers to administration of half of the preparation the evening prior to the colonoscopy and the second half the morning of the colonoscopy.

Peer-review

This is an interesting article that the authors made a study to review and meta-analyze colon cleansing preparations in patients with inflammatory bowel disease IBD. They concluded that in patients without contraindications, low-volume PEG preparation with adjuvants in split-dosing may represent a valid alternative to standard high-volume PEG with at least a similar efficacy and a better acceptability.

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Postoperative inflammation as a possible cause of portal vein thrombosis after irreversible electroporation for locally advanced pancreatic cancer

Jun-Jun Su, Ming Su, Kai Xu, Peng-Fei Wang, Li Yan, Shi-Chun Lu, Wan-Qing Gu, Yong-Liang Chen

Jun-Jun Su, Division of Gastroenterological Surgery, Department of Surgery, Shanxi Provincial People's Hospital, Taiyuan 030012, Shanxi Province, China

Jun-Jun Su, Ming Su, Kai Xu, Peng-Fei Wang, Li Yan, Shi-Chun Lu, Wan-Qing Gu, Yong-Liang Chen, Department of Hepatobiliary Surgery, Chinese People's Liberation Army General Hospital, Beijing 100853, China

Author contributions: Su JJ collected the case data, prepared the photos, and wrote the manuscript; all authors proofread the pathologic materials; Su M, Xu K, Wang PF, and Yan L proofread and revised the manuscript; all authors approved the final version to be published.

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Correspondence to: Dr. Yong-Liang Chen, Department of Hepatobiliary Surgery, Chinese People's Liberation Army General Hospital, 28 Fuxing Road, Beijing 100853, China. chenyongli301@163.com
Telephone: +86-13601021099

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Abstract

Portal vein thrombosis (PVT) is a rare but serious postoperative complication associated with irreversible electroporation (IRE). We report a case of postoperative PVT in a 54-year-old woman who underwent IRE for locally advanced pancreatic cancer. Drain removal and discharge of the patient from the hospital were scheduled on postoperative day (POD) 7; however, a magnetic resonance imaging scan revealed the presence of PVT. We suspected postoperative inflammation in the pancreas as the main cause of PVT. However, the patient did not undergo any medical treatment because she did not have any clinical symptoms, and she was discharged on POD 8.

Key words: Irreversible electroporation; Portal vein thrombosis; Locally advanced pancreatic cancer; Safety

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Core tip: Irreversible electroporation (IRE) is a medical technique that utilizes high voltage pulses to create permanent nanopores in the cell membrane, which in turn induces apoptosis of the targeted cells. Portal vein thrombosis (PVT) is a rare but serious postoperative complication associated with IRE. This review focuses on the mechanism of PVT after IRE for locally advanced

pancreatic cancer.

Su JJ, Su M, Xu K, Wang PF, Yan L, Lu SC, Gu WQ, Chen YL. Postoperative inflammation as a possible cause of portal vein thrombosis after irreversible electroporation for locally advanced pancreatic cancer. *World J Gastroenterol* 2017; 23(32): 6003-6006 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i32/6003.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i32.6003>

INTRODUCTION

Irreversible electroporation (IRE) is a medical technique that utilizes high voltage pulses to create permanent nanopores in the cell membrane, which in turn induces apoptosis of the targeted cells^[1-4]. The main advantage of IRE over other approaches is the avoidance of thermal injury to the surrounding structures, thereby sparing essential structures such as the nerves, vessels, and bile ducts^[5-8]. Although there are benefits of IRE, many adverse events should be taken into consideration before its use, including mild hypertension and hemodynamically relevant arrhythmia. Initial long-term survival data in animals have confirmed the safety of IRE as it does not show vascular thrombosis as a related complication^[9,10]. We report a case of successful IRE in a patient with locally advanced pancreatic cancer who developed PVT shortly after IRE.

CASE REPORT

We report the case of a 54-year-old woman who was diagnosed with tumor in the body of the pancreas, using computed tomography (CT). Magnetic resonance imaging (MRI) of the abdomen also revealed a tumor mass located at the pancreatic body; hence, pancreatic cancer was suspected. Artery invasion was seen near the celiac axis (Figure 1).

The patient underwent abdominal vascular ultrasonographic scanning of the hepatic vein, inferior vena cava, superior mesenteric vein, splenic vein, and portal vein, which showed that the blood flow was unobstructed prior to the surgery. The blood flow volume of the splenic vein was 14.5 cm/s and that of the superior mesenteric vein was 18.8 cm/s. The diameter of the portal vein was 1.1 cm and its blood flow volume was 28.8 cm/s. The patient underwent IRE ablation under balanced general anesthesia for pancreatic cancer because the tumor mass was considered unresectable because of tumor infiltration of the celiac axis root and major abdominal blood vessels. Furthermore, multiple metastases were established in the lymph nodes of different regions. Before the IRE ablation, we performed tumor mass biopsies. The patient was admitted to the hospital after routine blood tests, which showed normal levels

of leukocytes, neutrophils, erythrocytes, leukocytes, platelets, and amylase. These levels increased on postoperative day (POD) 2. The levels of C-reactive protein (CRP), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor (TNF) were also increased on POD 2. The amylase level of the drain from the IRE was 922.8 U/L on POD 3, which was 6 times higher than the upper limit of the normal serum amylase level, indicating the presence of postoperative pancreatic fistula according to the International Study Group on Pancreatic Fistula classification^[11]. The drain amylase level reached the normal serum range on POD 5 and after the drain removal. The levels of cancer antigen 199 in the serum were decreased after the surgery. The re-evaluation MRI scan following the IRE ablation demonstrated thrombosis of the portal vein trunk on POD 7 and revealed that the tumor size had decreased (Figure 2).

Non-occlusive PVT did not affect the patient's general condition, and the patient was discharged from the hospital on POD 8 because she showed no other complications. The patient currently has good quality of life.

DISCUSSION

There is no effective treatment available for patients with pancreatic cancer. Therefore, we put forward a novel method of minimally invasive IRE for the treatment of pancreatic tumors, which was reported recently in 2008. Although IRE is now regarded as an attractive treatment option for locally advanced pancreatic cancer, there are some complications associated with the use of this method. PVT is a rare but serious postoperative complication of IRE^[12]. Although the mechanism is still unclear^[13], medical literature on the occurrence of PVT reports that it is associated with three factors: endothelial cell injury, slow blood flow, and hypercoagulable state of the blood. In pancreatitis, various inflammatory mediators are released with concomitant thrombosis, resulting in high levels of IL-6, IL-8, and TNF. These factors stimulate the hepatic cells, which in turn produce large quantities of CRP. CRP is a sensitive indicator of the severity of inflammation. The initial clinical signs of PVT are often subtle and similar to those observed in postoperative pancreatitis. PVT can be detected using CT, MRI, and Doppler ultrasonography. Doppler ultrasonography is a non-invasive, easily available bedside examination with an 89% sensitivity and 92% specificity in detecting PVT^[14]. Early diagnosis of PVT might provide clinicians an opportunity for intervention before severe damage occurs. Once the thrombus is formed, thrombolysis and anticoagulation must be performed as soon as possible, including surgical treatment if necessary. In this case, the patient developed PVT, which did not completely block the portal vein. No clinical symptoms such as abdominal pain and portal hypertension were observed; hence,



Figure 1 Computed tomography and magnetic resonance imaging photographs. A: Computed tomography scan showed a locally advanced malignant pancreatic mass of 34 mm in diameter surrounding and narrowing the coeliac trunk (arrow); B: Magnetic resonance imaging demonstrating the mass located in the pancreatic body (arrow).

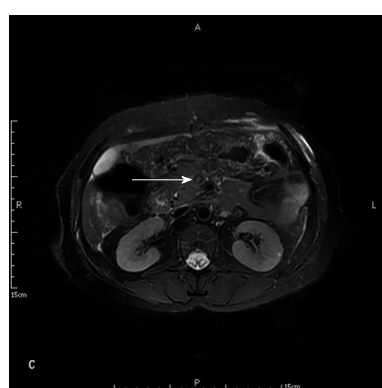


Figure 2 Magnetic resonance imaging findings. Magnetic resonance imaging showed thrombosis of the portal vein trunk.

anticoagulant was not administered. However, the case was closely followed. The patient was administered no special medical treatment during the follow-up period because she recovered from acute pancreatitis and showed no signs of thrombus. In this case, the levels of prothrombin time, activated partial thromboplastin time, fibrinogen, and thrombin time were normal before the operation. But after surgery, the patient developed abnormal blood coagulation and significantly increased white blood cells, CRP, IL-6, IL-8, and TNF, which explains that PTV occurred due to abdominal surgery.

COMMENTS

Case characteristics

A 54-year-old woman was referred to our hospital because of a tumor in the body of the pancreas found by physical examination.

Clinical diagnosis

The abdomen was soft, lax, and nondistended without evidence of a palpable mass.

Differential diagnosis

Abdominal sarcoma, abdominal neurogenic tumor, and cholangiocarcinoma.

Laboratory diagnosis

Before surgery, laboratory results were normal.

Imaging diagnosis

Computed tomography scan showed a locally advanced malignant pancreatic mass of 34 mm in diameter that surrounded and narrowed the coeliac trunk. MRI demonstrated the mass located in the pancreatic body.

Pathological diagnosis

The tumor was diagnosed as a low differentiated adenocarcinoma in the pancreas.

Treatment

The patient underwent irreversible electroporation (IRE) for locally advanced pancreatic cancer.

Related reports

Portal vein thrombosis (PVT) is a rare but serious postoperative complication associated with IRE. To date, only a few cases have been reported in the English literature, including our case presented in this report.

Experiences and lessons

PVT is a rare but serious postoperative complication associated with IRE. A patient with locally advanced pancreatic cancer who developed PVT shortly after IRE makes us suspect postoperative inflammation in the pancreas as the main cause of PVT.

Peer-review

This study highlights the mechanism of PVT after IRE in locally advanced pancreatic cancer and the authors also conducted a literature review so as to deepen the understanding of the subject. The information of this paper is valuable to the readers.

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Comment on "Efficacy and adverse events of cold vs hot polypectomy: A meta-analysis"

Huan-Huan Sun, Si-Lin Huang, Yang Bai

Huan-Huan Sun, Si-Lin Huang, Yang Bai, Department of Gastroenterology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China

ORCID Number: Huan-Huan Sun (0000-0002-7762-3260); Si-Lin Huang (0000-0003-4120-6307); Yang Bai (0000-0002-6991-9010).

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Correspondence to: Yang Bai, MD, Professor, Department of Gastroenterology, Nanfang Hospital, Southern Medical University, No. 1838, North Guangzhou Avenue, Guangzhou 510515, Guangdong Province, China. 13925001665@163.com
Telephone: +86-20-61641036
Fax: +86-20-61641049

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Abstract

This is a comment on a meta-analysis of published studies comparing cold vs hot polypectomy. We believe that the conclusion of this meta-analysis that "cold polypectomy is a time-saving procedure for removing small polyps with markedly similar curability and safety to hot polypectomy" needs more rigorous evidence.

Key words: Cold polypectomy; Hot polypectomy; Colon adenoma; Meta-analysis

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Core tip: This is a comment on a meta-analysis of published studies comparing cold vs hot polypectomy. We believe that the conclusion of this meta-analysis needs more rigorous evidence.

Sun HH, Huang SL, Bai Y. Comment on "Efficacy and adverse events of cold vs hot polypectomy: A meta-analysis". *World J Gastroenterol* 2017; 23(32): 6007-6008 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i32/6007.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i32.6007>

TO THE EDITOR

We read with interest the article by Fujiya *et al*^[1] entitled "Efficacy and adverse events of cold vs hot polypectomy: A meta-analysis", which compared cold and hot polypectomy with respect to efficacy and adverse events. The authors attempted to perform a systematic review and meta-analysis of the "randomized controlled trials (RCTs)" from several databases, one

of which is actually a retrospective study^[2]. In addition, among the six included studies, two (one article^[3] and one abstract^[4]) actually utilized the same data, which is another serious issue.

Colorectal polyps can be divided into three groups based on size: diminutive (≤ 5 mm), small (6–9 mm), and large (≥ 10 mm). The American Society for Gastrointestinal Endoscopy recommends that cold snare polypectomy should be the primary modality used for resection of diminutive polyps. However, polyps that are 6 to 9 mm in size can be resected by cold snare polypectomy or hot snare polypectomy because the optimum technique is not defined^[5]. In this meta-analysis, the authors demonstrated that cold polypectomy is a time-saving procedure for removing small polyps with markedly similar curability and safety to hot polypectomy. However, among the six included studies, one compared hot snare, cold snare and cold forceps polypectomy for diminutive colorectal polyps^[6], and the other five studies compared hot snare with cold snare polypectomy for small polyps (10 mm or less in diameter, and most were 8 mm or less)^[2–4,7,8]. Hence, we believe that the conclusion is not sufficient.

All six included studies reported the rate of adverse events, including bleeding. The study by Horiuchi *et al.*^[8], however, focused on small colorectal polyps in patients receiving anticoagulation therapy. Thus, it should be excluded from this meta-analysis, or sensitivity analysis should be done to explore whether it was biased.

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